Facultad de Veterinaria

Departamento de Genética

Integrated analysis of miRNA expression in response to *Salmonella* Typhimurium infection in pigs

Tesis Doctoral

Presentada por:

Juber Herrera Uribe

Dirigida por:

Prof. Dr. Juan José Garrido Pavón

Dra. Sara Zaldívar López

Córdoba, España - Febrero de 2017

TITULO: *Integrated analysis of miRNA expression in response to Salmonella Typhimurium infection in pigs*

AUTOR: *Juber Herrera Uribe*

© Edita: UCOPress. 2017
Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

www.uco.es/publicaciones
publicaciones@uco.es

TÍTULO DE LA TESIS: Integrated analysis of miRNA expression in response to Salmonella Typhimurium infection in pigs.

DOCTORANDO/A: Juber Leonardo Herrera Uribe

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La idea directriz del trabajo de tesis de D. Juber Leonardo Herrera Uribe ha sido la caracterización del papel que juegan los microARNs en la regulación post-transcripcional en el íleon y nódulo linfático mesentérico de cerdos infectados con Salmonella Typhimurium, con el fin de contribuir al conocimiento de la respuesta del hospedador. Para ello, se usó un enfoque integrador, basado tanto en el empleo de modelos in vitro como técnicas de microscopia, inmunológicas, moleculares, proteómica y de genómica funcional. Los resultados obtenidos se ajustan a los objetivos inicialmente previstos y son de gran interés, parte de ellos han sido ya publicados y otras partes están en proceso de preparación de manuscrito. Los resultados han sido también presentados en congresos nacionales e internacionales especializados.

Publicaciones:

Uribe JH, Collado-Romero M, Zaldívar-López S, Arce C, Bautista R, Carvajal A, Cirera S, Claros MG, Garrido JJ. Transcriptional analysis of porcine intestinal mucosa infected with Salmonella Typhimurium revealed a massive inflammatory response and disruption of bile acid absorption in ileum. 2016. Vet Res. 7; 47(1):11.

Trabajos en congresos:

Comunicacion oral: XXXIX Congreso de la Sociedad Española de Genética. microRNA expression in the porcine ileum in response to Salmonella Typhimurium infection using a Human heterologous microarray. Girona, Spain. 18-20/09-2013.

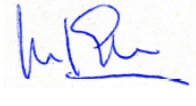
Comunicacion oral: 5TH European Veterinary Immunology Workshop. microRNA expression analysis using small RNA-Seq of ileum and mesenteric lymph node of pigs infected with Salmonella Typhimurium. Vienna, Austria. 2-4/09/2015.

Comunicacion oral: VII Jornadas de divulgación de la investigación en Biología Molecular, Celular, Genética y Biotecnología. Pig intestinal immune response to Salmonella enterica serovar Typhimurium by microRNA expression profiling. Córdoba, Spain. 27-28/06/2016.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 9 de Febrero de 2017

Firma del/de los director/es



Fdo.: Juan José Garrido Pavón Fdo.: Sara Zaldívar López

El presente trabajo ha sido realizado en el Departamento de Genética de la Universidad de Córdoba y financiado por fondos de la Unión Europea (proyectos EADGENE y SABRE), Ministerio de Ciencia e Innovación (AGL2008-00400 y AGL2011-28904) y Junta de Andalucía (P07-AGR-02672). **Juber Herrera Uribe** ha sido beneficiario de una beca del Programa de Formación de Personal Investigador (FPI) del Ministerio de Economía y Competitividad.

A mis padres.

ÍNDICE

LIST OF ABBREVIATIONS

ALT	Alanine transaminase
AMP	Antimicrobial peptides
APC	Antigen presenting cells
AST	Aspartate aminotransferase
BA	Bile acid
BUN	Blood urea nitrogen
CABIMER	Andalusian Center for Molecular Biology and Regenerative Medicine
CARD	Caspase Recruitment Domain
CDS	Coding sequence
CFU	Colony forming units
CHO	Chinese hamster ovary cells
DE	Differentially expressed
Dpi	Days post infection
ECDC	Disease Prevention and Control
EFSA	European Food Safety Authority
EMT	Ephitelial-mesencgymal transition
EU	European Union
FAE	Follicle associated epithelium
FC	Fold-change
FDR	False discovery rate
GALT	Gut associated lymphoid tissue
HDL	High density lipoprotein
HEV	High endothelial venules
HMDD	Human miRNA disease database

ILCs	Innate Lymphoid Cells
IPEC-J2	Porcine jejunum epithelial cell line
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat domain
LT	Lymphoid toxin
LTi cells	Lymphoid tissue-inducer cells
M cells	Microfold cells
MHC	Class major histocompatibility complex
MiRISC	microRNA induced silencing complex
MiRNAs	microRNAs
MLN	Mesenteric lymph nodes
MRE	miRNA recognition elements
NK cells	Natural killer cells
NLRs	NOD-like receptors
NOD	Nucleotide oligomerization domain
Nt	Nucleotides
PAB-UM	Andalusian Platform of Bioinformatics of the University of Malaga
PAMPs	Pathogen associated molecular patterns
PFA	Paraformaldehyde
PFP	Percentage of false predictions
PPs	Peyer's patches
PRRs	Pattern-recognition receptors
PYD	Pyrin-like domain
qPCR	Quantitative PCR
RMA	Robust multi-array analysis
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
S. Typhimurium	*Salmonella* Typhimurium:
SCV	*Salmonella* containing vacuole
SED	Sub epithelial dome
SPI	*Salmonella* pathogenicity island
sPLA2	Phospholipase A2
T3SS	Type III secretion system
TCRs	T cell receptors
Th cells	T helper cells
TLRs	Toll-like receptors
TNF	Tumor Necrosis factor
Treg cells	T regulatory cells
UTR	Untranslated region

ABSTRACT

Salmonellosis is a gastrointestinal disease caused by *S*. Typhimurium, which is transmitted by ingestion of contaminated food sources from animal origin. It is considered a major public health concern, and pork meat is one of the most important reservoirs of disease. Given that the pig is considered a good animal model for the study of human diseases, investigation of the molecular mechanisms involved in the response to infection in pigs is needed for human health. MicroRNAs (miRNAs) are a class of non-coding RNAs that interfere with mRNA stability and/or protein translation. miRNAs post-transcriptionally regulate biological processes such as those caused by bacteria. Although the porcine molecular response to *S*. Typhimurium infection has been studied, the role of miRNAs in gene regulation and protein expression during infection is still poorly known. The objective of this study was to identify miRNAs involved in gene and protein regulation during *S*. Typhimurium infection in porcine gastrointestinal tract and mesenteric lymph node, using next generation "*omics"*.

Sixteen piglets were orally challenged with S. Typhimurium. Samples from jejunum, ileum and colon, collected 1, 2 and 6 days post infection (dpi) were hybridized to mRNA and miRNA expression microarrays and analyzed. Jejunum showed a reduced transcriptional response indicating mild inflammation only at 2 dpi. In colon, genes involved in epithelial adherence, proliferation and cellular reorganization were down-regulated at 2 and 6 dpi. In ileum inflammatory genes were over expressed (e.g., IL-1B, IL-6, IL-8, IL1RAP, TNFα), indicating a strong immune response at all times of infection (particularly at 2 dpi) that tends to resolve at 6 dpi. Infection also down-regulated genes of the FXR pathway (e.g., NR1H4, FABP6, APOA1, SLC10A2), indicating disruption of the bile acid absorption

in ileum. This result was confirmed by decreased high-density lipoprotein cholesterol in serum of infected pigs. miRNA analysis of ileum at 2 dpi revealed 62 miRNAs potentially regulating target genes involved in this inflammatory process, such as miR-374 and miR-451.

To gain knowledge about the regulatory role of miRNAs during S. Typhimurium infection we used a small RNA sequencing strategy to investigate what happens in ileum and mesenteric lymph nodes at 2 dpi. We found 30 miRNAs differentially expressed in ileum. Target genes of these miRNAs were compared with those from the gene expression microarray, finding predictive target genes differential expressed in ileum. Target genes were validated using miRNA mimics (miR-223-3p, miR-220a-3p y miR-194b-5p) in porcine intestinal epithelial cells IPEC-J2. We found that down regulation of miR-215, miR-194, miR-192, miR-200 family (miR-200a/b/c, and miR-141) regulates expression of target genes involved in inflammatory response such as YWHAZ, P53, FZD4, FOXA1, IL-6, IL-8, IL-1A, IL-1β, and TLR4. Also, over expression of miR-146a/b and miR-223 regulates genes from the epithelial–mesenchymal transition (ZEB1/2, SNAIL1/2, CDH1/2, VIM, TGFBR1/2).

In mesenteric lymph node at 2dpi we discovered 110 miRNAs differentially expressed using small RNA-seq. Target genes predicted from those differentially expressed miRNAs were integrated with proteomic data, predicting 46 miRNA-protein interactions. We found that over expression of 13 miRNAs (miR-210-3p, miR-221-3p, miR-23a-3p, miR-23b-3p, miR-106a-5p, miR-20a-5p, miR-20b-5p, miR-378a-3p, miR-30b-5p, miR-181b-5p, miR-92b-3p, miR-363-3p and miR-155-5p) may be repressing 5 proteins (STMN1, LASP1, VIM, YWHAZ y ACTR3), and that down regulation of 18 miRNAs (miR-30d-5p, miR-182-5p, miR-204-5p, miR-128-3p, miR-125a-5p, miR-125b-5p, miR-451a, miR-148a-3p, miR-29b-3p, miR-144-3p, miR-148b-5p, miR-1-3p, miR-143-3p, miR-217, miR-96-5p, miR-130a-3p and miR-

122-5p) were likely up regulating 10 proteins (PPID, PSMB8, PDIA3, GRB2, HSPA8, SYNCRIP, HSP90B1, PCMT1, FKBP4 and ALDOA). This miRNA-protein interaction network suggested that deregulated miRNAs could be regulating the expression of proteins involved in the increase of inflammatory response, cytoskeletal reorganization, antigenic presentation MHC-I and MHC-II, apoptosis inhibition and necroptosis induction.

In summary, findings of this work suggest that miRNAs play an important role in regulation of biological processes during porcine *S*. Typhimurium infection. These results have generated new knowledge about miRNA interactions with genes and proteins, subsequently altering biological pathways. This information will be used in future studies of miRNAs regulating the host response to pathogens such as S. Typhimurium.

RESUMEN

La salmonelosis es una enfermedad gastrointestinal causada por *S*. Typhimurium, la cual es transmitida mediante ingesta de comida de origen animal contaminada. Se considera un problema grave de salud pública, y la carne de cerdo es uno de los reservorios más importantes de la enfermedad. Como el cerdo es considerado un buen modelo animal para estudiar enfermedades humanas, la investigación sobre mecanismos moleculares involucrados en la respuesta a la infección en cerdos es necesaria para la salud humana. Los microRNAs (miRNAs) son un tipo de RNA no codificante que interfiere con la estabilidad y traducción de la proteína. Los miRNAs regulan post-transcripcionalmente procesos biológicos como aquellos causados por bacterias. Aunque la respuesta porcina a la infección por S. Typhimurium ha sido estudiada previamente, se sabe poco del papel que juegan los miRNAs en la regulación génica y expresión de proteínas durante la infección. Por tanto, el objetivo de este estudio fue la identificación los miRNAs que regulan la respuesta a la infección por S. Typhimurium en el tracto gastrointestinal y nódulo linfático mesentérico porcino, usando herramientas "*ómicas*" de nueva generación.

Dieciséis cerdos fueron desafiados oralmente con S. Typhimurium. Muestras de yeyuno, íleon, y colon recogidos los días 1, 2 y 6 post infección (dpi) fueron hibridados en microarrays de expresión de mRNA y de miRNA y analizados. En yeyuno observamos una respuesta transcripcional reducida que indicaba inflamación leve a 2 dpi. En colon vimos represión a 2 y 6 dpi de genes involucrados en adherencia epitelial, proliferación y reorganización celular. En íleon se vio sobreexpresión de genes inflamatorios (ej. IL-1B, IL-6, IL-8, IL1RAP, TNFα), lo cual indica una fuerte respuesta inflamatoria en todos los puntos post infección estudiados (particularmente a 2 dpi) que tiende a resolverse a 6 dpi. La

infección provocó también una represión de genes de la ruta de FXR (ej. NR1H4, FABP6, APOA1, SLC10A2), indicando una interrupción de la absorción biliar en íleon. Este resultado se confirmó con los bajos niveles de colesterol HDL en suero de los animales infectados. El análisis de los miRNA en íleon a 2 dpi reveló 62 miRNAs regulando potencialmente genes diana implicados en el proceso inflamatorio, como por ejemplo miR-374 and miR-451.

Con intención de investigar más a fondo el papel regulatorio de los miRNAs en la infección, usamos una estrategia de secuenciación de nueva generación de pequeños RNA para investigar lo que ocurre en íleon y nódulo linfático mesentérico a 2 dpi. Se encontraron 30 miRNAs diferencialmente expresados en íleon, cuyos genes diana fueron enfrentados a los del microarray de expresión génica. De esta manera se identificaron genes diana diferencialmente expresados en íleon, que fueron validados usando mimics de miRNAs (miR-223-3p, miR-220a-3p y miR-194b-5p) en células epiteliales intestinales porcinas IPEC-J2. Resultó que la represión de miR-215, miR-194, miR-192, familia de miR-200 (miR-200a/b/c, and miR-141) regula la expresión de genes implicados en respuesta inflamatoria, como son YWHAZ, P53, FZD4, FOXA1, IL-6, IL-8, IL-1A, IL-1β, and TLR4. Por otro lado, la sobreexpresión de miR-146a/b and miR-223 regula genes de la transición epitelial-mesenquimal (ZEB1/2, SNAIL1/2, CDH1/2, VIM, TGFBR1/2), que desempeñan un papel importante durante la infección por S. Typhimurium.

En nódulo linfático mesentérico a 2 dpi descubrimos 110 miRNAs diferencialmente expresados usando RNA-seq de pequeños RNA. La predicción de sus genes diana se integraron con datos proteómicos, lo que llevó a identificar 46 interacciones miRNA-proteína. La sobreexpresión de 13 miRNAs (miR-210-3p, miR-221-3p, miR-23a-3p, miR-23b-3p, miR-106a-5p, miR-20a-5p, miR-20b-5p, miR-378a-3p, miR-30b-5p, miR-181b-5p, miR-92b-3p, miR-363-3p and miR-155-5p) puede estar provocando la represión de 5 proteínas (STMN1, LASP1, VIM,

YWHAZ y ACTR3), y la represión de 18 miRNAs (miR-30d-5p, miR-182-5p, miR-204-5p, miR-128-3p, miR-125a-5p, miR-125b-5p, miR-451a, miR-148a-3p, miR-29b-3p, miR-144-3p, miR-148b-5p, miR-1-3p, miR-143-3p, miR-217, miR-96-5p, miR-130a-3p and miR-122-5p) está probablemente sobreexpresando 10 proteínas (PPID, PSMB8, PDIA3, GRB2, HSPA8, SYNCRIP, HSP90B1, PCMT1, FKBP4 and ALDOA). Este mecanismo de interacción miRNA-proteína sugiere que la alteración de miRNAs puede estar regulando la expresión de proteínas que incrementan la respuesta inflamatoria, reorganización del citoesqueleto, presentación antigénica MHC-I y MHC-II, inhibición de la apoptosis e inducción de necroptosis.

En resumen, los hallazgos de este trabajo sugieren que los miRNAs juegan un papel importante en la regulación de procesos biológicos durante la infección de cerdos con *S*. Typhimurium. Estos resultados proporcionan nuevas bases para futuros estudios de la función de los microRNAs en la regulación post-transcripcional de la respuesta del hospedador a bacterias patógenas como *S*. Typhimurium.

INTRODUCTION

1. Host defense mechanisms against infection

1.1. Innate immune system.

The mechanism of innate immunity forms the first line of defense to infection. The physical, cellular and biochemical defenses of innate immunity are in place prior to infection and ready to response. One of the most remarkable adaptations of the immune system is its ability to recognize self. The mechanism of innate immunity only reacts to invading pathogens [1]. The primary elements of innate immunity are: physical and chemical barriers including epithelia and mucus at the epithelial surface; phagocytes, including macrophages, dendritic cells and neutrophils as well as natural killer cells; the complement system which consist of blood borne proteins that recognize infection, and respond by promoting microbe destruction and increasing inflammation; and cytokines that regulate and direct many of the innate immune cell responses to infection (Figure 1) [2].

The genuine first lines of defense against pathogens are the barriers provided by the skin and the epithelial surfaces, such as the respiratory or gastrointestinal epithelium. Due to these internal epithelia are more vulnerable than the rigid and keratinized skin, they constitute the primary sites of infection by microbial and viral pathogens. As a countermeasure, the surfaces of the gastrointestinal and respiratory tracts and genitourinary tracts are covered by mucus, a thick layer of fluid that is rich in glycoproteins and antimicrobial peptides. To increase its capacity of defense against pathogens, the surfaces of the mucosal membranes are rich in a diverse array of immune cells that cooperate in the rapid recognition and elimination of invading microbes through phagocytosis mediated killing and the induction of inflammation [3].

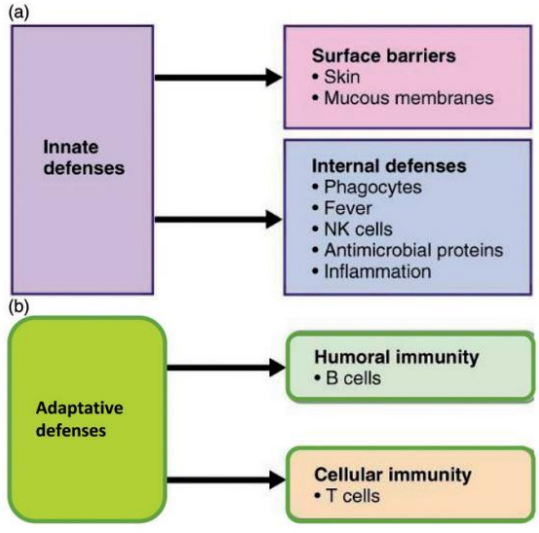


Figure 1. Biological defense mechanisms that protect the body. The immune system is a functional system rather than an organ system involving hematopoietic, vasculature and lymphatic system. A) Innate defenses, b) Adaptive defenses [4].

Detection of invading microbes by innate immune cells is based on germline-encoded pattern-recognition receptors (PRRs), which recognize conserved microbial molecules called pathogen associated molecular patterns (PAMPs). Several families of pattern-recognition receptors have been discovered thus far and are known to engage in the surveillance of both the extracellular and intracellular space. These families include the membrane bound Toll-like receptors (TLRs) that recognize PAMPs in the extracellular compartment and in endosomes, and recognize the cytosolic NOD-like receptors (NLRs) that monitor the intracellular space. These receptors allow the discrimination of various types of tissue insults and launch appropriate inflammatory responses. Acute inflammation is characterized by an increase blood flow, rapid influx and activation of immune cells, and the release of pro-inflammatory cytokines. This results in heat, swelling and redness of the affected area and serves to prevent the spread of the infection and to promote pathogen clearance and tissue repair.

The inflammatory response needs to be tightly regulated and quickly terminated to prevent unnecessary damage to tissues and to the organism [1].

1.2. Adaptive immune system.

Adaptive immunity is so called because, the mechanisms that respond to infection increase in magnitude with successive exposures to a particular pathogen or infectious agent. Lymphocytes are the predominant cell type responsible for the mechanisms of adaptive immunity. Lymphocyte display antigen receptors on their cell surface. There are two kinds of lymphocytes, T and B. These cells are able to provide the antigenic specificity and memory that defines adaptive immunity [5]. The type of effector T-cell reaction is determined by a complex interaction of antigen-presenting cells (APC) with naive T cells and involves genetic and environmental factors, including the type of antigen, cytokines, chemokines, co-stimulatory molecules, and signalling cascades. T cells are characterized by the presence of T cell receptors (TCRs) and can be categorized in two main subsets by the cell surface expression of their CD4 or CD8. CD4+ cells, which recognize antigens in the context of Class II major histocompatibility complex (MHC), are mainly regulatory cells, whereas CD8+ cells are mainly cytotoxic cells that recognize antigen presented within Class I MHC molecules. Both functions are of vital importance in the adaptive and innate immune responses [6]. Viruses growing within infected cells as well as intracellular bacteria are facing the killing of their host cells by $CD8^+$ cytotoxic T cells. Most microbial components are endocytosed by APC, processed and presented preferentially to $CD4^+$ T helper (Th) cells. Th cells help B cells to produce antibody and to undergo class switching and affinity maturation; they recruit and activate CD8 T cells, macrophages, neutrophils, eosinophils, basophils and other effector cells. They also directly act on many tissue cells, including

epithelial cells and mucosal cells, during the process of pathogen clearance. Based on their functions, their pattern of cytokine secretion and their expression of specific transcription factors, Th cells, differentiated from naïve $CD4^+$ T cells, are classified into four major lineages, Th1, Th2, Th17 and T regulatory (Treg) cells, although other Th lineages may exist [7]. Th1 cells mainly produce IFNγ, which is important for macrophage activation and clearance of intracellular pathogens, whereas Th2 cells produce *IL-4*, *IL-5*, *IL-10* and *IL-13*, later shown to be critical for IgE production, eosinophil recruitment and clearance of extracellular parasites. Th17 cells, which produce many cytokines including *IL-17a*, *IL-17f*, *IL-22* and *IL-21* are involved in autoimmune diseases and also play critical roles during immune responses against extracellular bacteria and fungi [8, 9]. Finally, Treg cells produce TGFβ and *IL-2* and are involved in self-tolerance, immune modulation and promoting immune responses under certain circumstances [10].

1.3 The gastrointestinal immune system.

In the gut, the epithelial barrier that separates the luminal content from the underlying internal environment and the gut associated lymphoid tissue (GALT) is permeable and allows the absorption of molecules of different size by using various active and passive mechanisms. The epithelium of the small intestine has many important functions among which the digestion, absorption and transport of nutrients, water and electrolytes, as well as inflammatory and immunological defense against external aggression. In this latter case, the role of the underlying GALT is crucial in protecting the mucosa [11]. Each epithelial cell maintains intimate association with their neighbors and seals surf gut tight junctions. Non hematopoietic cells in the epithelial layer help maintain this barrier by secreting mucus (Goblet cells) or antimicrobial peptides (Paneth cells) [1]. In summary,

intestinal epithelial monolayer provides both intrinsic and extrinsic barriers to potentially harm-full pathogens and antigens. However, pathogenic microorganisms routinely circumvent this physical barrier. For example, antigen may be taken up by microfold (M) cells found within the follicle-associated epithelium of Peyer's patches (PPs) [12]. In addition, antigen may be sampled directly by dendritic cells, which open tight junctions between epithelial cells to extend dendrites into the intestinal lumen [13], and certain species of bacteria (i.e. *Salmonella*) overcome the epithelial barrier by using specialized invasion strategies such as the Type III secretion system [14]. Pathogens and other antigens within the gut lumen that traverse the epithelial barrier eventually interact with phagocytic cells (e.g., macrophages and dendritic cells) as well as B and T lymphocytes within the GALT. These interactions provide the necessary signals for the initiation of an adaptive immune response and the generation of effector mechanisms [15]. GALT is considered a component of the mucosal immune system and is composed of aggregated tissue including Peyer's patches and solitary lymphoid follicles, and non-aggregated cells in the lamina propria, intestinal epithelial cells, intraepithelial lymphocytes, as well as mesenteric lymph nodes (Figure 2) [15].

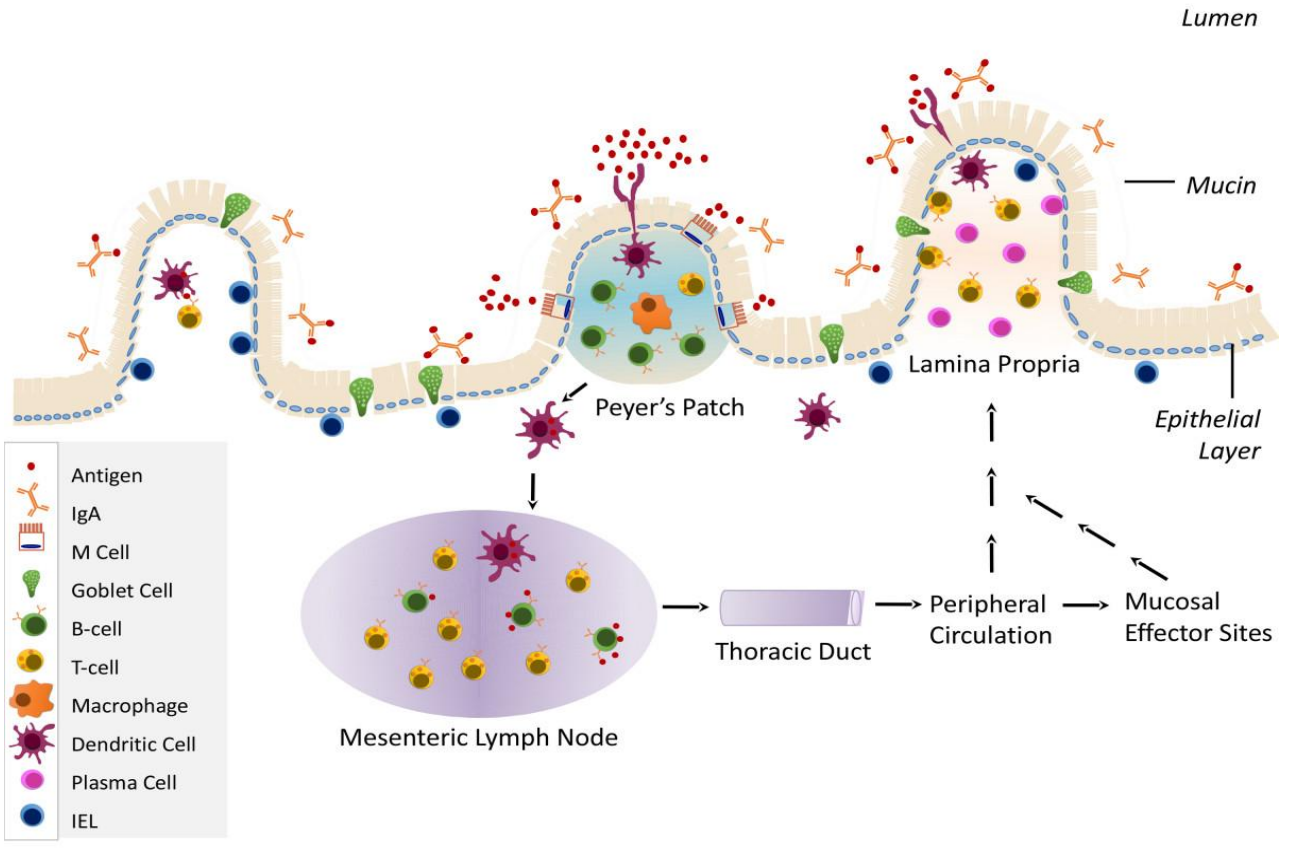


Figure 2. Diagram of the Gut-Associated Lymphoid Tissue [15]

In addition to the physical barrier that intestinal epithelial cells provide, they are considered as part of the innate immune system. Thus, intestinal epithelium has evolved receptors, such as the extracellular TLR and the intracellular NLR, to identify pathogens that have breached the epithelial barrier. This will to elicit the activation of signaling cascades that regulate the expression of pro-inflammatory cytokines and chemokines leading to the recruitment of macrophages, lymphocytes and polymorphonuclear leukocytes [16].

The Peyer´s patches (PP) are macroscopic lymphoid aggregates that are found in the submucosa along the small intestine, predominantly in the ileum. Mature Peyers´s patches consist of a collection of large B cells follicles and intervening T cell areas. B naïve cells from the germinal centre of the follicle are supported or connected by follicular dendritic cells. These follicular dendritic cells present antigens to the naïve T cells [17]. Peyer´s patches differ from lymph nodes elsewhere in the body because they lack afferent lymphatic vessels. This characteristic is in keeping with the notion that antigen is sampled from the lumen via the overlying epithelium [18].

The lymphoid areas are separated from the intestinal lumen by a single layer of columnar epithelial cells, known as the follicle associated epithelium (FAE), and a more diffuse are immediately below the epithelium, known as the sub epithelial dome (SED). The FAE differs from the epithelium that cover the mucosa as it has lower levels of digestive enzymes and a less pronounced brush border. It is infiltrated by large numbers of B cells, T cells, macrophages and dendritic cells. The most notable feature of the FAE is the presence of M (microfold) cells. M cells are specialized enterocytes with poorly developed brush borders and a thin overlying glycocalix, and thus are adapted to antigen uptake function [19]. Subsequently, antigens encounter numerous professional APC in the SED that prime naïve T and B cells. These lymphocytes become memory or effector cells

and migrate from PP to the mesenteric lymph nodes and then via the thoracic duct to peripheral blood for subsequent extravasation at mucosal effector sites [20].

Mesenteric lymph nodes (MLN) are the largest lymph nodes in humans and other animals and play an important role in immune defense against bacterial pathogens [21]. The structure of MLN is similar to that the peripheral lymph nodes and consist in a outer cortex containing a paracortex that surrounds a large medulla. The cortex is mainly composed of T-cell areas and B-cell follicles. It is within T-cell area where circulating lymphocytes enter the lymph nodes and dendritic cells present antigens to T-cells. Lymph is collected from intestinal mucosa and reaches MLN via the afferent lymphatics. In the majority of mammalian lympn nodes, lymph fluid leaves MLN through efferent lymphatics to reach the thoracic duct that drain to the blood. The pig lymph nodes – both peripheral and mucosa-associated – have a specific structure that is called inverted. The tissue lacks a larger medullary area and is preferentially composed of cortical areas and paracortex [19]. The pig shares this inverted lymph node structure with the rhinoceros, the dolphin and the elephant [19]. Lymphocyte trafficking in pig also differs from that in other animals. Whereas most lymph node structures allow lymphocytes to exit via the efferent ducts into the thoraci duct, the density of the outer medulla in the inverted structure inhibits lymphocytes exiting in the lymph. Instead, they leave the lymph node via the high endothelial venules (HEV), resulting in largely acellular lymph emptying into the thoracic duct of pigs [22].

2. *Salmonella* and salmonellosis

2.1 Salmonella.

Salmonella are Gram negative bacteria consisting of non-spore forming bacilli and are a member of the family *Enterobacteriaceae*, considered a major cause of disease in cold- blooded and warm-blooded animals [23, 24]. *Salmonella* spp are non-fastidious as they can multiply under various environmental conditions outside the living host. Most *Salmonella* serotypes grow at temperature range of 5 to 47º C with optimum temperature of 35 to 37ºC but some can grow at temperature as low as 2 to 4ºC or as high as 54ºC [25].

The genus *Salmonella* comprises three species, *Salmonella* enterica, *Salmonella* bongori, and *Salmonella* subterranean. *S.* enterica subdivides into the subspecies enterica (subsp. I), arizonae (subsp. IIIa), diarizonae (subsp. IIIb), houtenae (subsp. IV), indica (subsp. VI), and salamae (subsp. II). *Salmonella* strains belong to over 50 serogroups based on the O antigen, and to over 2500 serotypes (each having a unique combination of somatic O, and flagelar H1 and H2 antigens). Most of these serotypes belong to one single *Salmonella* subspecies, enterica, and are associated with >99% of *Salmonella* caused diseases in human, including gastroenteritis and enteric fever [26]. In the European Union (EU), over 100.000 human cases of salmonellosis are reported each year. The European Food Safety Authority (EFSA) has estimated that the overall economic burden of human salmonellosis could be as high as EUR 8 billion a year [27].

2.2. Pathogenesis of *Salmonella*.

The most common mode of *Salmonella* infection is acute gastroenteritis. The incubation period may vary from 4 hours to 72 hours after ingestion of contaminated food or water. Symptoms are acute onset of fever and chills,

nausea and vomiting, abdominal cramping, and diarrhea. If a fever is present, it generally subsides in 72 hours. Diarrhea is usually self-limiting, lasting 3-7 days, and may be grossly bloody. *Salmonella* is excreted in feces after infection, a process that may last for a median of 5 weeks [26].

Salmonella ingestion occur by oral route, although it was found that airborne *Salmonella* transmission over short distances is possible, but may be serotype dependent [28]. During ingestion, *Salmonella* enters the tonsils in the soft palate and persists in the tonsillar crypts. Following ingestion, *Salmonella* must survive the low pH (5-7) of the stomach by transcription of genes that encode acid shock proteins [23].

Bacteria that survive passage through the stomach, travel to the small intestine where they encounter other antibacterial factors including bile salts, lysozyme and defensins. In the distal parts of the intestine, adherence to the intestinal mucosa is generally accepted as the first step in the pathogenesis of *Salmonella* infections. Several adhesins and fimbriae are necessary to mediate adherence and, following adhesion *Salmonella* cross the intestinal epithelium using various mechanisms, including the invasion of adsorptive enterocytes, M cells and even globet cells. Fimbriae attachment triggers transcription of the *Salmonella* pathogenicity island 1 (SPI-1) that encodes the Type III secretion system 1 (T3SS-1) required for invasion into the host cell. T3SS-1 functions like a molecular syringe, injecting effector proteins into the host cell cytoplasm allowing manipulation of the host-cell signalling cascades and facilitating *Salmonella* engulfment and transport through the intestinal epithelial barrier [29]. Effector proteins are also encoded by genes within SPI-1 and serve to promote bacterium uptake by the host cell. Among them, SopB and SopE2 are involved in the rearrangement of the cytoskeleton through the activation of the host Rho GTPases and the consequent stimulation of signal transduction pathways that

result in the recruitment of actin reorganization complexes. Others such as SipA acts to increase the stability of actin filaments, while SipC bundles actin. Together, these actions result in membrane ruffling of the host cell, and Salmonella uptake [30, 31]. Of the 23 SPIs known to date, SPI-1 and SPI-2 are the most studied. SPI-2 contains the genes for TSS2 that is required for intracellular survival, transferring effector proteins from *Salmonella* across the *Salmonella* containing vacuole (SCV) membrane to interact with targets in the host cells, leading to systemic spread. The role of genes from others SPIs remains unclear [31, 32]. After crossing the intestinal barrier, bacteria are taken up by phagocytic immune cells such as macrophages, neutrophils or dendritic cells. Once phagocytosed, *Salmonella* replicates within a vacuolar compartment in the cytoplasm, spreading to enterocytes and mesenteric lymph node, and increasing the loss of epithelial cells and the inflammatory response observed during infection [1, 33-35].

2.3 Salmonellosis

Salmonellosis is an important zoonotic disease of world-wide significance. In particular, food animals with subclinical infection constitute a vast reservoir for disease in human [36]. Disease is caused by various *Salmonella* serovars and it occurs in a wide variety of forms presenting a broad clinical spectrum (from gastroenteritis to systemic infections) [37]. Depending on the clinical syndromes caused in this host, strains can be classified into two groups: typhoid *Salmonella* and non-typhoid *Salmonella*. Human infections are commonly caused by non-typhoidal *Salmonella* spp. Serotypes (predominantly *Salmonella* enterica serovar Typhimurium) and rarely by typhoidal *S*. enterica serotype Typhi [38, 39]. Gastroenteritis by *Salmonella* is one of the major concern in developed and developing countries [40]. The last report of the EFSA and the European Centre

for Disease Prevention and Control (ECDC) in 2014 showed that, a total of 88,715 confirmed salmonellosis cases were reported by European countries (Figure 3), resulting in a EU notification rate of 23.4 cases per 100,000 population, which has impacted in 9,830 hospitalised cases and 65 reported deaths [27]. EFSA has estimated that the overall economic burden of human salmonellosis could be as high as EUR 3 billion a year.

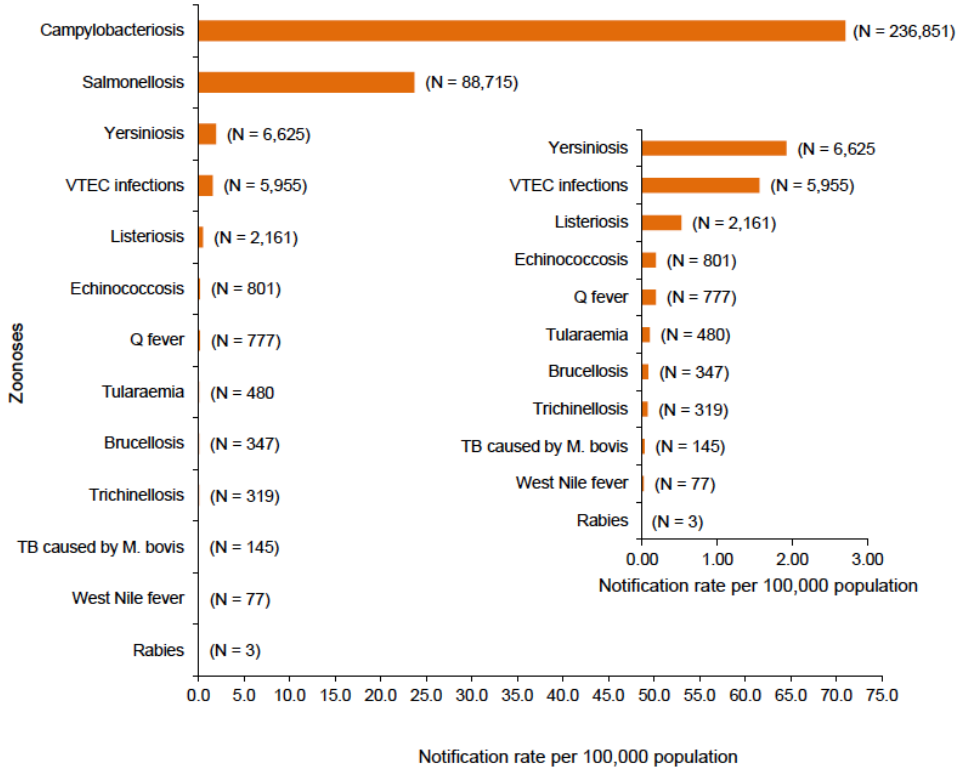


Figure 3. Total number and notification rates of confirmed human zoonoses cases in the 2014 [27].

Salmonellosis is easily spread, and consequently, it is a worldwide public health problem. It is among the most commonly isolated food borne pathogens associated with pig and meat pig [41]. The control of *Salmonella* enterica in pig production is necessary for both public and animal health. The persistent and frequently asymptomatic nature of porcine *Salmonella* infection and the organism´s abilities to colonize other animal species and to survive in the environment mean that effective control generally requires multiple measures, increasing the difficulty detection and control [42].

Almost half (48.9%) of the meat consumed in the EU is of pig meat [27]. Thus, pig meat plays an essential role as reservoir of *Salmonella*. The pig and pig meat are considered the second most important source of human salmonellosis in Europe. Source attribution studies for the European Union implicate poultry and pig meat as the commonest sources, after eggs, for human salmonellosis [43]. However, if only considered the south Europe countries, the pig could be considered as first infection source. The pig meat would responsible of the 43,6% of the outbreaks of *Salmonella* in these countries and the 31,8% in Spain. In the whole European Union, the pig meat and their products are involved in the 48,1% of the outbreaks caused by *S*. Typhimurium (Figure 4). For all that, salmonellosis is covered as an infection under surveillance and control to avoid economic losses [27, 44].

2.4 Host response to *Salmonella*

2.4.1 Innate immune response to *Salmonella*

One of the first obstacles faced by *Salmonella* is a thick layer of mucous that covers the surface of the gut epithelium and provides an initial barrier, which must be penetrated in order to gain direct contact with the epithelium. This mucus layer is formed by mucins, a family of glycoproteins secreted by

specialized types of epithelial cells, called Goblet cells. In addition to the mucins, cells in the gastrointestinal tract secrete several types of antimicrobial peptides, which are small amphipathic proteins that function like peptide antibiotics by disrupting the integrity of the bacterial cell membrane. The production and release of antimicrobial peptides and mucins by the epithelial cells of the gut represents a major barrier against microbial invasion and an important part of the innate immune response. While some antimicrobial peptides are expressed constitutively, many are induced as part of the inflammatory response to invading pathogens. Paneth cells of the intestinal crypts are the most abundant producers of constitutively expressed antimicrobial peptides in mammals, such as α and β-defensins, CRS peptides, calprotectin lysozyme and phospholipase A2 (sPLA2) in response to microbial PAMPs [45].

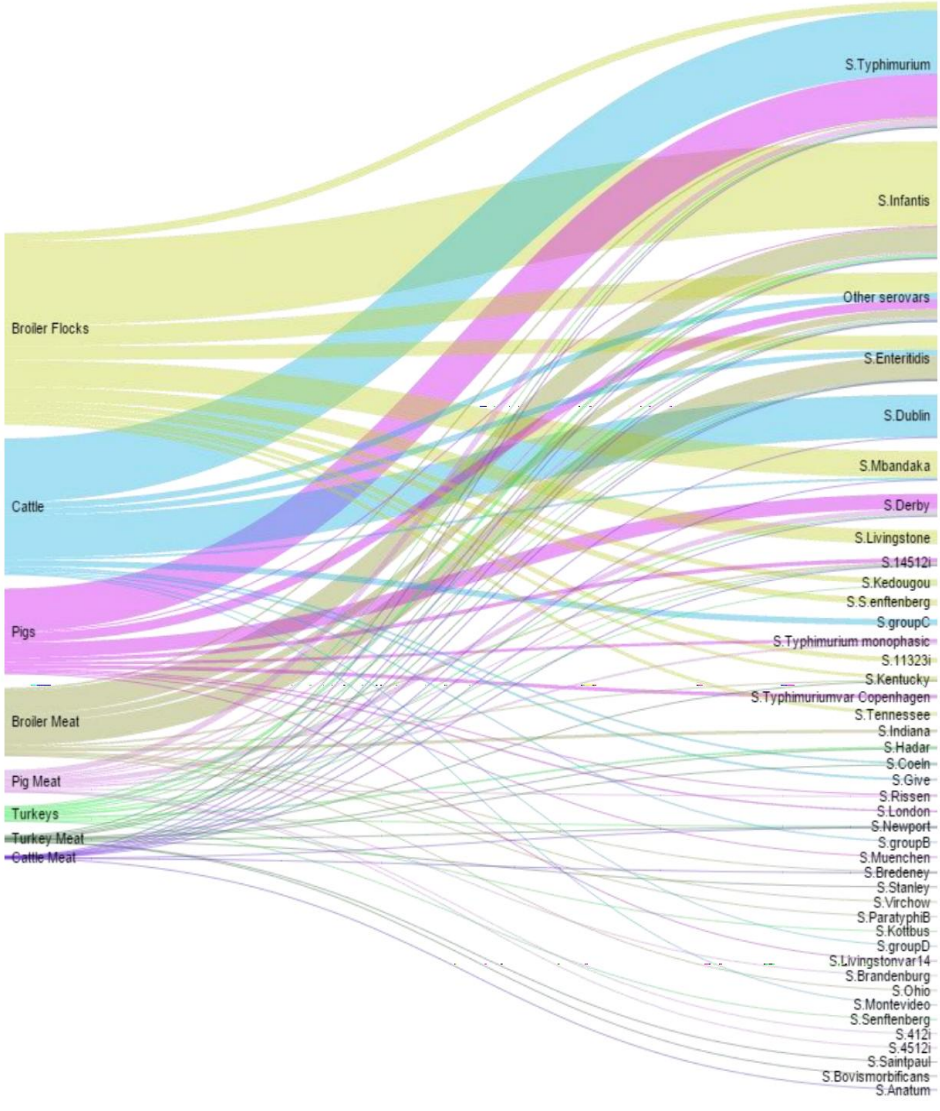


Figure 4. Sankey diagram of reported Salmonella serovar isolates, in animal species, food animal origin and animal feedingstuff, by matrix [27].

Crossing the epithelial barrier allows *Salmonella* to escape the inhospitable environment at the surface of the intestinal mucosa and to evade many antimicrobial defenses. However, once *Salmonella* has passed through M cells or enterocytes, it encounters the next layer of innate immune defenses, the monocyte-derived phagocytic cells of the GALT: macrophages and dendritic cells. The main function of these cells is to remove invading microbes by phagocytosis and to alert other immune cells of the infection, either directly or by secreting pro-inflammatory cytokines. Following phagocytosis, *Salmonella* express their virulence-associated SPI-2 T3SS to establish themselves in SCV. Within this intracellular compartment, *Salmonella* can replicate to high numbers before exiting the cell and infecting new host cells. Although *Salmonella* remains partially hidden within its intracellular niche, it cannot completely escape host cell sensing [46].

All monocytic cells express an array of germline-encoded pattern recognition receptors (PRR), which enable them to detect PAMPs. TLRs, which are located on the outer membrane of the cell (TLR1, 2, 4, 5, 6, 10) or in intracellular vesicles (TLR-3, 7, 8, 9, 11, 13 early and late endosome, lysosomes), are the first PRRs to detect the presence of *Salmonella*. TLRs can detect a variety of extracellular and endosomal PAMPs such as LPS, bacterial lipoproteins, peptidoglycan, flagellin DNA, RNA and others. Upon ligand binding, TLRs engage the signaling adaptors MyD88 and TRIF, which initiate signaling cascades leading to the activation of the transcriptional factors NFκβ and IRF3 that induce the production of inflammatory cytokines (*IL-8, IL-10, pro-IL-1β, pro-IL18* and others) as well as a type I IFN response, respectively [47].

Once *Salmonella* has established itself within the SCV, it is hidden from many extracellular detection mechanisms. Nevertheless, macrophages and other leukocytes have evolved mechanisms to recognize the presence of PAMPs in the

cytosol. The NOD-like receptor (NLR) family of PRR is a surveillance system that can detect the presence of PAMPs in the cytosol. NLRs contain an N-terminal protein-protein interaction domain [either a Caspase Recruitment Domain (CARD) or a Pyrin-like domain (PYD)], a central nucleotide oligomerization domain (NOD), and a C-terminal Leucine-rich repeat domain (LRR). Upon ligand binding, NLRs multimerize and initiate different signaling cascades. For example, NOD1 and *NOD2* interact with RIP2 kinase, which is a potent activator of *NFkB* [48].

In addition to NODs, there is a family of NLRs that do not initiate a transcriptional program, but induce the assembly of a large multiprotein signaling complex, called the inflammasome. Assembly of the complex usually also requires an adaptor protein called ASC (apoptosis-associated speck-like protein with a CARD), which recruits the cysteine protease pro-Caspase-1 to the inflammasome. Within this complex pro-Caspase-1 is activated by dimerization and autoproteolytic cleavage [49]. Activated Caspase-1 cleaves the pro-forms of interleukin *(IL)-1β* and *IL-18*, leading to production and secretion of the mature cytokines. Caspase-1 activation also initiates a pro-inflammatory cell-death program called pyroptosis. Pyroptosis is similar to apoptosis in that it is a programmed form of cell death controlled by caspases. Nevertheless, pyroptosis is solely dependent on Caspase- 1 and, in contrast to apoptosis, pyroptosis is not a silent form of cell death. During pyroptosis, there is formation of pores in the cell membrane and subsequent release of cellular contents and proinflammatory cytokines, which serve to amplify the inflammatory response by signaling the recruitment of other mediators of inflammation. Inflammasome induced pyroptosis likely benefits the host during microbial infections by eliminating the intracellular niche of the pathogen and re-exposing it to extracellular immune defenses (Figure 5) [50, 51].

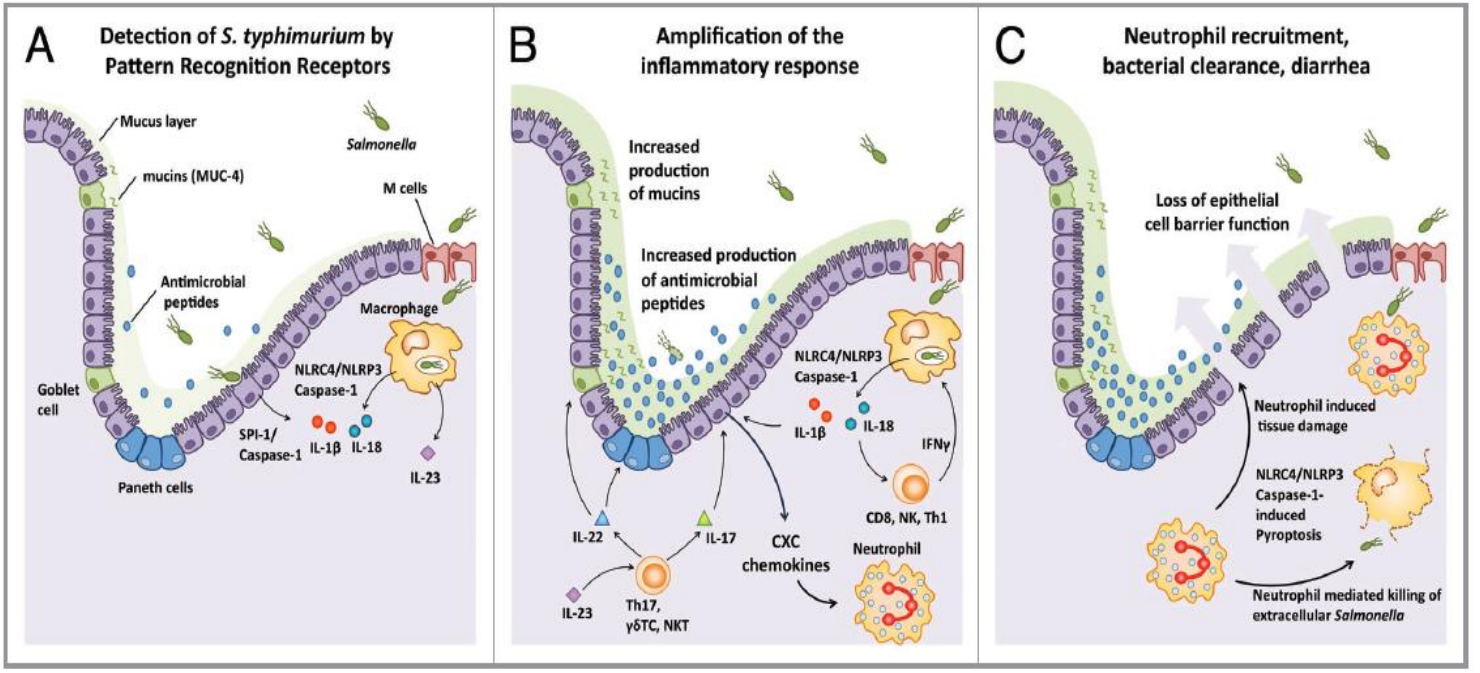


Figure 5. The gastric innate immune response to *Salmonella*. A) Recognition of *Salmonella* by TLRs and NLRs induce expression of proinflammatory cytokines such us IL-23, IL-1β and IL-18 through inflammasome activation and Caspase 1. B) Inflammatory response by paracrine signaling is induce by IL-18 and IL-23, increasing production of mucins, antimicrobial peptides and CXC chemokines, leading to an influx of neutrophils into the mucosa. C) Infiltrating neutrophils is necessary for killing of extracellular *Salmonella*, but can also lead to loss epithelial cell and promoting diarrhea [1].

Mice lacking Caspase-1 are much more sensitive to *Salmonella* infection and have higher bacterial burdens in systemic organs compared with wild-type mice. This is due at least in part to the production of *IL-1β* and *IL-18*, since mice lacking either cytokine are also more susceptible to *Salmonella* infection than wild-type mice [52]. In pigs, whose immunophysiology is closer to humans than mice, *S.* Typhimurium infections rapidly activates *NLRC4*, a cytoplasmic flagellin sensor, which initiates inflammasome assembly and Caspase-1 activation [53]. In addition, activated Caspase-1 has been revealed as a very important mediator of the porcine response to Salmonella [54].

The activation of innate immune responses in the mucosa results in the induction of an inflammatory response, leading to MAPK-signaling and nuclear translocation of transcription factors, such as *NF-κB* and activator protein-1 (*AP-1*), resulting in production of proinflammatory mediators. Thus, *Salmonella* activation of PRRs leads to the expression and secretion of key cytokines such as *IL-18* and *IL-23*, which amplify the inflammatory response by paracrine signaling mechanisms, inducing the massive secretion of *IFNγ*, *IL-22* and *IL-17* by mucosa-resident T cells. In particular, *IL-18*, which is released in an inflammasome dependent manner, is critical to induce T cells to secreted *IFNγ* release in vivo [55]. Consistently, Caspase-1-deficient mice are deficient for gamma interferon expression in the murine cecum early (12 h) after *S.* Typhimurium infection [56].

Another major inflammatory pathway in the mucosa is controlled by the release of *IL-23*. Such an early amplification of host inflammatory responses could involve the stimulation of antigen-experienced T cells by *IL-23*, inducing the release of *IL-17* and *IL-22*. In addition to T cells, Innate Lymphoid Cells (ILCs), which include natural killer cells (NK cells) and lymphoid tissue-inducer cells (LTi cells), could be potential sources of *IL-22* [57, 58]. In particular CD4+ LTi cells play a critical role in the development of lymphoid tissues and in inflammatory responses. This

response was dependent on *IL-23* and was induced in response to Lymphoid toxin (LT), a member of the Tumor Necrosis factor (TNF) core family that is expressed mainly by lymphocytes, including T, B, NK and LTi cells [59] However, a role of LTi cells in *S*. Typhimurium infections remains to be demonstrated. Distinct T cell populations express the IL-23 receptor on their surface, including Th17 cells, γδT cells and NK T cells. The combination of these cytokines induces a strong inflammatory environment in the intestine, which is characterized by an increased production of anti-microbial peptides (IL-22/23 axis) and a recruitment of neutrophils by the IL-17/23 axis, leading to diarrhea and the deprivation of nutrients, which affects commensals as well as the pathogen [60-62].

The activation of innate immune responses in the mucosa and the resulting inflammation serves as an activator of further innate immune responses. One of these is the recruitment of neutrophils to the mucosa, which is mostly mediated via the IL-23/ IL-17 axis. The production of IL-17 stimulates the secretion of CXC chemokines by intestinal epithelial cells and granulopoiesis in the bone marrow by inducing the production of G-CSF (granulocyte colony-stimulating factor) [63]. *IL-1β* production could also contribute to neutrophil recruitment, since Caspase-1- deficient mice exhibit reduced levels of CXC chemokines in the cecal mucosa early after *S*. Typhimurium infection [56].The recruitment of neutrophils is crucial to prevent the dissemination of *Salmonella* from the gut, since neutropenia increases the risk of systemic infections [64]. Although *S.* Typhimurium is mainly considered an intracellular pathogen, the role of neutrophils in containing the infection is likely to involve ingestion and killing of extracellular bacteria. *Salmonella* is susceptible to neutrophil mediated killing when it exits epithelial cells and transits to phagocytes or when it spreads to new host cells [65].

Consistent with this idea, it has been shown recently that Salmonellae constitutively expressing flagellin are released from host cells via *NLRC4*/Caspase-

1-induced pyroptosis and are subsequently removed by neutrophils [51]. In addition, *Salmonella* replicate extracellularly after depletion of neutrophils. Although neutrophils are important for host defense against Salmonella, infiltrating neutrophils are also a major cause of tissue damage in the mucosa, which is sometimes associated with necrosis in large areas of the terminal ileum and colon [66, 67]. This tissue damage leads to a loss of epithelial barrier function, resulting in an increase in inflammation and contributing to diarrhea. In addition, neutrophils might contribute to diarrhea by stimulating chloride secretion from epithelial cells. Thus, although neutrophil recruitment prevents the systemic spread of the infection, it might nevertheless be a key determinant for the development of gastroenteritis. Although *S*. Typhimurium cannot resist the onslaught of neutrophils in intestinal tissue, the pathogen appears to be custom-built to bloom in the lumen of the inflamed intestine, because its numbers in this niche increase dramatically during inflammation. Considering the significant growth advantage that *Salmonella* gains from intestinal inflammation, it would not be surprising if *Salmonella* would actively induce host innate immune pathways to cause inflammation [68].

2.4.2 *Salmonella* infection in mesenteric lymph nodes.

Mesenteric lymph nodes (MLN) are the largest lymph nodes in humans and other animals and play an important role in immune defense against bacterial pathogens as one of the main components of GALT [21]. After *Salmonella* have gained entry into intestinal lymphoid tissues, it is believed that they travel through afferent lymphatics to the draining MLNs, and subsequently disseminate through efferent lymphatics to the blood and systemic tissues. The evidence for this step-wise migration is largely based on the understanding of the flow of lymph and the circumstantial finding that bacteria are initially detected in PPs,

followed by the MLN [69]. The trafficking of *Salmonella* from PPs to MLNs is most likely accomplished by migration of infected DCs, macrophages and neutrophils. To prevent systemic infection, MLN form a life-saving firewall that protects the host from rapid pathogen dissemination beyond the intestine. By contrast, S. Typhimurium can persist for a long time in the MNL and is capable of establishing a persistent infection in the host [70]. Persistently infected carriers serve as the reservoir for the pathogen, excrete large numbers of the bacteria in their feces and transmit the pathogen by contaminating water or food sources. The carrier state has also been described in livestock animals and is responsible for food-borne epidemics. In pigs, *Salmonella* Typhimurium is able to express some of its major virulence effectors in MLN. In spite of that, a combination of early innate and adaptive immunity mechanisms overcome virulence strategies employed by the pathogen, enabling the host to protect itself against bacterial spread beyond gut-associated lymph-nodes [54, 71]. Persistence of *Salmonella* Typhimuriun in the porcine MLN from 2 h [34] up to 6 weeks after oral inoculation [72] has been reported and sustain this organ as immune inductive site during pig salmonellosis.

2.4.3 Adaptive immune response to *Salmonella* infection.

As a facultative intracellular pathogen, adaptive immunity to *Salmonella* requires both T-cells and B cells responses. Some studios have shown that early *Salmonella* specific CD4 T cell activation occurs in PP and it is started around 3 to 6 h of infection. *Salmonella* specific T cells appear also activated in the MLN, but this response usually occurs after that in PP. Interestingly, *Salmonella* specific T cell are not found on any other secondary lymphoid tissue suggesting that, at early time, the adaptive immune response to *Salmonella* takes place exclusively within the gut-associate lymphoid tissues [73]. The cells of the innate immune

system, however, play a crucial part in the initiation of the adaptive immune response. Bacteria encounter phagocytes during penetration of the mucosal epithelium [1]. *Salmonella* specific Ig produced by B cells can opsonize extracellular bacteria for phagocytic uptake. *Salmonella* then spread systemically within phagocytes to colonize target organs [52]. Inside these professional antigen presenting cells (APCs), major histocompatibility complexes (MHCs) serve as receptors surveying the host cellular cytosol and vacuolar compartments for the presence of foreign peptides. When bound by MHC, these peptides are presented on the APC surface for recognition by T-cell antigen receptors (TCRs). The development of *Salmonella*-specific CD4 effector responses has been mainly examined in the mouse model (Mittrucker et al 2002). These studies suggest a massive expansion of specific CD4 T cells and a rapid acquisition of the Th1 effector function by secretion of interferon-γ, *IL-2* and *TNF-α*. In addition, recent data have shown that cytokines associated with Th17 cells, *IL-17* and *IL-22*, are secreted in the intestinal mucosa after *Salmonella* infection [74] suggesting an important additional contribution of the Th17 response to protection against *Salmonella*. A Th17 response might be protective by initiating or enhancing neutrophil infiltration to intestinal tissues. Also, Th17 cytokines induce production of antimicrobial peptides by epithelial cells that are effective against bacteria within the lumen [69]. In addition to T CD4 response, *Salmonella* infection also induces antigen-specific CD8 T-cell, and B-cell responses, all of which can contribute to protective immunity [75]. B cells express cytokines that appear to participate in the differentiation and function of effector CD4+ T cells [76]. Activated CD4+ T cells in turn provide critical stimulation for B cells to undergo expansion and Ig class switching, as well as for activation and growth of cytotoxic CD8+ T cells and phagocytic cells such as macrophages.

Although CD4+ T cells are known for their critical function during the infection with *Salmonella*, the role of CD8+ T cell responses have been controversial. Nevertheless, the involvement of non-polymorphic MHC-I during the response against *Salmonella* has recently gained attention due to their role as presentation molecules for *Salmonella* antigens [77]. It is generally accepted that peptides present in cytoplasmic compartments are presented on MHC class II molecules. However, antigens from intracellular pathogens including *Escherichia coli*, *Salmonella* Typhimurium, *Brucella abortus* and *Leishmania* localized in endophagocytic compartments of APCs can be efficiently presented by MHC-I molecules [78] to elicit an MHC class-I-dependent CD8+ T-cell response. This process is known as cross-presentation or cross-priming and requires peptide translocation from the phagosomes to the cytosol for their proteosomal processing and subsequent ER translocation [79].

In pigs, it has been observed [80, 81] that *Salmonella* Typhimurium infection do not produce an up-regulation of cytokines involved in T helper 1 (Th1) response, on the contrary to previous reports in mice. These findings could be related to the ability of pathogens to limit antigen presentation to CD4 restricted T cells by reducing MHC-II levels in infected cells. Additionally, *Salmonella* Typhimurium antigens appears to be cross-presented via MHC-I in a proteasome-dependent manner and this mechanism probably triggers an early cytotoxic response against bacteria [71].

Despite innate and adaptive immunity are rapidly initiated after *Salmonella* infection, these effector responses can be hindered by bacterial evasion strategies. Thus, *Salmonella* are capable of destroying both macrophages and DCs by pyroptosis [82-84], which abrogates their important APC function and thereby antagonizes initiation of the adaptive immune response. In addition, *Salmonella* can impair antigen processing and presentation steps at multiple levels to

prevent activation of T cell responses. Nevertheless, the mechanisms by which Salmonella evades primary immune responses to generate lethal infections in some animal host, including human remain are still poorly understood [85].

2. MicroRNAs.

2.1. History of miRNAs.

In 1993 Lee et al. [86] discovered that the gene lin-4 is transcribed into a 22 nt long RNA that inhibits lin-14 by RNA interference in the nematode *Caenorhabditis elegans*. The author concluded that lin-4 may belong to a class of small, regulatory non-translate RNA, albeit for years there was no evidence for the lin-4 line RNAs in *C. elegans*, or other organisms. The identification of a second miRNA, let-7 in *C. elegans, Drosophila* and humans was identified in 2000 [87], along with the discovery of additional 22 nt RNAs in 2001 [88-90], prompted their characterization as microRNA. In 2006, miRBase was established to provide a database of miRNA sequences and organize nomenclature [91]. Computational approaches predicted the existence of approximately 1000 miRNAs in the human genome in 2007 [92]. To date 1881 human miRNA sequences have been registered on mirBase release 21 [93], these follow nomenclature rules outlined in Table 1.

Table 1. Mature miRNA terminology, used by miRBase. There are 5 sections to the nomenclature [89].

Example miRNA = has-miR-146a-5p	
hsa	Tells us it is human, homo sapian
miR	Capitalisation of the "R" infers that the miRNA is the mature sequence, opposed to "r" which refers to the miRNA precursor, the genomic locus, the primary transcript, or the extended hairpin that includes the precursor.
29	Named sequentially, this miRNA belongs to the 29 family. The same number is used for miRNA of different species that are orthologous, but conveys no information about functionality.
b	Paralogous miRNAs that differ in 1 or 2 nt.
-5p/-3p	the same mature miR originate from opposite arms of the same pre-miRNA

2.2. MiRNA Biogenesis.

MiRNA biogenesis begins with the transcription of primary miRNA transcripts, which are encode either by miRNA genes or in the introns of mRNA genes. Figure 6 illustrates the canonical biogenesis and processing pathway. It represents the standard maturation pathway for most miRNAs, although literature reports numerous exceptions along that way. For example, miRNA editing, differential trimming (isomer generation) or an alternative branch that escapes Dicer processing in the cytoplasm can take place for certain miRNA [94, 95].

Transcription of pri-miRNAs results in single or, in the case of miRNA clusters, polycistronic miRNA [95]. Following transcription, the so-called *microprocessor* complex, consisting of endonuclease Drosha and RNA binding protein Phasa (DGCR8 or partner of Drosha), cleaves the pri-miRNA into an approximately 60 nt long stem loop structure temed pre-miRNA. After its export into the cytoplasm by the Exportin 5 Ran GTP complex, further cleavage through interaction with endonuclease Dicer together with double stranded RNA binding protein TRBP finally results in an approximately 22 nt short, double stranded RNA made up of two mature miRNAs. The generated duplex is then loaded onto an Argonaute

(AGO) protein, where on mature miRNA strand is selected to act as the guide strand. This strand subsequently directs AGO as the center of the miRISC (microRNA induced silencing complex) to its complementary mRNA target site, teaming up to exert target regulation. The second miRNA, also termed passenger strand, is released and degraded [94].

Ultimately, miRNA binding results in changing the expression of the target, mediated by the interaction of AGO with various other proteins such as GW182 during translation [96]. Down regulation of gene expression seems to be the predominant case, although reports do exist about stimulation of gene expression induced by miRNAs [97-99]. There is still an ongoing discussion about how down regulation of expression is actually achieved, i.e. if translational repression precedes mRNA degradation or not [100]. Several recent publications, however, support the notion that translational repression is the initial event, followed by mRNA degradation [101-103].

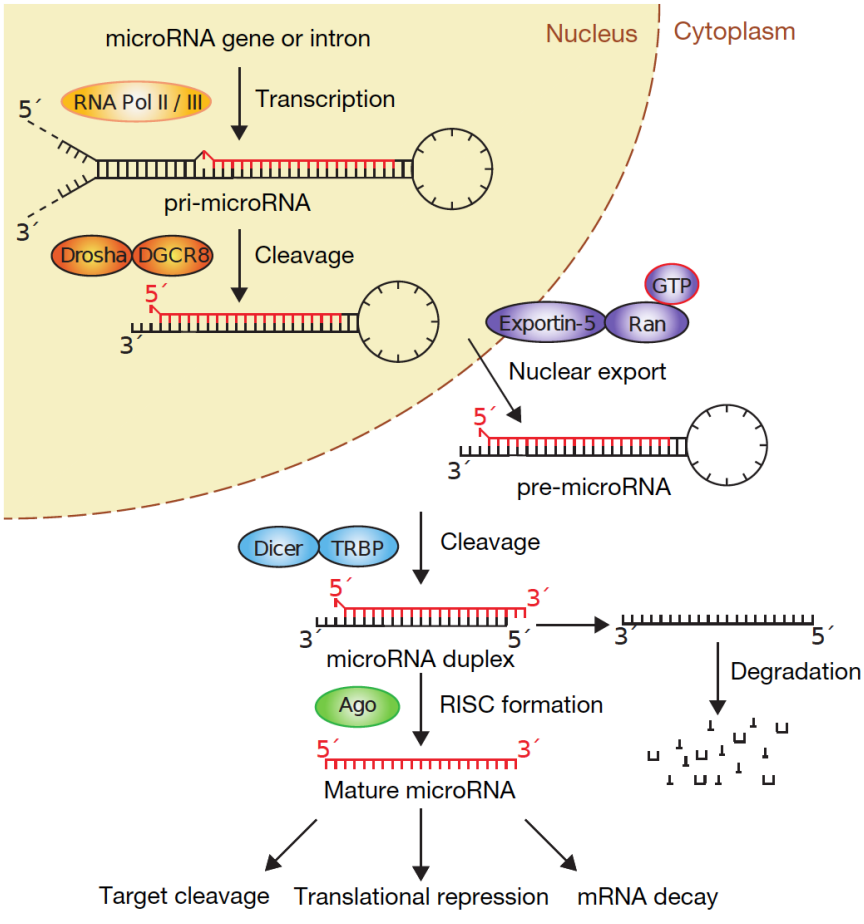


Figure 6. Canonical view on animal miRNA biogenesis and processing [94].

2.3. Gene regulation by miRNAs.

In order to exert their regulatory role, miRNA need to be bound by AGO proteins [96]. There are four distinct AGO proteins in human that participate in miRNA mediated regulation of gene expression, with AGO2 being the most abundant one [104]. In addition, AGO2 is unique among the others for this slicing activity, enabling cleavage of highly complementary miRNA targets site. The extent of cleavage in animals in unknown but expected to be rather uncommon, since

there usually is only limited miRNA target complementary [95, 105]. Other than this difference, all four AGO proteins are reported to bind highly similar sets of transcripts, indicating substantial functional redundancy [106, 107]. Moreover, miRNA sorting to individual Argonautes seems to be mostly random, although there might be sorting mechanism for at least some miRNAs [104, 108, 109].

After joining forces, miRNA guide their associated Ago proteins to complementary mRNA target sites. One particularly important factor that stabilizes miRNA target interactions involves strong base pairing between the target and the 5´ end (position 2 to 7) of the miRNA, generally referred to as the seed region of the miRNA.

Such miRNA seed also play a pivotal role in animal computational target prediction, since extensive base pairing between the seed and the target alone is often functionally sufficient. Up to recently, miRNA target sites were thought to be located primarily in the 3´ untranslated region (UTR) of the target mRNAs [105]. Lately however, there have been various reports about biologically functional target sites in the coding sequence (CDS), with smaller but still measurable effects on gene expression [107, 110, 111]. Apart from sequence complementary and target site location, there are many more target site features that influence the affinity of the site towards miRNA binding. Since these features are also considered in computational target prediction.

2.4. MiRNA targeting.

In animals, miRNA target interactions normally feature a limited amount of sequence complementarity between the miRNA and its target. The miRNA seed region constitutes an exception in this case, since strong base pairing between the target and the seed is the most prominent feature of animal miRNA target

interactions [105]. As expected, the seed region happens to be the evolutionary most conserved sequence part of animal miRNAs. Moreover, miRNA seed motifs (seed complements) are significantly enriched in regulated target sequences [112, 113]. According to miRNA target databases, one miRNA may regulate many genes as its targets, while one gene may be targeted by many miRNAs [114], however, all these methods of prediction assume a general pattern underlying these interactions and therefore tolerate reduced prediction accuracy and a significant number of false predictions [115]. The functional significance of each miRNA is mostly unknown due to the difficulty in identifying experimentally target genes and the lack of genome-wide expression data combining miRNAs, mRNAs and proteins, leading to that so far vast majority of miRNA targets are unknown [116]. We introduce a integrative analysis, that integrates the miRNA identification, target prediction, gene and protein expression data and functional analysis for identifying the direct and indirect relationship between miRNA and their target genes. Searching for seed motifs in mRNA sequences marks the first step taken by almost all popular miRNA prediction programs in order to find interaction sites, thus constituting the main prediction feature in animal miRNA target prediction [117].

2.4.1. Thermodynamic stability.

Thermodynamic stability of the miRNA-mRNA duplex (MRE-miRNA recognition elements) is commonly utilized by prediction programs [110, 118, 119], to extend the sequence complementarity measure. This stability is expressed in the minimum free energy of the duplex secondary structure and can be calculated by RNAhybrid software [120]. That way, the interaction is evaluated by considering the stability of the duplex, using the calculated energy as a quality measure for the interaction. By setting a reasonable energy cutoff, hybrids with less stable

secondary structure are removed from the initial list of predicted interactions. Although thermodynamic stability is utilized in many predictions programs for ranking as well as removing false positive candidates, the fact that animal miRNA target interactions rely on hybrids with partial complementarity also implicates that interactions do not need a particularly stable secondary structure in order to be functional. Indeed, it has been shown that ranking perfect 7mer seed containing sites by their hybridization energy does not perform significantly better than random ranking. Besides, the impact of target secondary structure on hybrid formation is not considered. This impact, which can be approximated by measuring target site accessibility [121].

2.5. MiRNAs and disease.

MiRNA are important regulatory molecules in many biological processes including metabolism, cell proliferation, apoptosis, developmental timing, and neuronal cell fate [122]. Numerous studies over the past decade have been reported the assessment of miRNA expression and have shown considerable changes in their expression profiles affects the regulation of many cellular functions and gene networks in various disease. The most remarkable changes were observed in all types of cancer [123, 124], viral infections, hematological system, nervous system disorders, cardiovascular disorders, muscular disorders, diabetes, and other diseases [125, 126]. MiRNAs deficiencies or excesses have been linked to a number of other clinically important diseases and several studies have been specifically designed to validate miRNAs as biomarkers and clarify their role in regulating physiological and pathological processes [127, 128].

The second version of human miRNA disease database (HMDD) contain 10,368 entries for 572 miRNAs and 378 diseases, with 3511 manuscripts. Focusing on circulating miRNA markers from this data base, founding 556 interactions

between miRNAs and diseases. Among the least specific miRNAs are miR-20a, miR-122, miR-155, miR-17, miR-21 and miR-126 (Figure 7) [129, 130].

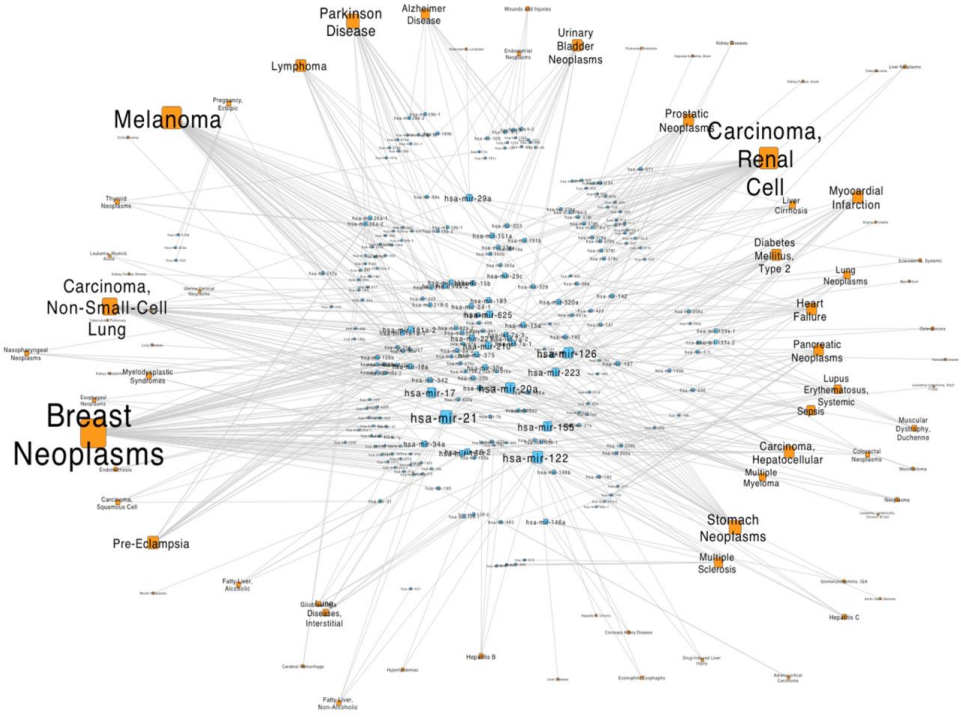


Figure 7. Human miRNA Disease Database (HMDD) disease network. All circulating markers were downloaded from the HMDD [130].

2.6. The role of miRNA in infection.

The response of host cells to microbial infection or immune activation is among the most well studied examples of cellular responses to external stimuli. This response is characterized by marked changes in gene expression [131-134], which require precise coordination to establish appropriated immunological outcomes, ensuring maximal protection against infection while avoiding tissue damage. The crucial role of miRNAs regulate the development and function of immune cells

and can have pro-inflammatory or anti-inflammatory effects [135, 136]. Furthermore, experimental data indicate that microbial infection alters the miRNA repertoire of host cells [137] and that, when aberrantly expressed, miRNAs can contribute to immunity related pathological conditions, such as infectious or inflammatory diseases, autoimmunity or cancer [138]. The host´s miRNAs are important for coordinating responses against infectious agents, some pathogens also benefit from miRNA encoded by the host or their own genomes [135].

The most known miRNAs in bacterial infection are miR-155 and miR-146. These miRNAs are induced by the NF-κB pathway through patter recognition receptors (PPR) sensing of pathogen motif, in particular LPS. Expression of miR-146 is induced by sub-inflammatory levels of *NF-κB* activity. It acts as an anti-inflammatory regulator, by targeting *TRAF6* (TNF Receptor associated factor 6) and *IRAK1* (IL-1R associated kinase 1), which are involved in the *NF-κB* pathway, thus promoting tolerance to low doses of LPS. This desensitized state is necessary to protect the organism against septic shock [137, 139-142]. In contrast, miR-155 is induced by higher doses of LPS, at levels which results in pro-inflammatory *NF-κB* activity, as well as by *TNF-α* and interferon *β*, via *TAB2*. miR-155 is known to amplify the expression of pro-inflammatory factors, thereby acting in defense against pathogens, and also to exert negative feedback on the immune system, thus protecting the host from potentially damaging overreaction [140, 143, 144].

The involvement of this miRNA in the pro-inflammatory response has been thoroughly investigated, among others, miR-155 targets SHIP1, a negative regulator of the *NF-κB* pathway [145], and *SOCS1* (suppressor of cytokine signaling 1), an effector involved in the homeostasis of Treg cells [146]. This is turn, stimulates the expression of the pro-inflammatory cytokines *TNF-α, IL-6, IL-1β, IL-8 and IL-12,* while it reduces the expression of the anti-inflammatory

cytokine *IL-10* [147, 148]. In line with these data, miR-155 is essential for an efficient immune response to several bacterial pathogens. However, this miRNA has also been shown to repress genes such as *NIK*, *IKK*ε and *TAB2*, which encode proteins involved in the inflammatory pathway, accordingly, it is proposed that miR-155 could acts as a limiter of inflammation [140, 144, 149]. Interestingly, miR-155 expression is also stimulated by the intracellular *NOD2* receptor. In tolerant macrophages, where miR-146 prevents inflammation, stimulation of NOD2 can restore the *NF-*κ*B* pathway activation, as well as the feedback control by miR-155 [140].

Over the past 10 years, mechanisms and effects of miRNAs mediated regulation on gene expression in response against pathogen infection have been broadly investigated. Many miRNAs have been reported to be involved in bacterial infection, given that their expression and functionality depends of the tissue and cell typed examined. Upon pathogen exposure, miRNA variability occurs at different levels. The abundance of miRNAs is modified to face infection, and miRNAs are up or down regulated early or late in response to pathogens, leading to the expression of a core temporal response common to all pathogens, as well as a specific response [138, 150]. There are many other miRNAs reported in response to bacterial infection, but here we will emphasize the miRNAs related to infection with *Salmonella* Typhimurium.

2.7. MiRNA expression by *Salmonella* infection.

MiRNA expression has been studied in different biological models, since cell lines, until different tissues or fluids from animals (Table 2) [151]. Modulation of the host miRNA repertoire by *Salmonella* infection was first described in mouse macrophages, where the *NF-*κ*B* dependent miRNAs miR-155, miR-146a/b and miR-21, were shown to be strongly induced upon infection [152]. Up regulation

of these miRNAs was also observed in human monocytes [153]. Members of the Let-7 family were shown to be down regulated upon *Salmonella* infection both in macrophages and epithelial cells [152], suggesting that repression of this miRNA family constitutes a common signature of the infection of phagocytic and non-phagocytic cells by Salmonella. The Let-7 family targets two major immunomodulatory cytokines: the pro-inflammatory cytokine *IL-6* and the anti-inflammatory cytokine *IL-10* [152, 154]. Thus, *Salmonella* infection, through the inhibition of Let-7, induces the expression of both cytokines resulting in a balanced inflammatory response.

Table 2. MicroRNAs altered by *Salmonella* Typhimurium

	miRNAs	Cell/Tissue examined	References
Up	miR-155, miR-146a/b, miR-21	Raw264.7, macrophages, HeLa epithelial cells	Schute et al. 2011
Down	Let-7 family		
Up	miR-1308	Raw264.7, macrophages, HeLa epithelial cells	Schute et al. 2011
Down	Let-7 family		
Up	miR-146a/b	THP-1 (monocyte cells)	Sharbati et al. 2012
Up	miR-29	Piglet ileal samples	Hoeke et al. 2013
Up	miR-128, miR-135, miR-21, miR-29a/b, miR-146a/b	Zebrafish embryos	Ordas et al. 2013
Down	Let-7b, miR-30e, miR-92b, miR-129, miR-153, miR-181a, miR-430a		
Down	miR-15 family	Hela cells	Maudet et al. 2014
Up	miR-128	Mouse intestinaltissue	Zhang et al. 2014
Up	miR-30c/d	HCT-8, Hela, J774A.2	Verma et al. 2015

	miRNAs	Cell/Tissue examined	References
Up	Let-7 family, miR-769, miR-30 family, miR-339, miR-451, miR-17, miR-4334, miR-16, miR-331, miR-532, miR-363, miR-221, miR-98, miR-20a, miR-27a/b, miR-29c, miR-374b, miR-582, miR-15a, miR-18a, miR-101, miR-142, miR-340, miR-144, miR-133a, miR-21, miR-127	Whole blood of piglets	Bao et al. 2015
Down	miR-214, miR-99b, miR-125a, miR-100, miR-146a, miR-664, miR-215, miR-20b, miR-99a, miR-155, miR-191, miR-24		
Up	miR-26, miR-9839	Whole blood of piglets	Yao et al. 2016
Down	miR-143, miR-4335, miR-125a/b, miR-221, miR-27b		

Salmonella can modulate the miRNA production, manipulating their host cell physiology and defences, with favourable consequences for pathogen survival. For example, down regulation of miR-15 family by Salmonella in Hela cells is used to allow cell cycle progression and intracellular bacterial replication, through repression of Cyclin D1 (*CCND1*), a protein required for G1/S transition [155]. Another example of this is the reduction of host SUMOylation by *Salmonella*, via the stimulation of two miR-30 family miRNAs, and subsequent down regulation of their target Ubc9, the only cellular E2 SUMO-conjugating enzyme [156].

The effect of *Salmonella* infection on miRNA expression has also been studied in vivo, in piglets and zebrafish embryos. In the ileum of infected piglets, Salmonella induces miR-29 expression as early as 3 h post-infection [157]. MiR-29 was shown to target Caveolin-2 (*CAV2*), an inhibitor of the small Rho GTPase *CDC42*, which plays an essential role in Salmonella invasion of epithelial cells [158]. On the other hand, whole blood of piglets infected with Salmonella has shown up and down regulation of several miRNAs of which two miRNAs have been characterized [159]. These two miRNAs, miR-124 and miR-331, target genes *SLC11A1* and PIGE-108A11.3 and *VAV2*, respectively, and products of these genes are associated with the regulation of immune responses. Moreover, Salmonella infection up regulates the expression of miR-128 in mouse intestinal tissue. It targets macrophage colony stimulating factor (M-CSF) to down regulate its expression, which in turn leads to decrease recruitment of macrophages to clear Salmonella infection [160]. Infection of zebrafish embryos with Salmonella induces expression of miR-146a/b, miR-21 and miR-29a [161]. In zebrafish embryos, miR-146a/b down regulation by Salmonella infection had no major effect on canonical pro-inflammatory genes, except for the up regulation of apolipoprotein genes that have been previously linked to immunoregulation and host defense [162].

Recently, there have been reported new miRNAs in *Salmonella* infection, from whole blood of piglets showed the down regulation of miR-143 (*ATP6V1A* and *IL13RA1*), miR-221 (FOS), miR-125b (*MAPK14*) and miR-27b (*INFG*), and up regulation of miR-26 (*BINP3L* and *ARL6IP6*) affected the expression of their peripheral blood specific gene targets, indicating that dysregulation of these miRNAs might contribute to the increase the risk of *Salmonella* infection and establishment of a carrier status and proposed the whole blood sample as good and low cost means for measuring miRNA expression patterns in *Salmonella* infection [163, 164].

OBJECTIVES

The present study aims to determine host miRNA expression changes and to establish interactions with their targets genes/proteins in ileum and mesenteric lymph node in pigs infected with *S*. Typhimurium. To achieve this, the following specifics objectives establish:

To characterize the host transcriptional response in pig intestinal tract after *S*. Typhimurium infection.

To determine miRNA expression profile in ileum and mesenteric lymph node from pigs infected with *S*. Typhimurium using high throughput sequencing.

To determine changes in expression of miRNAs in ileum and mesenteric lymph node during infection with *S*. Typhimurium.

Finally, this study aims to dissect the global functions of miRNAs in the host responses to *S*. Typhimurium, contributing to a better understanding of the roles of miRNAs in immunity against microbial infection, given that the vast majority of miRNA targets are still unknown.

MATERIALS AND METHODS

1. Experimental infection and sample processing.

Sixteen male and female crossbreed weaned piglets, approximately 4 weeks of age, were used in this study. All piglets were derived from a *Salmonella*-negative herd and were serologically negative before the experiment. Pigs were housed in an environmentally controlled isolation facility at 25 °C and under constant light with ad libitum access to feed and water. After an acclimation period of 5 days, four piglets were necropsied (control group), 2 hours prior to experimental infection of the other animals. Then, the 12 remaining piglets were challenged orally with 10^8 colony forming units (cfu) of a *S*. Typhimurium phagetype DT104 strain isolated from a carrier pig [165]. Fever, lethargy and diarrhea were monitored every day. Four randomly chosen infected pigs were necropsied at three different time points: 1, 2 and 6 days post infection (dpi). Fecal samples were collected for bacteriological cultures the day of arrival and the day of sacrifice. Fecal sample processing and bacteriological analysis were performed following the current EN-ISO standard methodology 6579:2002/Amd 1:2007. Before euthanasia, blood was collected from each animal and placed into non-anticoagulated tubes, letting it clot and centrifuging to obtain sera. Obtained samples were sent for analysis to an external laboratory (Laboratorio Veterinario Garfia S.L., Cordoba, Spain), from where the following measurements were obtained: total proteins, albumin, blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase, total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, triglycerides, glucose, immunoglobulins (G, A and M), complement component C3, and Salmonella spp. antibodies.

Additional information regarding detection methods is included in Additional file 1. Descriptive statistics and normality tests were followed by ANOVA using Dunnet's post-test. Statistical significance was set at $P<0.05$

Segments from jejunum, ileum, colon and MLN were aseptically collected right after euthanasia, sectioned into pieces of around 10 cm and immediately frozen in liquid nitrogen for future mucosa and MLN isolation and RNA purification. All procedures involving animals were previously approved by the institutional bioethical committee, and performed according to European regulations regarding animal welfare and protection of animals used for experimental and other scientific purposes.

2. *Salmonella* detection in tissues.

Immunohistochemical analysis of intestinal tissue samples of jejunum, ileum and colon at 0, 1, 2 and 6 dpi was performed as previously described by our group, using a S. Typhimurium specific antibody [53].

3. RNA isolation.

For mucosa and MLN isolation and RNA purification, intestinal tissue samples stored at −80 °C were treated with RNAlater®-ICE (Ambion Inc, Austin, TX, USA) and cut into 2 cm pieces, according to manufacturer's instructions. Intestinal mucosa was scraped from the intestinal luminal surface with a razor, and it was immediately disrupted and homogenized in lysis buffer (Rneasy Mini Kit, QIAGEN, Valencia, CA, USA) using a rotor–stator homogenizer. Similarly, MLN samples were homogenized in lysis buffer using a rotor–stator homogenizer. RNA extraction was done using the Rneasy Mini Kit following manufacturer instructions. For miRNA studies, RNA from ileum (0 and 2 dpi) was isolated using

mirVana miRNA isolation kit (Ambion Inc, Austin, TX, USA). Eluted RNA was treated with DNase using TURBO DNA-free™ Kit (Ambion Inc, Austin, TX, USA) to eliminate traces of DNA. RNA integrity was assessed in the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA), and only samples with RNA integrity numbers (RIN) ≥7 were used for further analysis.

4. Microarray hybridization and analysis.

Gene expression analysis was carried out using the GeneChip Porcine Genome Array (Affymetrix, Inc., Santa Clara, CA, USA) at the Unidad Científico-Técnica de Apoyo (UCTS) of the Institut de Recerca del Hospital Universitario Vall d'Hebron (Barcelona, Spain). The One-Cycle Eukaryotic Target Labeling Assay (Expression Analysis Technical Manual, Affymetrix, Inc., Santa Clara, CA, USA) was used to obtain biotinylated cRNA from individual mucosal mRNA samples. Then, they were hybridized to the GeneChip Porcine Genome Array and processed using manufacturer's instructions. Data analysis was conducted using in-house algorithms in R (v. 2.7.0). Quality control analysis of the mRNA array was performed using the robust multi-array analysis (RMA) [166] included in the *affy* library of Bioconductor package [167]. Differentially expressed (DE) genes were obtained by paired comparisons using the limma package (moderated t test of linear models after an empirical Bayes correction [168]); three independent comparisons were carried out for each combination of control vs. infected samples (i.e., 0 dpi vs 1 dpi, 0 dpi vs 2 dpi and 0 dpi vs 6 dpi). Only genes with a fold-change (FC) >1.5 or <1.5 were considered for further investigation. Due to the lack of complete annotation of the GeneChip Porcine Genome Array, the entire data set was re-annotated using Blast2GO [169].

RNA samples from ileum for miRNA analysis were initially hybridized to the human miRNA Microarray (V3) 8 × 15 K (Agilent Technologies, Inc., Santa Clara,

CA, USA), and processed at the Andalusian Center for Molecular Biology and Regenerative Medicine (CABIMER) Genomics Core Facility. Background correction and quantile normalization were performed [170-172]. Differential expression of miRNAs was calculated using the RankProd method [173], which is a non-parametric method based on the estimated percentage of false predictions (PFP). *P*-values were adjusted for multiple testing using the Benjamini and Hochberg method for false discovery rate [174], and adjusted $P < 0.05$ were considered to be statistically significant. As in the mRNA array, miRNA threshold was set at a FC=1.5.

5. Small library preparation and Next Generation Sequencing.

Four samples of ileum and four samples of MLN were used for next generation sequencing (2 controls and 2 S. Typhimurium infected per tissue). For each sample, 500 ng of total RNA were used for library preparation, using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina (Set 1) (Figure 8). Libraries were quantified (Qubit dsDNA HS assay) and quality was assessed (Agilent Bioanalyzer 2100, Agilent Technologies, Palo Alto, CA, USA). Then, single-end next generation small RNA sequencing of (50 nucleotides-long reads) was performed using HiSeq2000 sequencing platform (Illumina Inc., San Diego, CA, USA).

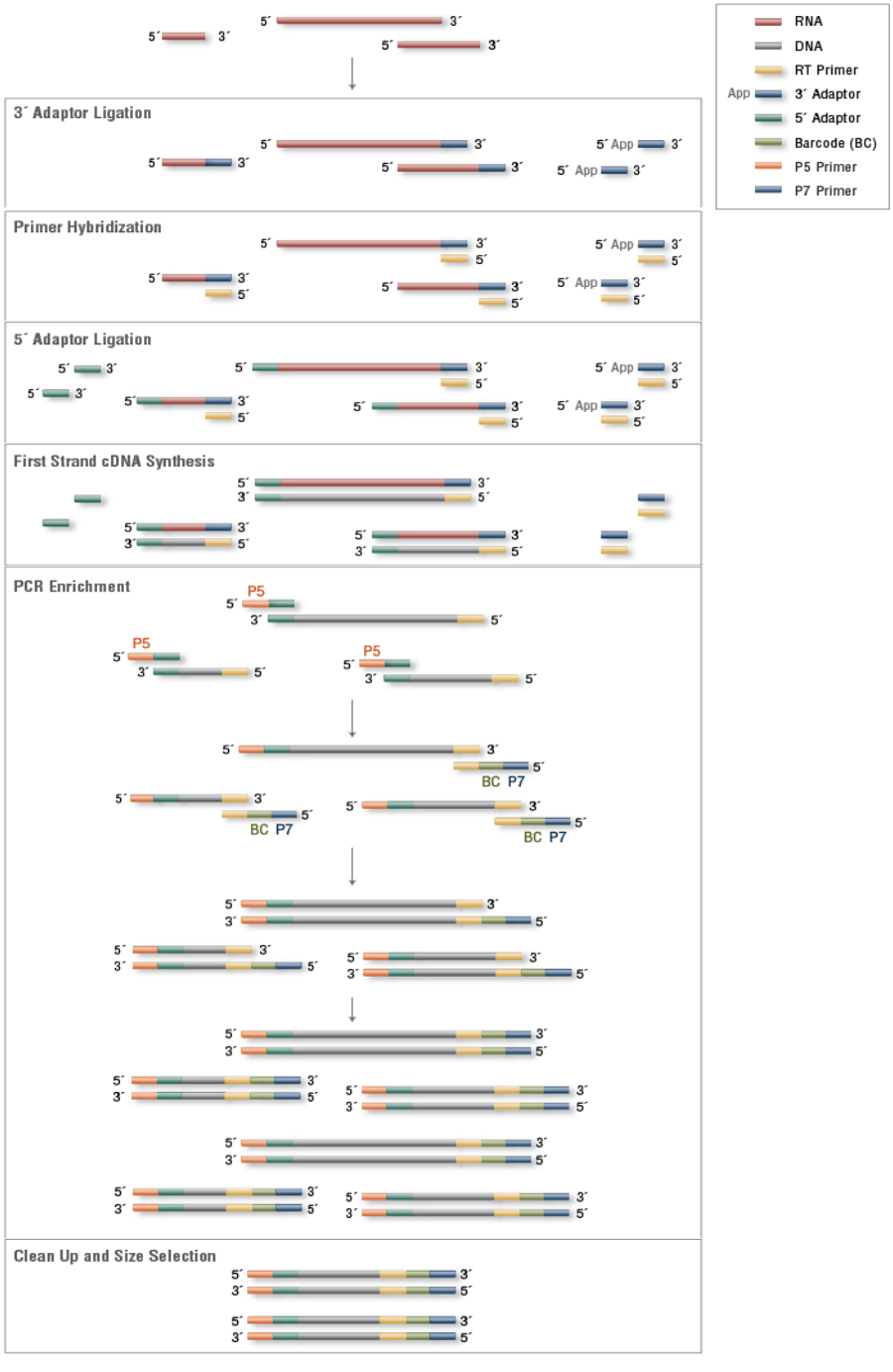


Figure 8. NEBNext® Small RNA workflow (www.neb.com).

6. Sequencing data analysis.

Sequencing data analysis was performed at the Andalusian Platform of Bioinformatics of the University of Malaga (PAB-UM). Raw reads were pre-processed using SeqTrimNext (an in-house developed customizable pre-processing pipeline) [175]. Contaminants, sequencing adapters, short (<17 nucleotide) and bad quality reads (Phred score <20) were removed, so only high quality sequences were used for further analyses. Databases used included Rfam (version 11.0 August 2012, 2208 families; http://rfam.xfam.org/), miRBase (v20; (http://www.mirbase.org), human genome Homo sapiens (GRCh37 -hg19-, http://grch37.ensembl.org/Homo_sapiens/Info/Index) and Sus scrofa (Sscrofa10.2.75 -susScr3-, http://www.ensembl.org/Sus_scrofa/Info/Index). In the Rfam database there are all known small RNA (383,004 total sequences), including 524 miRNA families with a total of 27,475 sequences. We excluded miRNAs from the Rfam file since we used this database for the other small RNAs, so we ended up with 355,529 sequences. We will use the miRNAs from miRbase database, selecting porcine and human miRNAs. Total number of mature miRNAs are 24521, from which 2578 are human and 326 are from pigs. Given that the majority are human miRNAs and the homology is high, these were used for further analyses. We used the CAP-miRSeq pipeline (Figure 9, [176]) for the analysis of deep sequencing data (alignment, miRNA detection, quantification, and differential expression analysis between control and infected group) [176]. This pipeline includes alignment with bowtie1 [177] and miRDeep2 algorithms [178], and shown in Figure 9. Determination of differential expression between control and infected samples was performed using edgeR (Bioconductor, http:www.bioconductor.org) [179], and we considered for further investigations only the miRNAs differentially expressed with a corrected *P*-value ≤0.05.

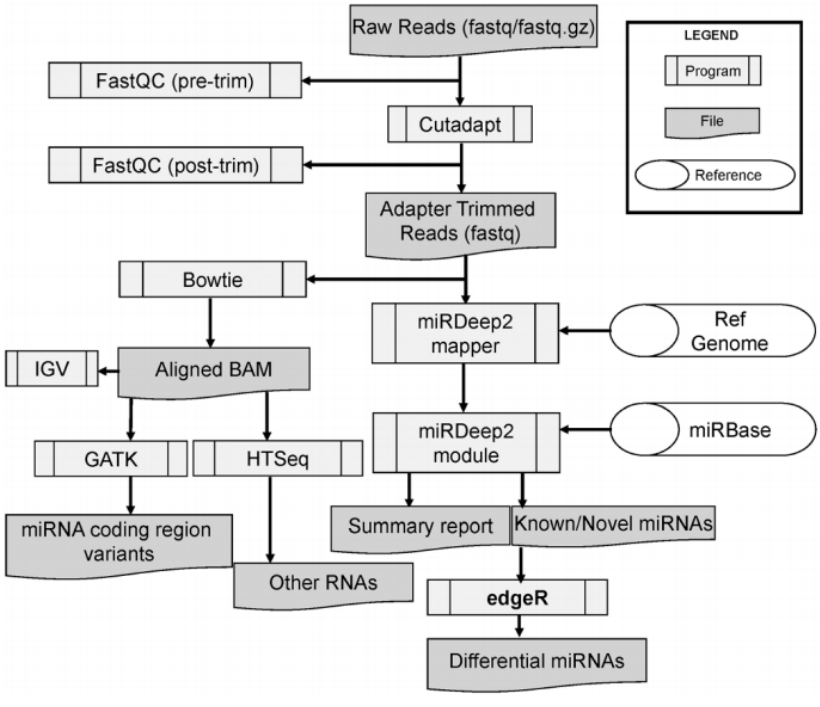


Figure 9. Workflow of miR-CAP-miRSeq [176]

7. Systems biology analysis.

Functional analysis of DE genes was carried out using Ingenuity Pathway Analysis (IPA, Ingenuity Systems® Inc, Redwood City, CA, USA). Genes differentially expressed in each intestine section/time point were uploaded

into IPA and analyzed separately. Obtained results included biological functions, canonical pathways, and networks involved, all of which were filtered by setting a threshold of $P < 0.05$. Additionally, we used this software for investigating the role of DE miRNAs and their targets in biological processes.

8. Quantitative real-time PCR (qPCR)

All expression analyses were performed following the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE)" guidelines. Selected genes and miRNA target genes differentially expressed were validated using gene-specific primers (showed in Additional file 2). Briefly, cDNA from each investigated sample was synthesized using qScript™ cDNA synthesis kit (Quanta Biosciences Inc.) from 1 microgram of RNA, following manufacturer's instructions. The final 20uL PCR reaction included 2 µL of 1:10 diluted cDNA as template, 2uL of 5x PyroTaq EvaGreen qPCR Mix Plus with ROX (Cultek Molecular Bioline, Madrid, Spain), and transcript-specific forward and reverse primers at a 10 µM final concentration. Real time PCR was carried out in a QuantStudio 12K system (Applied Biosystems) under the following conditions: 5 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 57 °C and 45 s at 72 °C. Melting curve analyses were performed at the end, in order to ensure specificity of each PCR product.

For the miRNA expression analysis, 100 ng of total RNA per animal was reverse transcribed to cDNA as previously reported [180, 181] and diluted 1:8 times. The 10 µL final PCR reaction mix contained 1 µL of cDNA, 2 µL of 5x PyroTaq EvaGreen qPCR Mix Plus with ROX (Cultek Molecular Bioline, Madrid, Spain), and 10 µM of each primer. Cycling conditions were 10 min at 95 °C followed by 40 cycles of 5 s at 95 °C, and 60 s at 60 °C; a final melting curve analysis was performed (60–99 °C). The miRNA-specific primers were designed according to guidelines set by Balcells et al. [180], and using publicly available software miRprimer [182] (Additional file 2).

Expression results were calculated using GenEx5 Pro software (MultiD- Göteborg, Sweden), based on the Cq values obtained. First a set of potential reference genes was tested for stability using NormFinder and geNorm softwares [183,

184]. Although for analysis we used miRNAs mapped to the human genome, we designed primers of their pig-specific counterpart for qPCR validation. After evaluation, five miRNAs (miR-26a, let-7a, miR-103, miR-17-5p and miR-16-5p) were selected as reference to normalize expression [185]. Relative gene expression was measured in control and infected pigs. Expression ratios were calculated according to the $2^{-\Delta\Delta Ct}$ method [186]. Statistical differences in expression values among groups were assessed using a Kruskal–Wallis test (mRNA) or Student's t test (miRNA) (Graphpad Prism 6, Graphpad Software Inc, La Jolla, CA, USA). Statistical significance was set at $P < 0.05$. Additionally, a Pearson correlation analysis was performed between small RNA-Seq and qPCR results in order to validate the sequencing results.

9. Target gene analysis.

MiRNA targets were selected using the miRNA target database miRTarbase (release 6.0) and TargetScan (release 7.0) [187, 188]. In order to increase the confidence of the findings, we selected only targets that have been validated by strong evidence (reporter assay, western blot or qPCR), and those miRNA targets with highly conserved seed regions.

9.1. Integrative analysis of miRNAs and genes DE in ileum.

We compared all miRNA targets predicted from miRNAs DE in ileum at 2dpi with differentially expressed mRNAs from our study to previously published gene expression data sets in ileum at 2dpi [189]. MiRNA targets (miRNA-up/mRNA-down-up and miRNA-down/mRNA-up) were selected and analyzed using QIAGEN´s Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City). Most significant biological functions (top 5 - *P*-value ≤0.05) were selected to analyze

miRNA interactions, and using that information we chose most relevant miRNAs and their target candidates for functional analysis.

9.2. Integrative analysis of miRNAs and proteins DE in MLN.

We compared all miRNA targets predicted from miRNAs DE in MLN at 2dpi with differentially expressed proteins from a previous study from our group, which contained protein expression data from MLN at 2dpi [71]. Based on the mechanism of action of miRNAs, we selected only miRNA targets that were inversely expressed (miRNA up-regulated, protein down-regulated and vice versa). Most significant biological functions (top 5, P-value ≤0.05) were selected to elucidate the miRNA role in MLN after S. Typhimurium infection.

10. In vitro miRNA target validation using miRNA mimics in porcine cell lines.

MiRNA mimics hsa-miR-194-5p, hsa-miR-200a-3p, and hsa-miR-223-3p (for ileum validation), hsa-miR-125b-5p (for MLN validation) and a mimic negative control were obtained from Dharmacon, (Thermo Scientific). Mirnas were transfected into porcine cell lines (epithelial cell line IPEC-J2 for ileum and macrophage 3D4/31 for MLN validation) using a standard reverse transfection protocol, at a final miRNA concentration of 50nM. The transfection reagent (Lipofectamine RNAi-MAX, Life Technologies) was diluted in OPTI-MEM medium (Life Technologies) and added to the miRNA mimics. After 30 minutes, 7.5 x 10^4 cells (per well) were resuspended in DMEM/F-12 (Life Technologies), supplemented with 5% fetal bovine serum (Life Technologies), mixed with transfection solution and seeded on 24-well plates (Thermo Fisher Scientific). Cells transfection were incubated at 37ºC in 5% CO2 humidified atmosphere for 48 h. After transfection, cells were infected with S. Typhimurium DT104 until OD 0.6-0.8 (MOI 1:25) was

reached. After 1 h of infection, the medium was replaced with fresh medium containing gentamicin (100 µg/mL) to kill extracellular bacteria. After 2 h of incubation, RNA lysate from cells were obtained and RNA were isolated as mentioned above.

11. MiRNA- target validation by luciferase assay.

11.1. Identification of MREs in 3ÚTR gene region.

miRNA recognition elements (MREs) were predicted with TargetScan and RNAhybrid [188, 190]. TargetScan prediction criteria is based on strong pairing in the seed region, thermodynamic stability, number of target sites on the 3´UTR of a given mRNA, sequence context (location of MRE with respect to stop codon and to the poly A tail or presence of AU-rich clusters) and accessibility of the target site to the RISC complex [191, 192]. Besides strong/moderate pairing in the seed region and thermodynamic stability, RNAhybrid lets you change the search parameters (e.g. seed length, presence of G:U wobble in seed pairing), useful features for MRE identification. In addition, the MREs were ranked based on their score. The hybridization energy required for the formation of miRNA-MRE duplex was calculated by uploading to RNAhybrid the sequence of 3´UTR segments containing the MREs and their respective miRNA. Only the duplexes with favorable hybridization energy of ≥-15kcal/mol were chosen as potential MREs.

11.2. Cell culture and 3´UTRs cloning.

Cell culture of epithelial CHO cells (Chinese Hamster Ovary cells) was performed. We selected this cell line because they are widely used for cell transfection given their stability and other properties such as adhesion and growth [193]. Cells were cultured in RPMI (Biowest) supplemented with 10% of heat inactivated fetal calf

serum (Gibco, Life Technologies) and 2mM L-Glutamine (Biowest). Cell culture was performed at 37ºC and 5% CO2.

The 3´UTR of three selected mRNAs (TLR4, ABCB1, PSMB8) predicted to be targeted by our miRNAs of interest were amplified by PCR. Then, the 3´UTR was cloned into the firefly luciferase in the psiCHECK2 vector (Figure 10; Promega). Primer sequences and restriction enzyme used for cloning of the 3´UTR of porcine genes are shown in Table 3, and design is detailed in additional file 3.

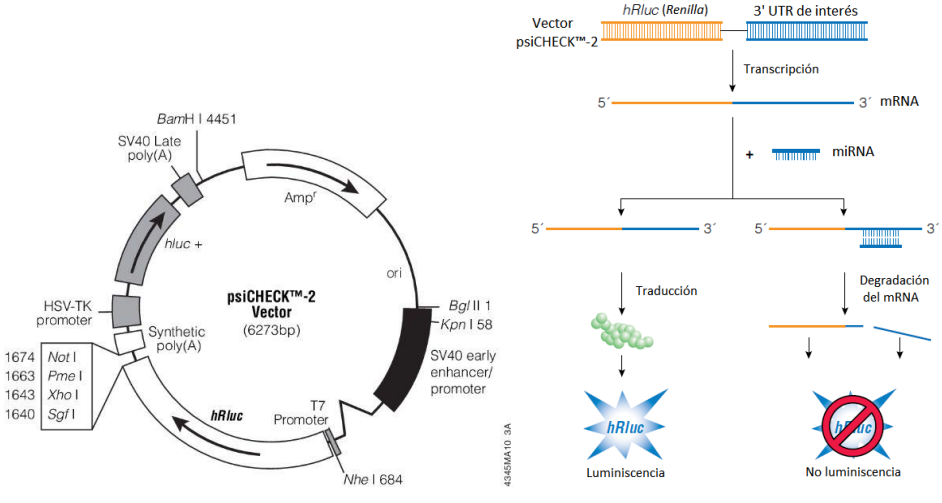


Figure 10. psiCHECK2 vector map and mechanism of action.

Table 3. Primers used for 3'UTR amplification of target genes. Restriction sites of XhoI is underlined.

Primer	Sequence	Tm (°C)	Product
3'UTR_TLR4_F	5' **GGTCTCGAG**GCAACCAGCATGATACAACAG 3'	53,9	698 pb
3'UTR_TLR4_R	5' **GGTCTCGAG**TTCTCAAAAGATAGTGGTTAATTATGG 3'		
3'UTR_ABCB1_F	5' **GGTCTCGAG**CAAAGCGCTCATCAACTGTG 3'	50,5	441 pb
3'UTR_ABCB1_R	5' **GGTCTCGAG**GGCATTTTAGACAAGATGACTCG 3'		
3'UTR_PSMB8_F	5' **GGTCTCGAG**ACACCTACGCTTATGGGGTCA 3'	60,2	377 pb
3'UTR_PSMB8_R	5' **GGTCTCGAG**ACAAAAGGATAAACTCCGCCCTG 3'		
3'UTR_PSMB9_F	5' **GGTCTCGAG**TGGTGTGGACCATCGAGTAA 3'	56,6	292 pb
3'UTR_PSMB9_R	5' **GGTCTCGAG**ACATGATGGCCTGTCACGTA 3'		

E. coli cells transformed with recombinant miRNA target expression vector (psiCHECK2) were grown overnight in appropriated volume of LB medium with ampicillin (100μg/mL). Plasmid DNA was isolated using the JetStar 2.0 Plasmid purification kit system (Genomed) according to the manufacturer´s protocol..

11.3 Transfection and luciferase activity measurements

Each miRNA mimic and negative control were transfected individually into CHO cells using a standard reverse transfection protocol, at a final miRNA concentration of 75 nM. The transfection reagent (Viromer Blue, Lipocalix) was diluted in OPTI-MEM (Life Technologies) and added to the miRNA mimics; 30 min after, 20000 cells (per well) were resuspended in RPMI medium with 10% of heat-inactivated fetal calf serum (Gibco, Life Technologies) and 2mM L-Glutamine (Biowest), mixed with transfection solution and seeded on 96-well plates (Thermo Fisher Scientific). Transfected cells were incubated at 37ºC in 5% CO2

humidified atmosphere. After 24h, cells were co-transfected with 250ng of the psiCHECK™-2 vector constructions, using Lipofectamine 3000 transfection kit (Invitrogen, Life Technologies). After 48h of incubation, cells were washed twice in PBS, and lysed with 50 μL of 1X passive lysis buffer (Promega). An aliquot of 20 μL was assayed for firefly and renilla luciferase activity using the dual luciferase reporter assay system (Promega) according to the manufacturer´s protocol. Luciferase activity values were obtained using a Varioskan Lux luminometer (ThermoFisher Scientific). For each putative target, control experiments were performed, including a plasmid which did not contain the 3´UTR fragment and negative controls of miRNA mimic. Statistical differences in expression values among groups were assessed using a Student's t test (Graphpad Prism 6, Graphpad Software Inc, La Jolla, CA, USA). Statistical significance was set at $P < 0.05$.

12. Invasion assays.

12.1. Gentamicin resistance assay.

The invasion ability of S. Typhimurium during the over expression of miRNAs candidates (using miRNA mimics) was determined in porcine jeunum epithelial cell line (IPEC-J2) using the gentamicin survival assay as described in a previous work from our group [194]. Briefly, miRNA mimic transfection in IPEC-J2 culture and infection with S. Typhimurium was performed as mentioned above. After infection, the monolayers were washed twice with PBS containing gentamicin (100 μg/mL), then the medium was replaced with fresh medium containing gentamicin (100 μg/mL) to kill extracellular bacteria. After 2 h of incubation, monolayers were washed twice with PBS and lysed with 1% Triton X-100 solution, breaking cells and releasing the bacteria. The lysates were vigorously vortexed for

1 min, diluted and spread in TSA medium (Tripticase soy agar) in triplicate. Invasiveness was calculated by counting the c.f.u. (colony forming units). The assay for each strain was conducted in triplicate on three different days. Statistical differences in c.f.u. among control (transfected with mimic negative control and infected with *S.* Typhimurium) and miRNA transfected group (infected with *S.* Typhimurium) were assessed using a Student's t test (Graphpad Prism 6, Graphpad Software Inc, La Jolla, CA, USA). Statistical significance was set at $P < 0.05$.

12.2. Confocal microscopy.

Before infection, *S.* Typhimurium culture was stained with 0.1 μg/μL of FITC (Fluorescein isothiocyanate-lipopolysaccharide from *Salmonella* enterica serotype Typhimurium, Sigma Aldrich, St Louis, USA) for 30 min. Transfection with miRNA mimics of IPEC-J2 cells, and infection with *S.* Typhimurium for invasion assays are described above. After the 2h of incubation, infected monolayers were washed twice with PBS and then fixed with paraformaldehyde (PFA) for 20 min at room temperature. Monolayers were then washed with PBS, followed by nucleic acids staining with 0.5 μg/mL of DAPI (4´,6-diamidino-2-phenylindole solution, Roche, Barcelona, Spain) for 2 minutes. Finally, monolayers were washed twice with PBS and prepared for display using fluorescent mounting medium (Dako, Agilent Technologies). The samples were visualized in a LSM EXCITER confocal microscopy (Carl Zeiss, Jena Germany) and the images were analyzed with ImageJ software (National Institutes of Health, Bethesda, USA).

CHAPTER I

Transcriptional analysis of porcine intestinal mucosa infected with *Salmonella* Typhimurium revealed a massive inflammatory response and disruption of bile acid absorption in ileum

1. Results.

All infected animals tested positive for S. Typhimurium in feces, and developed clinical signs characteristic of the disease such as fever (a peak of fever at 2 dpi returning to normal values at 6 dpi), lethargy and diarrhea [195]. Control animals tested negative to S. Typhimurium in feces prior to their necropsy. The sera biochemistry profile along the time course of infection (Additional file 1) showed decreased albumin (at 1 dpi, $P = 0.039$), total proteins (at 1 dpi, $P = 0.009$), glucose (at 2 dpi, $P = 0.024$), high density lipoprotein cholesterol (HDL, at 1 dpi, $P = 0.046$; 2 dpi, $P = 0.003$ and 6 dpi, $P = 0.041$), alanine transaminase (ALT, at 1 dpi, $P = 0.030$ and 6 dpi, $P = 0.001$) and porcine IgM (at 2 dpi, $P = 0.012$ and 6 dpi, $P = 0.008$). Complete microarray results can be found in Additional file 4. In jejunum, expression changes were found only in day 2 after infection (2 dpi), and most genes were up-regulated (71%). Expression changes in colon occurred at 2 and 6 dpi, and most genes were found to be down-regulated (70 and 73%, respectively). Ileum was the gut portion where the vast majority of DE genes were found (over 2300 different genes), and the proportion of up- and down-regulated genes was similar at all points of the infection time course (Figure 11).

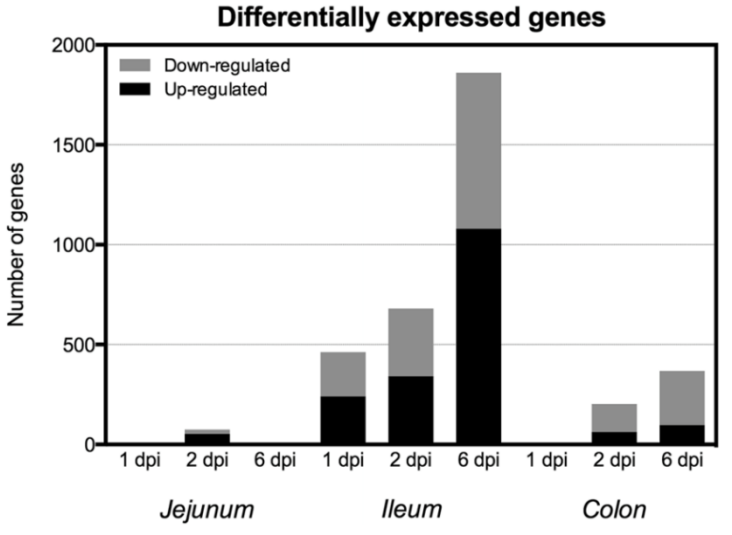


Figure 11. Number of differentially expressed genes in porcine jejunum, ileum and colon after *S.* Typhimurium infection. Number of genes differentially expressed compared to controls in the porcine gut (jejunum, ileum, colon) after 1, 2 and 6 days of S. Typhimurium infection.

1.1. Ileal transcriptomic response during *Salmonella* infection.

Salmonella colonization observed in ileum at 1 and 2 dpi (Figure 12) is in agreement with previous reports [53, 196]. Functional analysis of DE genes revealed that affected biological pathways included inflammation and immune response, lipid metabolism and cell death and survival (Figure 13; Additional file 5). Immune-related biological pathways were altered at all times of the infection time course, showing a chronological progress of events. The infection triggered an antimicrobial response at 1 dpi, and later the immune response was replaced by an alteration of the immune cell trafficking and inflammatory response (more pronounced at 1 and 2 dpi), cell-to-cell signaling and interaction, infectious disease and cell-mediated immune response.

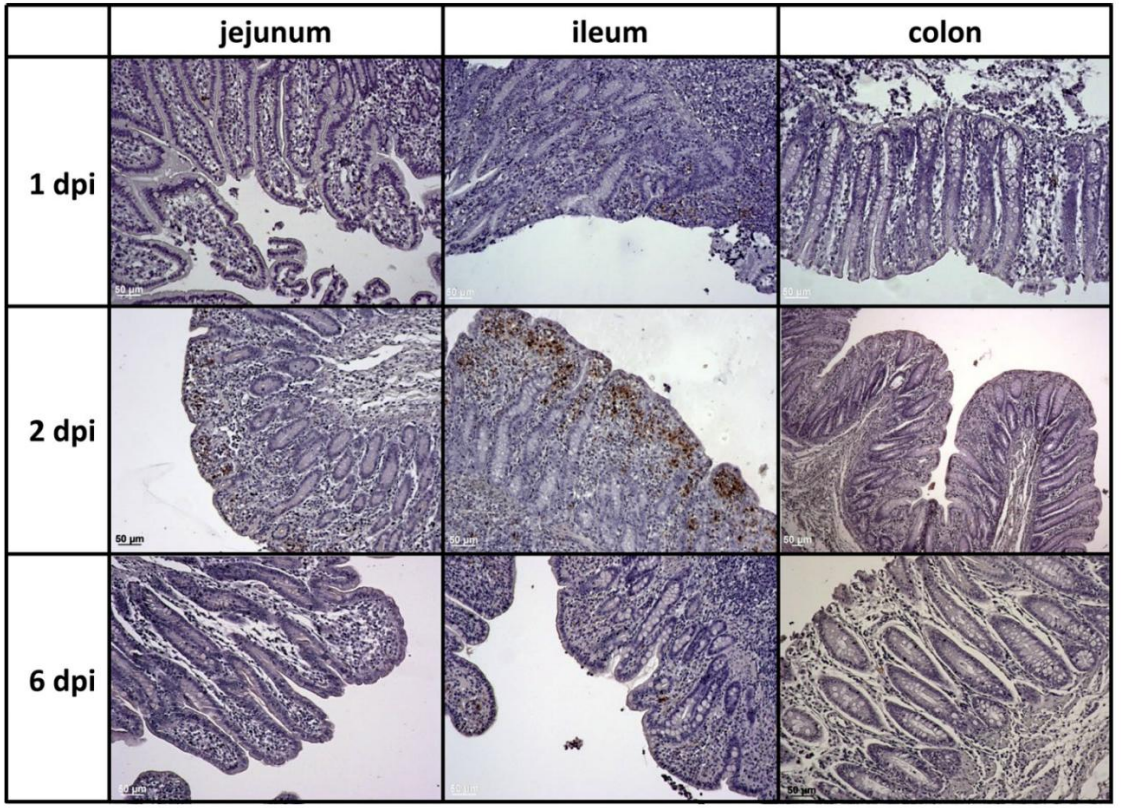


Figure 12. Intestinal colonization of S. Typhimurium in the porcine gut at 1, 2 and 6 dpi. Immunohistochemical detection of S. Typhimurium in intestinal tissue (i.e., jejunum, ileum, colon) at 1, 2 and 6 dpi.

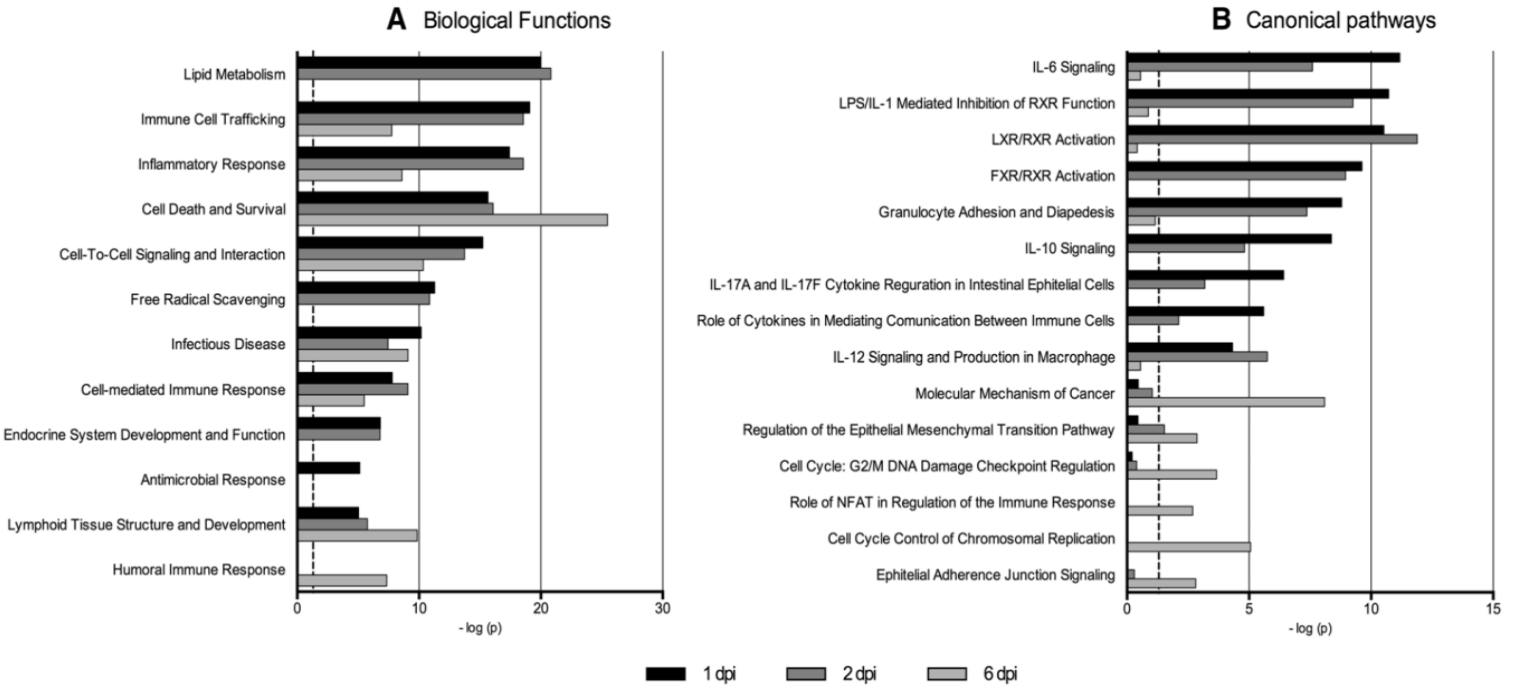


Figure 13. Biological functions impaired due to S. Typhimurium infection in porcine ileum. Biological functions (**A**) and canonical pathways (**B**) affected by S. Typhimurium infection in porcine ileum at 1, 2 and 6 dpi.

Genes affecting lymphoid tissue structure were impaired at all times, but changes were more evident at 6 dpi, where humoral response was also observed. This differential expression implicated dysregulation of inflammatory/immune processes such as *IL-6* signaling pathway, LPS/*IL-1* mediated inhibition of RXR function, granulocyte adhesion and diapedesis, *IL-10* signaling, differential regulation of cytokine production in intestinal epithelial cells by *IL-17A* and *IL-17F* and *IL-12* signaling and production in macrophages (Figure 13; Additional file 5). At 6 dpi, we observed a dysregulation of pathways related to proliferation and reposition of damaged tissue (Figure 13).

Along with inflammation and immune response, lipid metabolism and its related functions (e.g., free radical scavenging, endocrine system development and function) were highly impaired at 1 and 2 dpi after experimental infection, dysregulating retinoid X receptor (RXR) related pathways such as LPS/*IL-1* mediated inhibition of *RXR* function, *LXR/RXR* activation, *FXR*/ *RXR* activation and hepatic cholestasis. Genes showing the largest transcriptional changes at 1 and 2 dpi (Additional file 4) are involved in these pathways. DE genes described in Table 4 are involved in bile acid metabolism and intestinal absorption of bile through the ileum mucosa (e.g., *FABP2*, a fatty acid transporter, and *FABP6*, an ileum-specific bile acid transporter). In order to confirm the disruption of the normal lipid/bile acid absorption pathway in ileum due to S. Typhimurium infection and its impact in overall homeostasis, expression of genes involved in this pathway were further studied using qPCR (Table 4).

At 2 dpi, we confirmed up-regulation of *IL-1B, IL-6, TLR2, TLR4, TNFα* and *PPARG*, along with down-regulation of ASBT, FABP6, FABP2, FXR, RXRG and APOA1. The overall gene dysregulation found in this pathway tended to resolve at 6 dpi (Table 1), and some regulatory genes (*FXR* and *RXRG*) even changed direction of expression (from down to up-regulated).

Table 4. Validation (qPCR) of genes involved in bile acid metabolism after S. Typhimurium infection.

Gene	Day 2		Day 6	
	qPCR	Array	qPCR	Array
APOA1	-50**	-33,32**	-1,76	-
C-FOS	7*	-	1,87	-
C-JUN	1,87	-	1,33	-
FABP6	-200**	-56,24*	-2,28	-
IL-1B	30,22**	3,4*	2,92	-
IL-6	1,69	-	-1,91	-
NR1H4 (FXR)	-3,73	-4,68*	1,34	-
PPARG	1,70	1,88**	1,39	2,02*
RXRG	-4,09*	-2,92**	1,56	-
SLC10A2 (ASBT)	-1,92*	-	1,38	-
STAT3	1,64	-1,6	1,57	-1,6*
TLR2	2,99*	1,57*	3,43	2,41*
TLR4	10,44*	1,88*	2,25	3,52*
TLR9	-1,79	-	-1,29	-1,92*
TNF-α	8,68*	1,59*	-1,26	-

All values are expressed in fold change (FC) compared to controls. Asterisks indicate statistically significant values (compared to controls). *P<0.05;**P<0.01; ***P<0.001.

1.2. Transcriptomic response in jejunum and colon during *Salmonella* infection.

Jejunum showed a reduced transcriptional response, with absence of response at 1 and 6 dpi (Figure 11). At 2 dpi we observed scarce changes in genes involved in inflammatory response (e.g., *CXCL2* overexpression) but with few consequences in inflammatory/immune response signaling pathways (Additional file 5). In colon, we found no transcriptional changes at 1 dpi, while at 2 and 6 dpi a general down-regulation (70% of genes) of cellular proliferation pathways was found, especially actin-based Rho signaling pathways (Additional file 4).

1.3. MiRNA regulation of immune response against Salmonella in ileum at 2 dpi.

Based on the highest misregulation of gene expression (Additional file 4) and bacterial colonization found in ileum at 2 dpi (Figure 12), the miRNA expression profile was investigated at this time point. A total of 62 miRNAs (FC ≥ 1.5, P <0.05) were found DE in ileum after S. Typhimurium infection, from which 37 were up-regulated and 25 down-regulated (Additional file 6). The prediction of their target genes revealed that these 62 miRNAs are potential regulators of 880 genes (Additional file 7); these genes are involved in many biological functions such as cellular growth and proliferation, cell death and survival, inflammatory response, immune cell trafficking and gastrointestinal disease (Additional file 8). Although we validated the array results by qPCR (Table 5), we could observe that in general miRNA expression values were very moderate compared to mRNA results, and only miR-451 was found to be statistically significant and biologically meaningful (FC > 2, P<0.001).

Table 5. miRNA microarray validation by qPCR from ileum samples at 2dpi.

miRNA	qPCR	microArray
miR-374a-5p	1.18*	2,27*
miR-30a-5p	1,02	2,4*
miR-451	2,87***	2,3*
miR-454-3p	-1,09	-2,27
Let-7b-5p	1,11	3,22*
miR-27b-3p	1,07	2,62*

All values are expressed in fold change (FC) compared to controls. Asterisks indicate statistically significant values (compared to controls). *P<0.05;**P<0.01; ***P<0.001

2. Discussion.

The present experimental time course of infection demonstrated that S. Typhimurium infection in pigs lead to an inflammatory response and activation of immune mechanisms, as shown by massive transcriptional dysregulation. These changes were more pronounced at day 1 and day 2 after experimental infection, and tended to disappear by day 6 after bacterial challenge. Most gene expression changes occurred in the ileum, indicating an early immune response to infection (1 and 2 dpi) followed by cell proliferation (6 dpi).

Activation of *TLR2* and *TLR4* along the time course of the experimental infection triggered early intestinal (innate) immune response at 1 and 2 dpi. In parallel, initial exposure to pathogens also induced secretion of antimicrobial substances (at 1 and 2 dpi) such as antimicrobial peptides (AMP) and production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) at the site of infection. Antimicrobial elements differentially expressed in this study included *S100A9* and *S100A8* (highly over-expressed), as well as *DEFB1*, *EDN*, *S100A12* and *LTF*, showing the intense local response occurring in the porcine gut after S. Typhimurium infection (in agreement with previous reports [159]). Stimulation of TLR by bacterial recognition activated cytokine cascades such as *IL-6* and *IL-10* signaling pathways predominantly at 1 and 2 dpi, which lead to recruitment of other immune cells (e.g., neutrophils, dendritic cells) to the infection site in order to clear infection. This early inflammatory response disappeared at 6 dpi, and it was replaced by tissue regeneration and proliferation. Interestingly, and even though *TLR2* and *TLR4* expression remained elevated, the host inflammatory response weakened at 6 dpi. The role of *TLR2*, *TLR4* and *TLR9* in S. Typhimurium infection has been recently characterized by Arpaia et al. [197], who found that a failure in host cell TLR recognition of the bacteria impairs formation of the

Salmonella-containing vacuole (SCV) and activation of the Salmonella Pathogenicity Island-2 genes [197].

In the present study, we observed that intestinal inflammation at 1 and 2 dpi was associated with a down-regulation of the farnesoid X receptor (*FXR*, *NR1H4*), increasing expression of *NF-κB* dependent genes (*IL-1A*, *IL-1B*, *ILR*N, *IL1R1*, *IL1RAP*, *CD14*, *IL- 33*, *TNFα*, *TNFRSF1B, IL-6*) in agreement with previous reports [159, 198]. *FXR*, also called bile acid (BA) receptor, is a nuclear receptor that locally modulates intestinal immune response via regulation of cholesterol and BA metabolism. It has been described that *FXR* forms a heterodimer with the retinoid X factor (*RXR*), maintaining BA homeostasis in gut and liver [199]. Under physiological conditions, bile acids are synthetized in the liver and secreted into the intestine (i.e., duodenum) for digestion and absorption of dietary fat. Since over 90% of secreted BA are reabsorbed through the ileum to be recycled [200], *FXR* has an important role in regulation of the intestinal absorption for protecting the cells from biliary damage.

Most inflammatory *NF-kB* dependent genes were not differentially expressed at 6 dpi, which concurs with a *FXR* change to overexpression. Therefore, our findings support previous studies [198], claiming that modulation of the inflammatory process mediated by *FXR* prevents further tissue damage and avoids disease progression. Intracellular *FXR* activation by presence of BA in ileocytes has been described to inhibit further absorption by up-regulation of SHP (*NR0B2*), which represses the apical enterocyte BA transporter *ASBT* (SLC10A2) limiting additional entrance, and inhibits *CYP7A1*, the hepatic enzyme that synthetizes BA from cholesterol in the liver [201]. We report here that S. Typhimurium infection in pigs shut down the *FXR* pathway, subsequently downregulating *FXR* target genes; these results are in agreement with previous murine studies [202]. We observed that *FXR* repression at 1 and 2 dpi (along with

NR2B3 down-regulation, its heterodimer partner) during the early inflammatory response lead to a decreased expression of *ASBT* and the fatty and bile acid transporters *FABP2* and *FABP6*, impairing normal bile absorption in ileum. Repression of ileal BA transporters in the S. Typhimurium infected gut, contrary to the physiological regulatory mechanism, has been associated with decreased BA production in the liver (i.e., down-regulated *CYP7A1*) [202]. To our knowledge, changes in FXR expression had not been previously reported in pigs after S. Typhimurium experimental infection.

Down-regulation of the *RXR-LXR* pathway in ileum was evident at 1 and 2 dpi, with repression of its target genes *ABCG8*, *APOA1*, *APOC3*, and LPL. These findings indicate deficiency in cholesterol absorption, and are supported by serum concentrations of HDL cholesterol in the studied pigs, which was significantly lower during the infection time course, especially at 2 dpi, demonstrating a disruption in cholesterol carriage from tissues to the liver. Alteration of lipid metabolism (BA and cholesterol) is tightly linked to the inflammatory response triggered by the S. Typhimurium infection, since activation of the *IL-1* signaling cascade down-regulates *ASBT* [203], limiting BA absorption and therefore *FXR*-mediated transcription of the target genes previously mentioned.

After the initial period of non-specific immune response, the acquired pathogen-specific response is activated in order to clear bacteria (although this mechanism can fail, leading to persistence). Antigen presenting cells (e.g., dendritic cells, macrophages) will then stimulate T cells into different types, each of them pathogen- or toxin-specific. It has been shown that Salmonella infection decreases MHC class II molecules' expression (swine leucocyte antigen, SLA) by inducing polyubiquitination of SLA-DR in infected cells, limiting pathogen recognition [204]. Our data agrees with that, indicating a late overstimulation of

the antigen presenting function in ileum at 6 dpi, demonstrated by up-regulation of *SLA-DRA*, *SLA-DRB2, SLA-DRB4, SLA-B, SLA-DQA1,* and *SLA-DQB2*. In parallel, we found a down-regulation of B-cell differentiation and function suggesting impairment of a proper humoral immune response, as indicated by down-regulation of molecules involved in B-cell pathways such as *FOXO1*, *CD19*, *BLNK* and *EBF1* [205-208].

Transcriptional changes found in jejunum (e.g., overexpression of *CXCL2*, *S100A9*), which occurred only at 2 dpi, are product of the early inflammatory/acute phase response. This innate inflammatory response to Salmonella has been previously reported using experimental jejunal loop infection model in pigs, where overexpression of inflammatory molecules was detected from 2 to 8 h after Salmonella perfusion [209]. Colonic response to S. Typhimurium infection started at 2 dpi, and was still present at 6 dpi. Our results indicate a general repression of pathways related to cellular proliferation and disruption of cell junctions, which has also been reported in mouse colon 4 days post Salmonella infection [210]. Gastrointestinal epithelial turnover is a needed physiological process that helps maintaining gut homeostasis; although it can be accelerated due to cell injury [211], it has been demonstrated that bacterial infections counteract the renewal of epithelial cells due to the effect of bacterial proteins on different host genes [212]. Epithelial cell turnover is mainly triggered by cell oxidative burst that occurs in intestinal epithelial cells during infections (mainly in intestinal stem cells, located in the crypts and in charge of turnover), due to *JAK-STAT* and *JNK* pathways' stimulation [213]. In our study, we detected a down-regulation of *LIMS2* at 2 and 6 dpi. This gene encodes an integrin-linked kinase (*ILK*) binding protein. S. Typhimurium EspO1 protein deregulates cell shedding by acting on host ILK [214], therefore the down-regulation of LIMS2 could be explained by this phenomenon. Also, we found down-regulation of

tissue remodeling-related genes (especially those specific of tight junctions, such as *ACTC1*, *ACTG2*, and *ACTA1*), which can be explained by the action of Salmonella pathogenicity island 1 effector proteins (e.g., SopB, SopE, SopE2 and SipA) that disrupt cell–cell junctions [215]. AvrA has been shown to have an anti-inflammatory effect, acting on mitogen-activated protein kinase kinases (MAPKKs) [59], explaining the down-regulation of *MAPKAP1* (MAPK associated protein 1) found at 2 dpi.

Our microarray results on miRNAs revealed a total of 62 miRNAs differentially expressed in ileum 2 days after S. Typhimurium infection. Assessment of some of the best candidates by qPCR confirmed the microarray results but the fold changes obtained by qPCR were very modest compared with the fold changes found in the microarray data, with the exception of miR-451 which showed highly significant differential expression. In general, changes in expression levels are much more moderate in miRNAs compared to mRNAs. Nevertheless, several miRNAs can target the same mRNA, working cooperatively and making these fold changes accumulative. Moreover, target prediction analysis for the 62 miRNAs found DE in the microarray study revealed 880 genes. These target genes are mainly involved in biological functions such as cellular growth and proliferation, cell death, inflammatory response, immune cell trafficking and gastrointestinal disease, confirming the findings at the mRNA level. Agreeing with our present study, it has been previously described that let-7b overexpression is induced by *NF-kB* activation [216]. Similarly, we observed miR-374a up-regulation, which has been described as *CEBPB* regulator [217], controlling the expression of *IL-6, IL-8* and other acute phase inflammatory genes during S. Typhimurium infection [218]. We also found miR-451 overexpression in ileum, in agreement with a recent study of porcine blood miRNA profile after S. Typhimurium infection [40]. Target prediction analysis indicated that this miRNA controls the expression of

ATP-binding cassette B1 (*ABCB1*) [219, 220], highly expressed in the apical surface of epithelial cell in ileum, where it contributes to the luminal efflux of cholesterol [221]. Repression of miR-451 has been associated to *ABCB1* up-regulation, impairing S. Typhimurium ability to invade host cells by reducing adhesion to epithelial cells [66, 67]; on the other hand, *ABCB1* down-regulation (as in the present study) is associated with inflammatory reaction (TNF activation) in the gut in response to bacterial infections [222-224]. Additionally, it has been shown that overexpression of *ABCB1* is frequently associated with an increase in intracellular pH, therefore down-regulation of this gene could cause a decrease in intracellular pH, which is beneficial to intracellular survival of Salmonella [225].

CHAPTER II

Identification of differential microRNA expression in porcine ileum infected with Salmonella Typhimurium

1. Results.

1.1. Analysis of the small RNA (sRNA) sequencing data.

In order to investigate the composition and dynamic changes of sRNAs (miRNA expression in particular) expression in ileum from pigs infected with S. Typhimurium, four small RNA libraries were constructed using total RNA from ileum samples of two infected pigs and two non-infected controls. The four RNA sequencing libraries were sequenced on the Illumina HiSeq 2000 Platform. Sequencing yielded about 30 million raw reads and, after removing the adaptors, filtering the quality of the sequence (only sequences with a Phred score >20) and length of the reads, at least 29% clean reads (8,688,963 reads) remained. Approximately 22.6% of these reads were mapped onto the pig reference genome (Sscrofa 10.2) and about 39.2% aligned to the human reference genome (GRCh37-hg19) (Table 6).

Table 6. Classification of small RNA reads in ileum 2 dpi after *S.* Typhimurium.

Sample	Input reads	Clean reads	Mature miRNA Human (reads)	Mature miRNA Pig (reads)
Infected ileum1	7317722	2828863	816821	454598
Infected ileum2	8240996	2069705	855458	512045
Control ileum 1	7406318	1584859	620920	350729
Control ileum2	6956175	2205536	1116137	652839
TOTAL	29921211	8688963	3409336	1970211

Considering this and the availability of a better annotation of the human miRNAs in the mirBase, compared to porcine miRNAs, the clean reads mapped onto the human genome were used for further analysis. Clean reads of sRNA were aligned to the Rfam database and were classified on the basis of RNA type (Table 7). Approximately 41,5% of the aligned reads were classified in miRNA, 1,3% in tRNA, 4,7% in rRNA and 9,8% in snRNA, with a small number in other RNAs although with a 40% in unclassified RNA.

Table 7. Classification of clean small RNA sequencing reads obtained from mesenteric lymph nodes at 2 dpi into non-coding RNA types. Percentage was calculated from total number of clean reads.

Classification	Number of reads	Percentage of reads (%)
tRNA	130334	1.5
miRNA	3409336	39.2
snoRNA	842829	9.7
rRNA	408381	4.7
Other RNA types	217224	2.5
Unclassified	3519030	40.5

1.2. Profiles of miRNA expression in porcine ileal mucosa.

MiRNAs mapped at least once in each library (control or infected group) were selected for determining the miRNA expression profile in ileum. Detailed analysis suggested that 433 miRNAs were expressed in ileum from infected pigs while 413 miRNAs were expressed in ileal mucosa from non-infected control animals. (Additional file 9A). The twenty most abundantly expressed miRNAs in both

groups are shown in Table 8. The most abundantly expressed miRNA in both groups was miR-21, accounting for 30,8 and 19,5, respectively. Others miRNAs such as miR-143-3p, miR-192-5p, miR-215-5p and mir-148a-3p were also highly expressed in the porcine ileum miRNome. Interestingly, 19 miRNAs including miR-7134, miR-2320, and miR-1839 resulted exclusively annotated in the pig genome, while 106 miRNAs such as miR-223 and the miR-200 family, resulted exclusively annotated onto the human genome (Additional file 9B-C).

Table 8. Most abundant miRNAs in ileum of controls and infected pigs.

Control		Infected	
Mature miRNA	**(%)**	**Mature miRNA**	**%**
miR-21-5p	19,5	miR-21-5p	30,8
miR-192-5p	14,4	miR-143-3p	15,7
miR-143-3p	12,6	miR-148a-3p	4,1
miR-215-5p	5,0	miR-192-5p	3,7
miR-148a-3p	3,3	miR-26a-5p	3,6
miR-26a-5p	3,3	let-7f-5p	2,9
let-7f-5p	2,5	let-7g-5p	2,7
let-7g-5p	2,3	let-7i-5p	2,7
miR-194-5p	2,3	miR-10a-5p	1,6
miR-200a-3p	2,3	miR-126-3p	1,5
let-7i-5p	2,2	miR-146b-5p	1,5
miR-10a-5p	2,2	let-7a-5p	1,4
miR-200b-3p	1,6	miR-24-3p	1,3
miR-126-3p	1,4	miR-191-5p	1,2
miR-27b-3p	1,3	miR-27b-3p	1,2
let-7a-5p	1,1	miR-200a-3p	1,0
miR-191-5p	1,1	miR-100-5p	1,0
miR-7-5p	1,0	miR-99a-5p	1,0
miR-24-3p	1,0	miR-7-5p	0,8
miR-200c-3p	0,8	miR-199a-3p	0,8

miRNA percentage (%) was calculated respect to total reads obtain in each group.

1.3. Identification of differentially expressed microRNAs after S. Typhimurium infection.

Compared with control group, we defined microRNAs with a fold change (FC) > 2 and false discovery rate (FDR) < 0.05 as significantly differential expressed microRNAs (DE miRNAs). A total of 30 miRNAs were found significantly differentially expressed between the uninfected and Salmonella-infected samples. Of these, 20 were significantly down-regulated (FC ranging from -2.18 to -16.68) in infected samples while 10 were up-regulated (FC ranging from 2.14 to 6.25) in them (Table 9, Additional file 10). Notably, down-regulation of miR-200 family (miR-200a/b/c and miR-141), miR-215, miR-194, miR-192 and up-regulation of miR-146a, miR-146b and miR-223 were detected.

Table 9.MiRNA differentially expressed in S. Typhimurium-infected ileum compared to controls (using a threshold of ± fold change and corrected P-value <0.05).

miRNA ID	FC	Adjust P-value
hsa-miR-215-5p	-16,68	2,23E-14
hsa-miR-215-3p	-14,82	7,62E-13
hsa-miR-802	-14,39	1,08E-08
hsa-miR-424-3p	-8,89	0,00631597
hsa-miR-194-5p	-8,31	4,21E-13
hsa-miR-192-3p	-4,88	2,56E-05
hsa-miR-31-5p	-4,59	1,95E-06
hsa-miR-32-3p	-4,12	0,00386781
hsa-miR-192-5p	-4,05	5,07E-05
hsa-miR-96-5p	-3,60	1,70E-06
hsa-miR-141-3p	-3,16	2,56E-05
hsa-miR-141-5p	-3,08	0,00346382
hsa-miR-182-5p	-2,87	0,00224723
hsa-miR-375	-2,74	0,00371378
hsa-miR-183-5p	-2,71	0,01586962
hsa-miR-200a-3p	-2,51	0,00416330
hsa-miR-200c-3p	-2,37	0,01716978
hsa-miR-200b-3p	-2,34	0,02108066
hsa-miR-147b	-2,31	0,01306944
hsa-miR-151a-5p	-2,18	0,03896928
hsa-miR-146a-5p	2,14	0,02868059
hsa-miR-9-5p	2,35	0,00556520
hsa-miR-99a-5p	2,43	0,00084311
hsa-miR-223-3p	2,88	0,00005074
hsa-miR-449a	3,85	0,01291771
hsa-miR-206	4,01	0,00105414
hsa-miR-223-5p	4,34	0,00000089
hsa-miR-146b-5p	5,50	0,00000002
hsa-miR-4792	5,68	0,00227671
hsa-miR-216b-5p	6,25	0,03612284

To validate the reliability of the sequencing data, we conducted qPCR to compare the expression levels of the DEmiRNAs (Figure 14, Additional file 10). The differential expression of six abundantly expressed miRNAs (miR-9-5p, miR-99a-5p, miR-223-3p, miR-449a, miR-206, and miR-4792) was verified by qPCR analysis. In addition, qPCR analysis confirmed the differential expression of 19 significantly down-regulated miRNAs after Salmonella Typhimurium infection.

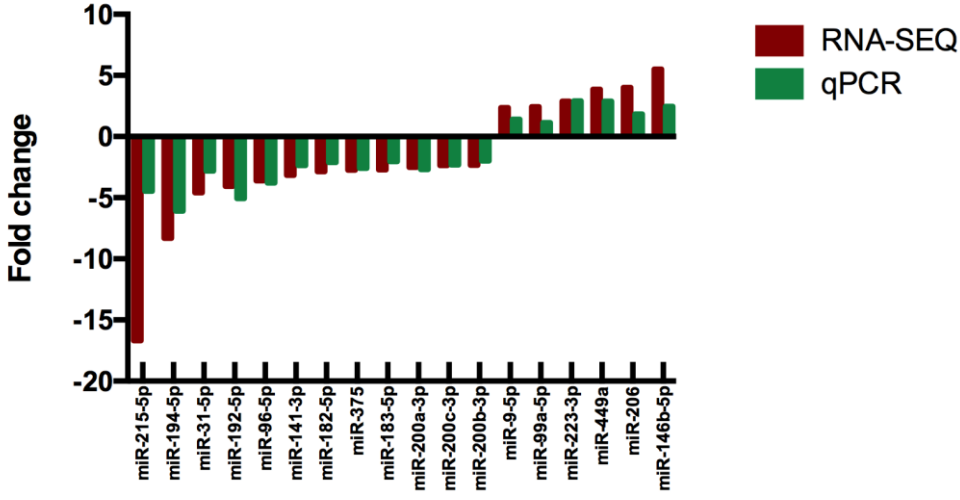


Figure 14. miRNAs differentially expressed in ileum 2 dpi, Red: miRNAs fold change results from RNA-Seq. Green: miRNAs fold change results from qPCR validation.

Expression levels of all of the selected DEmiRNAs were in concordance with the normalized deep sequencing results (Pearson correlation coefficient >0.9, Figure 15). In general, the results of the real-time RT-PCR validated the accuracy of the RNA-seq.

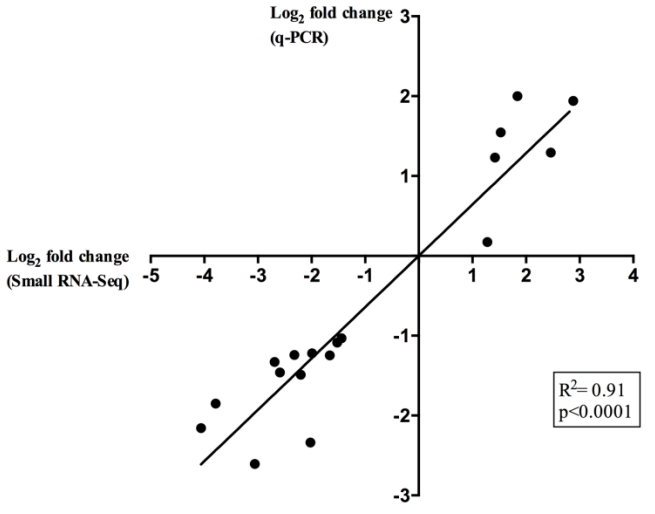


Figure 15. Pearson correlation analysis of differentially expressed miRNAs in RNA-Seq and qPCR validation in ileum at 2 dpi.

1.4. Target prediction and gene functional annotation.

To better understand the biological function of the DE miRNAs in the ileal mucosa of *Salmonella*-infected pigs, 37,560 putative target genes were predicted for 30 DEmiRNAs using TargetScan 7.0 and miRTarbase 6.0 databases (Additional file 11A). According to this result, each microRNA would regulate hundreds of genes since computational tools aim at identifying microRNA targets, usually selecting evolutionarily conserved microRNA binding sites. To provide an adequate set of target genes most biologically relevant, previous predictive data was compared with those obtained in Chapter 1 using microarrays. This integrative analysis revealed that 964 genes differentially expressed in the ileal mucosa were also potentially regulated by DEmiRNAs obtained in this study (Additional file 11B). Gene Ontology *enrichment* analysis reflected the regulatory roles that the pig-encoded miRNAs play in host physiological processes (Figure 16).

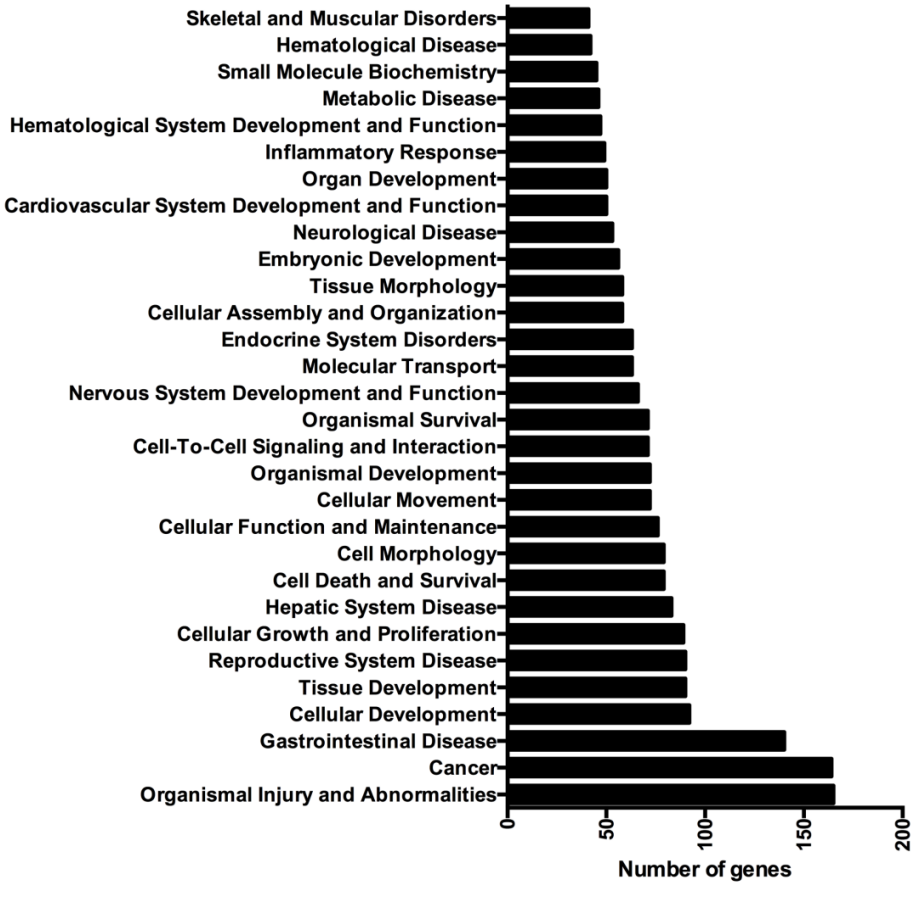


Figure 16. Gene Ontology *enrichment* analysis (P<0.05) associated to differentially miRNAs targets genes in ileum at 2dpi.

To identify biological processes predicted to be preferentially targeted by miRNAs, top ranked categories statistically significantly overrepresented in our target lists were established (Table 10). Within this top 5 functional categories, 76 genes predicted to be preferentially targeted by DEmiRNAs were indentify involved in function such as cellular development, cell death and survival, cellular growth and proliferation, molecular transport and inflammatory response (Additional file 11C).

Table 10. Top 5 of biological functions (P<0.05) associated to differentially miRNAs targets genes in ileum at 2pdi.

Biological functions: Ordered by significance according to IPA results.			
Category	**-log (P-value)**	**Nº Molecules**	**Molecules**
Cellular Movement	15,6	72	PHLDA1,ITGA8,CXCL12,TPM1,LAMC1,RHOB,ITGAV,PRLR,SERPINE1,MYO6,CXCL8,GUCY1A3,YAP1,RRAS,YWHAZ,ITGA5,IER3,TMOD3,VLDLR,ROR1,PTPRM,INHBB,TLR2,MARCKS,CORO1C,CDH1,ADAM12,FSCN1,ETV5,ANK3,MME,ADM,FLNB,SULF1,DPYSL3,FOXA1,CEBPD,PROX1,STYX,HIF1A,JAK2,AKAP11,DDX3X,TGM2,TM4SF5,TNC,MYO1B,IGF1,PLPP3,PRKAA2,STAT1,PRKCA,CELSR1,TIMP3,NRP2,TJP1,RGS16,HPSE,F13A1,S100A12,FRK,CYP1B1,GRN,TLR4,FAF1,FZD4,NFIA,MAP3K8,PTGS2,CXCL2,MSN,PSEN1
Cell Death and Survival	11,3	79	PHLDA1,SLC31A1,SLC7A11,PLRG1,INSIG1,DPYSL4,MAF,CXCL12,TPM1,LAMC1,RHOB,ITGAV,TFDP2,PRLR,SERPINE1,HK1,MYO6,CXCL8,STXBP1,GUCY1A3,YAP1,RRAS,XYLT1,YWHAZ,ITGA5,SLC1A1,TMOD3,IER3,ROR1,AGTRAP,PTPRM,TLR2,CDH1,ADAM12,IDH2,ENC1,ETV5,ANK3,MME,ADM,FLNB,SULF1,RASD2,DPYSL3,CEBPD,IGFBP7,HIF1A,JAK2,DDX3X,TGM2,SLC19A3,TNC,RGS5,IGF1,ANTXR2,PRKAA2,STAT1,CLYBL,PRKCA,CELSR1,TIMP3,ABCB1,GLS,SLC6A6,RGS16,HPSE,F13A1,SOAT1,KIF3A,CYP1B1,GRN,FAF1,TLR4,PRKAR2B,MAP3K8,PTGS2,CXCL2,PSEN1,MSN
Cellular Growth and Proliferation	10,8	89	PHLDA1,SLC7A11,SLC31A1,INSIG1,MAF,CA12,CXCL12,TPM1,LAMC1,RHOB,KCTD12,ITGAV,SRGAP2,PRLR,SERPINE1,HK1,CXCL8,YAP1,RRAS,CLTC,YWHAZ,ITGA5,IER3,VLDLR,ROR1,PTPRM,AGTRAP,INHBB,TLR2,MARCKS,CDH1,DLG5,ADAM12,FSCN1,GLUL,LFNG,IDH2,ENC1,ETV5,ADM,ANK3,FLNB,KCNH1,SULF1,MAP7,ENPEP,EPB41L3,FOXA1,DPYSL3,CEBPD,EXT1,PROX1,STYX,IGFBP7,HIF1A,JAK2,DDX3X,TGM2,TNC,RGS5,IGF1,STAT1,CLYBL,PRKCA,TIMP3,ABCB1,NRP2,ERBB2IP,TJP1,GLS,RGS16,PPL,F13A1,ERO1A,HPSE,SOAT1,FRK,KIF3A,CYP1B1,GRN,TLR4,FZD4,PRKAR2B,NFIA,KHDRBS3,MAP3K8,PTGS2,CXCL2,PSEN1
Molecular Transport	9,1	63	SLC31A1,SLC7A11,RIMS2,INSIG1,CXCL12,RHOB,GPD2,ITGAV,PRLR,SERPINE1,HK1,MYO6,CXCL8,STXBP1,ACSL3,CPT1A,YWHAZ,RAB10,SLC1A1,ATP6V1A,VLDLR,TLR2,MARCKS,SYT13,GLUL,TLK1,SYT1,SLC4A4,ADM,ANK3,KCNH1,FOXA1,CEBPD,JAK2,HIF1A,STYX,DDX3X,TGM2,SLC19A3,IGF1,PLPP3,AMN,PRKAA2,STAT1,PRKCA,SLC16A10,ABCB1,SLC6A6,F13A1,S100A12,SOAT1,CYP1B1,GRN,ATP1B3,TLR4,PRKAR2B,FZD4,MAP3K8,PTGS2,CXCL2,ATP6V1B2,MSN,PSEN1
Inflammatory Response	6,2	49	SLC7A11,INSIG1,MAF,METAP1,CXCL12,CA12,TPM1,RHOB,ITGAV,SERPINE1,CXCL8,STXBP1,GUCY1A3,RRAS,CLTC,RAB10,ITGA5,IER3,TLR2,CA7,CDH1,ADM,MME,ENTPD4,CEBPD,JAK2,HIF1A,TGM2,SLC19A3,TNC,IGF1,STAT1,PRKCA,TIMP3,ABCB1,NRP2,RGS16,HPSE,S100A12,SOAT1,GRN,TLR4,CNN3,ATRN,MAP3K8,PTGS2,CXCL2,PSEN1,MSN

Of these target genes, 28,2% are related to the five ranked processes, which suggests that, at intestinal level, the changes in the miRNA expression profile during *Salmonella* infection are preferentially associated with epithelial cell homeostasis, cell death, cell differentiation, mucosal immune response and inflammation. In this context, we found that expression levels of miR-200a, miR-200b, miR-200c and miR-141 were lower in infected samples compared to uninfected controls. All of these DEmiRNAs are members of the miR-200 family, highly expressed in normal epithelial cells and with a relevant function in epithelial-mesenchymal transition (EMT), metastatic tumour progression and chronic inflammatory states. In addition, miRNAs functional relevant in the control of the inflammatory response such as mir-194-5p and miR-223-3p resulted down and up-regulated in infected samples, respectively. These data demonstrate that *Salmonella* infection alters the expression of miRNAs that are critical to the maintenance regulation of the gut epithelial compartment as well as for generating inflammatory immune responses.

1.5. Validation of important target genes expression with qRT-PCR

With the purpose of verifying target gene expression and evaluating the expression of others genes related with miRNA expression profiling, qPCR gene expression profiling was performed. We found over expression of target genes as *NRP2, KIF3A, GLUL, FZD4, YWHAZ, CXCL2, FOXA1, RAB10* and down regulation of *ABCB1* among others. Expression of genes related with down regulation of miR-200 family (miR-200a/b/c and miR-141) was verified in ileum at 2dpi based on literature. Down regulation of this miRNA family has been associated to epithelial mesenchymal transition (EMT) by interaction of their inductors *SNAI2* and *ZEB1/2*, for this reason we confirmed the over expression of common EMT markers such as *TGFB1*, *TGFBR1/2*, *VIM*, and *CDH1/2* [226] (Figure 17).

Additionally, we confirmed the down regulation of TP53, known inductor of miR-200 family, miR-192/215 and miR-194 [227, 228]. Finally, we verified the expression of target genes of miR-223 involved in inflammatory response such as *IKKa* and *NLCR3* [229, 230]. Other genes involved in inflammatory response were tested in Table 4.

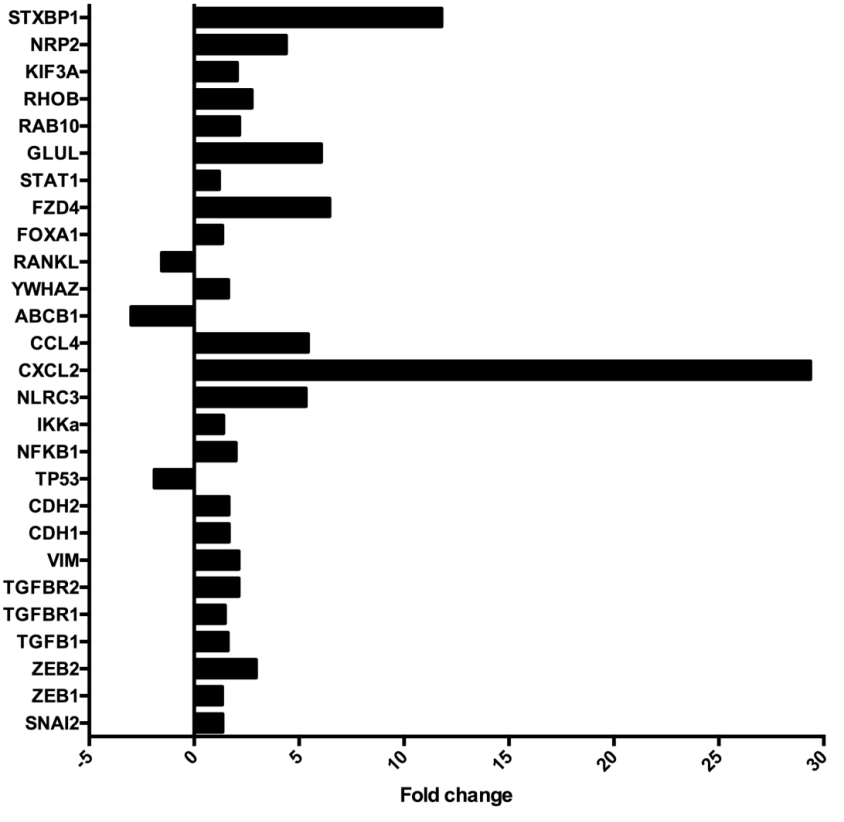


Figure 17. Gene expression profiling in ileum at 2dpi.

1.6. Changes in target genes expression in vitro after overexpression of mir-194-5p, miR-200a-3p and miR-223-3p.

To investigate the regulatory function of mir-194-5p, miR-200a-3p and miR-223-3p miRNAs during S. Typhimurium infection, the expression of target genes was evaluated following overexpression of the miRNAs into a porcine intestinal epithelium cell line (IPEC-J2) [231], mediated by corresponding miRNA mimics. Our in vitro experiments had focused solely on the effects of these three miRNAs because of their level expression and involvement in the biological functions above mentioned as well as because of their implication in the inflammatory response [232-234]. Similar to that observed in ileum mucosa, in the present study we found that expression of target genes and inflammatory mediators increased in Salmonella-infected cells, except in the case of ABCB1 and NRP2 mRNA whose expression was decreased (Figure 18). After transfection, the expression of *YWHAZ*, *FZD4*, *FOXA1* and *P53* decreased in IPEC-J2 cells transfected with mir-194-5p. Likewise, the expression of *P53*, *FZD4*, *NRP2* and *KIF3A* mRNA decreased in cells transfected with mir-200a-3p mimic. Finally, the overexpression of mir-223-3p resulted in a decrease of the *ABCB1*, *STAT3*, *GLUL* and *RAB10* mRNA expression when comparing with untransfected cells. In all cases, the expression of target genes was further reduced after infection with S. Typhimurium (Figure 18). The effect of mir-194-5p, miR-200a-3p and miR-223-3p in the inflammatory response was also evaluated. As shown in Figure 19, expression of gene coding for *TLR4*, *IL8*, *IL1A* and *IL6*, was downregulated in trasfected cells subsequently infected with S. Typhimurium.

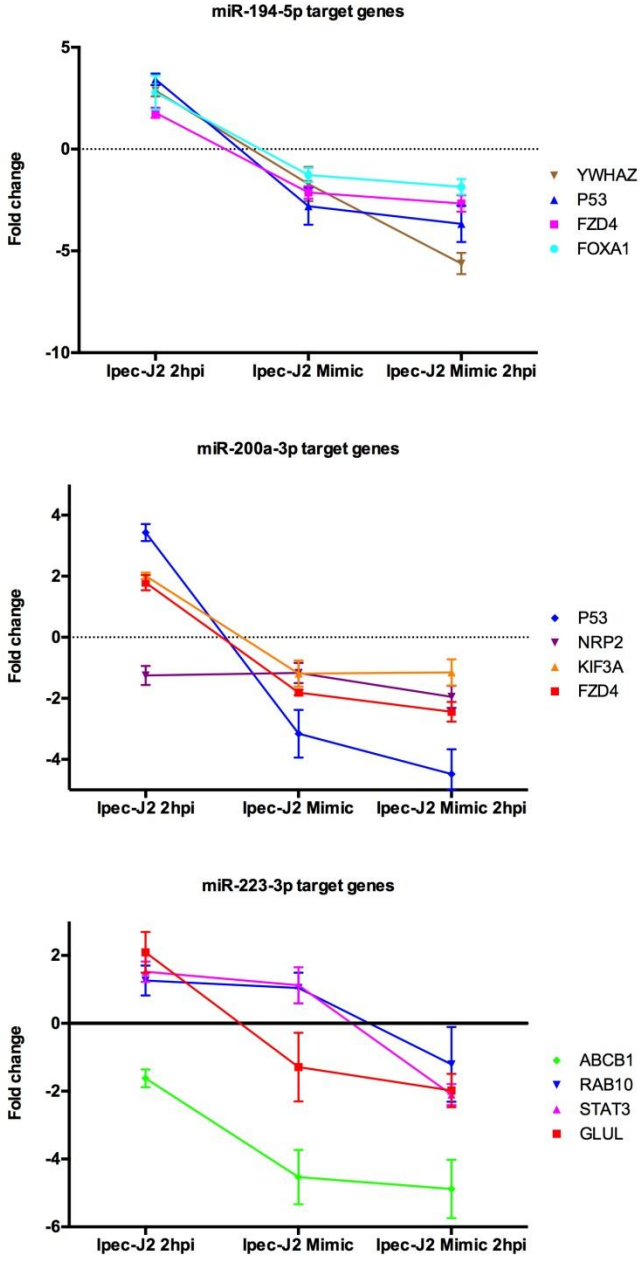


Figure 18. miRNA target gene expression of miR-194, miR-200 and miR-223 in: IPEC-J2 cells infected with *S*. Typhimurium (2hpi), IPEC-J2 cells transfected with each miRNA mimic and IPEC-J2 cells infected with *S*. Typhimurium and transfected with each miRNA mimic.

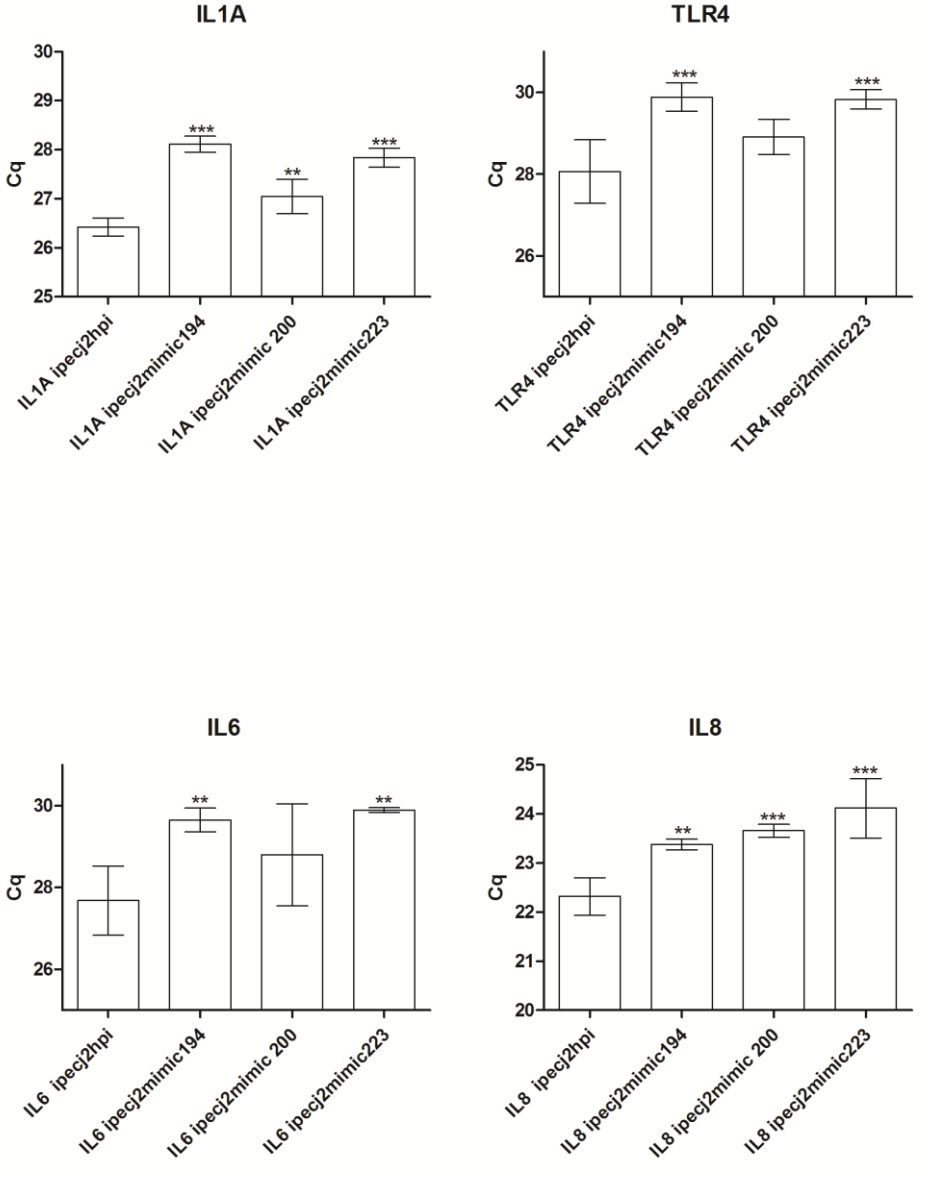


Figure 19. Gene expression (Cq) of IL1A, TLR4, IL6 and IL8 in IPEC-J2 transfected with each miRNA mimic separately and infected with S. Typhimurium compare to IPEC-J2 infected with *S*. Typhimurium.

1.7. Analysis of the target gene-mirRNA interaction.

1.7.1. Target gene binding-site prediction.

The miRNA–MRE interaction involves base pairing that requires favorable hybridization energy for the formation of a functional duplex. We computed the free energy of binding of the miRNA:MRE duplex by analyzing their sequences in RNAhybrid. Minimum free energy (MFE) of -15 kcal/mol was used as the scoring criteria to select stable duplexes. These criteria find the energetically most favorable hybridization of miRNA to a large RNA target [120, 235].

With this tool, we found multiple MREs along the 3´UTR region in all target genes (including the TargetScan prediction). Top 10 predictions of MREs of the interaction of miR-194b-5p, miR-223-3p and miR-200a-3p with *TLR4*, *ABCB1* and *TP53* are available in Additional file 3. Table 11 shows the highest scores from RNAhybrid prediction. 3´UTR region where were concentrated the majority of MREs were cloned into the psiCHECK2 vector for luciferase assay activity.

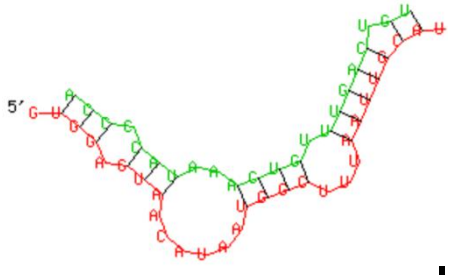
Table 11. miRNA target prediction performed by RNAhybrid. The highest score obtained from RNAhybrid prediction (-15 kcal/mol, scoring criteria) whit the probably structure of the interaction miRNA-MRE.

RNAhybrid results	Structure miRNA-3´UTR target

Target: *ABCB1*
length: 487
MiRNA: *miR-223-3p*
length: 22

mfe: -15.6 kcal/mol
p-value: 1.000000e+00

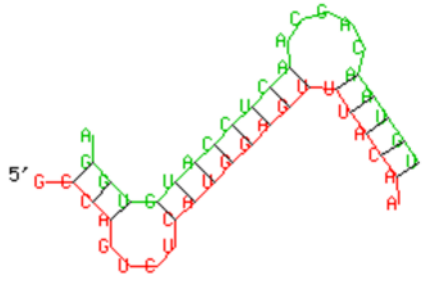
Position: 191
```
target 5' G   A   ACAUAA    UUU        U 3'
           UGG GUA      UGGC   AAUUG CA
           ACC CAU      ACUG   UUGAC GU
miRNA  3'     C   AA        U       U    5'
```



Target: *TLR4*
length: 806
MiRNA: *miR-194-5p*
length: 22

mfe: -21.7 kcal/mol
p-value: 1.000000e+00

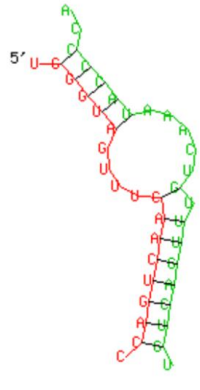
Position: 131
```
target 5' G   GUCU                   A 3'
           CCA    CAUGGAGU     UUACA
           GGU    GUACCUCA     AAUGU
miRNA  3' A               ACGAC      5'
```



Target: *TLR4*
length: 806
MiRNA: *miR-223-3p*
length: 22

mfe: -20.3 kcal/mol
p-value: 1.000000e+00

Position: 389
```
target 5'  U     GUUU          C 3'
             GGGUA      C AACUGAC
             CCCAU      G UUGACUG
miRNA  3' AC     AAACU U       U 5'
```



1.7.2. miRNA- target validation by luciferase assay.

Luciferase reporter assays were performed in CHO cells, with the objective of testing the interaction of these three genes with their predicted target miRNAs. Selection of these miRNAs were based on functional analysis (described in 1.5 section) and their biological implication during *S*. Typhimurium infection. We observed a down regulation of three of these genes by their respective miRNAs. For *TLR4* gene, we detected a down regulation of the luciferase activity of 19.2 % for miR-194-5p mimic, and 24% with the miR-223-3p mimic. The highest down regulation was observed for *ABCB1* gene upon miR-223-3p (54.1%). These results confirm the direct interaction between miRNA-3´UTR target gen and we could validate our bioinformatics predictions of the interaction between the sequences (Figure 20).

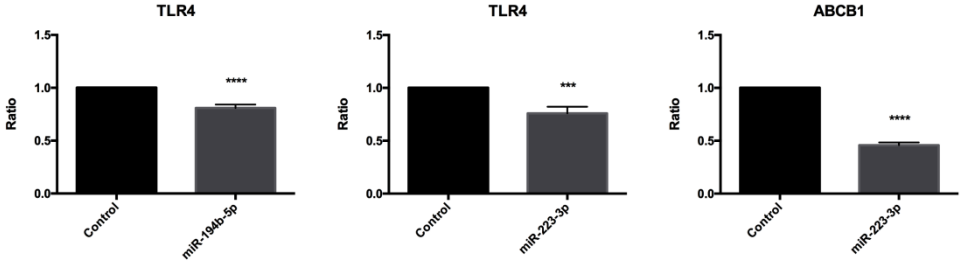


Figure 20. Luciferase reporter assay results. Firefly luciferase activity was measured and normalized by the Renilla luciferase activity. Data are represented as means ratio +/- SEM from four independent transfection experiments. Two-tailed Student´s tests were used to compare samples and significance was set at P<0.05. ***= P<0.0002, ****= P<0.0001.

1.8. Invasion assays.

The level of the interaction between *S*. Typhimurium and the IPEC-J2 cell line was evaluated by confocal microscopy and gentamicin resistance assay. The analysis by confocal microscopy showed that *S*. Typhimurium is able to adhere and invade intestinal epithelial cells IPEC-J2 *in vitro*. Quantification of bacteria in the images confirmed that the over expression of miR-194b-5p, miR-223-3p and miR-200a-3p induces an increase in adhesion and invasion of bacteria (Figure 21). Gentamicin resistance assay was used to quantify invasion of *S*. Typhimurium in intestinal epithelial cells. Figure 21 shows how invasion levels increase using the miRNA mimics' (194b-5p, 223-3p and 200a-3p) transfection in intestinal epithelial cells IPEC-J2. Thus, the results showed that although S. Typhimurium was able to invade the pig cells, the level of invasion was significantly greater when the miRNAs were over expressed.

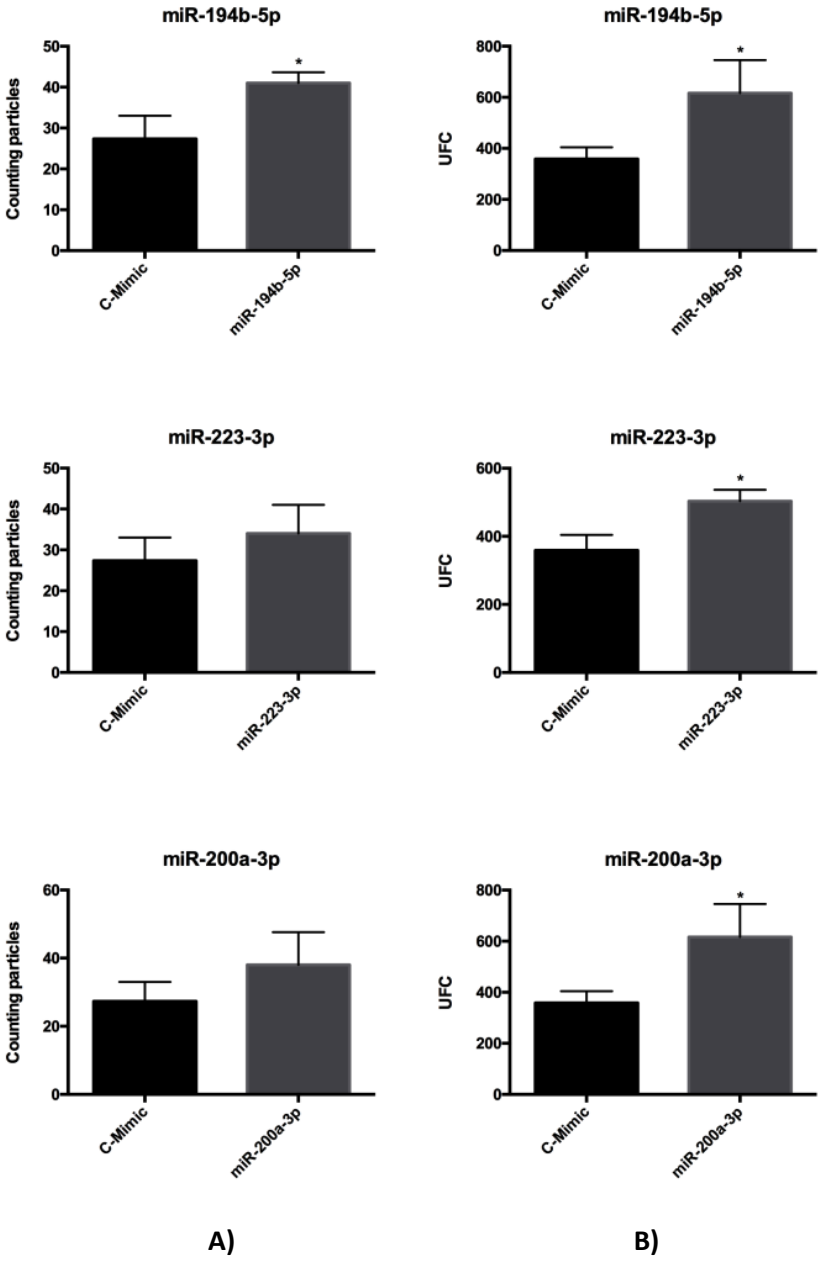


Figure 21. A) Confocal microscopy. Particle counting of confocal microscopy that represent the adhesion and invasion of *S*. Typhimurium in intestinal epithelial cell IPEC-J2. B) Gentamicin resistance assay. Data are represented as means ratio +/- SEM from three independent transfection experiments. Two-tailed Student´s tests were used to compare samples and significance was set at P<0.05. Asterisk means *= P<0.03.

2. Discussion

Specific roles in the innate immune response to living microbial pathogens had remained poorly characterized. Using a high- throughput sequencing approach, we identified miRNA-driven regulations in ileum from pigs exposed to the facultative intracellular microbial pathogen *S.* Typhimurium.

2.1 miRNAome and differentially expressed miRNAs in ileum after S. Typhimurium infection

Ileum samples were used to generate expression profiles of miRNA by high throughput sequencing technology. Although infected tissue had severe signs of microvilli loss (Figure 12), this epithelial loss did not affect the RNA quality obtained; however, we observed a decrease in the total reads obtained in infected samples. The read length distribution in all samples was comparable [236], even better to profiles generated in other studies [237], with the highest number of reads within the 19-25 nucleotides range, which suggests the reliability of the library construction and the sequencing.

The number of annotated miRNAs in domestic pig in the latest version of miRBase (v21) is still much smaller than in other organisms, featuring 1881 entries for human, 1193 for mouse and only 382 for pig [93]. Due to this fact, we obtained more miRNAs annotated when the reads were mapped to human miRNA database. Based on the assumption that the majority of miRNA sequences are conserved among species [192, 238], we used the human miRNA annotation. Which, it was good to find miRNAs as miR-200 family and miR-223, that are not mapped in pig miRNA annotation.

Previous studies using high throughput sequencing of small RNAs have shown that there are in most cases a few distinct miRNA reads, specifically in ileum [236,

237, 239, 240]. Our study reports highly abundant miRNAs such as miR-21, miR-192, miR-143, miR-200 family, miR-194 among others. These group of miRNAs has been previously shown to be abundant in mammalian ileum (Table 8) [236, 237, 239, 240]. miR-192, miR-194 and miR-215 were among the most significantly downregulated miRNAs in ileum after *S.* Typhimurium infection. These three miRNAs can be induced by p53 [241, 242] and they are also reported to be p53 positive regulators through an auto regulatory loop [243]. In our study we observed a down regulation of p53 in ileum at 2dpi (Figure 17), confirming that the down regulation of these miRNA cluster can be due to down regulation of their inductor p53. Numerous bacterial pathogens have been shown to inactivate the major tumor suppressor p53 during infection. This inactivation impedes the protective response of the host cell to the genotoxicity that often results from bacterial infection [244].

Recent reports reveal miRNA regulations in mammalian and its host cells challenged with various microbial pathogens. Almost all miRNAs dysregulated upon challenge of pigs with *S*. Typhimurium (e.g. miR-21, miR-146, miR-223, miR-200 family, miR194) [245, 246] have been recently implicated in the host response to other microbial pathogens as *Listeria* monocytogenes or *Helicobacter* pylori [158, 247, 248], but not all have been described in S. Typhimurium infection (miR-200, miR-194, miR-192, miR-215). This suggest that the host miRNA response to S. Typhimurium is not pathogen specific but rather reflects a basal host response to microbial challenge.

Several authors have observed small differences in miRNA expression by qPCR (FC 1.5 to 2.5) and have demonstrated that even very small changes in miRNA expression levels could have a direct impact on their target genes. However, we obtained FC from -6.09 to 2.88 between control and infected group, with highly significant P-values (up to $P<0.0005$).

The three DE miRNAs (miR-192, miR-194 and miR-215) belong to two clusters: the miR-215/miR-194-1 cluster and the miR-192/miR-194-2 cluster. miR-194 and miR-194-2 have the same mature sequence that are derived from two different precursors on two chromosomal locations. miR-192 and miR-215 are closely related, with similar seed sequence and their high conservation between species (28 species) indicates that these miRNAs may have vital functions that are maintained during their evolution. Sharbati et al. reported that the expression of these miRNA clusters in ileum are lower than jejunum and can be associated to a higher proliferative activity. The miR-200 family has showed the same sense of expression of miR-192, miR-215 and miR-194 cluster [249]; its expression is induced by p53 and its down regulation has been related with the increase of cell proliferation (epithelial growth and development) [240, 243, 250]. In fact, some studies describe that expression of these miRNAs (miR-192, 194, 215 and 200 family) are necessary for maintaining of the epithelial barrier [251, 252].

Several factors and pathways are known to drive EMT, primarily by converging on the SNAIL and ZEB families of transcriptional repressors of E-cadherin. These factors play a role in maintaining the mesenchymal phenotype [226], which is characterized by the polarity loss and tight junction between epithelial cells. Over expression of this miRNA cluster (miR-192, miR-194 and miR-215) and miR-200 family have been associated to decrease of ZEB1/2 and SNAIL1/2 expression [253, 254]. Besides the down regulation of these miRNAs in our study, we observed the over expression of their miRNAs targets (SNAI2 and ZEB1/2, known as inductors of EMT) and the over expression of typical markers of EMT as TGFB1, TGFBR1/2, VIM, CDH1/2.

One of the aims of the study was to identify the mRNA targets of dysregulated miRNAs using integrated analyses of miRNAs and mRNA expression profiling in ileum from pigs control with pigs infected with *S.* Typhimurium. Although

putative identification of miRNA targets using seed sequence complementarity and free energy predictions of miRNA-mRNA duplexes can be achieved using databases such as TargetScan, the false positive rate for such matches is unacceptably high. We have developed and validated an method to identify functional mRNA targets of miRNAs in S. Typhimurium infection using ileum tissue from infected and non-infected pigs, where the subset of correlated miRNA-mRNA (miRNA-up, mRNA-down; miRNA-up, mRNA-up; miRNA-down, mRNA-up) pairs is collected from a larger data set of miRNAs and mRNAs that are differentially expressed in infected and control groups. The principal merit our approach was to reduce the larger number of relatively speculative matches that are found when using seed sequences alone to a smaller set of functional, tissue specific targets. Some of these interactions were further supported by miRTarbase (the experimentally validated miRNA target interaction database). With this approach we found targets affected by translation inhibition. For such targets, changes in miRNA levels would not affect the mRNA levels, but would reduce the protein levels [255].

Our comparative mRNA-miRNA analysis revealed a large number of deregulated genes, which served to facilitate the identification of deregulated biological functions that might be regulated by miRNAs. Most interactions were between down regulated miRNAs and up regulated genes, given that the majority of miRNAs were down regulated. Biological functions affected by S. Typhimurium infection (cellular movement, cell death and survival, cellular growth and proliferation, molecular transport and inflammatory response) were significantly disrupted in ileum, based on microarray analysis, which is in agreement with previous studies [189, 256-259].

2.2 miR-194 and miR-200 regulate mesenchymal transition and inflammation in intestinal epithelium

The over expression of miR-194 leads to down regulation of YWHAZ, P53, FZD4 and FOXA1. Matta et al. found by immunoprecipitation of biopsies of oral cancer that YWHAZ protein binds to NFκB, suggesting a role in the inflammatory processes since YWHAZ facilitates the nuclear export of the IκBα-p65 complex [260]. The same implication in inflammatory response was reported by Rosenberg et al., who showed that over expression of YWHAZ leads to an increase of IL-6, TNFα, CCL5, INF and CCL3 by repression of FOXO3 and ZFP36, which binds to AU rich elements within 3´untranslated regions to destabilize cytokine mRNA [261]. Thus, we suggest that the down regulation in ileum of miR-194 allows the over expression of YWHAZ in S. Typhimurium infection. In fact, we evaluated the inflammatory response while over expressing miR-194 and we could see a down regulation of common inflammatory markers such as IL8, IL6, TLR4 and IL1A, suggesting that miR-194 has an indirect effect on inflammatory response, perhaps associated to the YWHAZ negative regulation. However, Tian et al. found that down regulation of miR-194 was a consequence of the TLR4 activation pathway, and they found a direct interaction between miR-194 and TRAF6, which is an intermediator of TLR inflammatory signal pathway in THP-1 cells [262]. In this study, we have found a direct inhibition between miR-194 and TLR4 by luciferase assay and we understand that the inflammatory regulation occurs by interaction of TLR4 (and not only by TRAF6 interaction).

The down regulation of miR-194 and over expression of its target gene FOXA1 has been related with cellular proliferation in many types of cancer [263, 264]. FOXA1 (forkhead box A1) is member of the FOX family of transcription factors, and expressed in many tissues, including in the epithelium of the gastrointestinal tract [265]. Loss of FOXA1 affects secretory capacity and differentiation of goblet cells,

affecting intestinal development [266]. Goblet cells are mucin producing cells residing throughout the length of the small and large intestinal epithelia, responsible of producing and maintaining the protective mucus layer [267]. We observe epithelial cell loss in ileum at 2dpi, evidenced by the decrease of microvilli, where goblet cells reside [268]; also, down regulation of miR-194 and over regulation of FOXA1 (a known inductor of mucins [269, 270]) and the MUC4 mucin gene suggest a necessity of renewal at the intestinal epithelial surface, that we observe at 6dpi. Mucin-deficient mice demonstrate increased permeability and bacterial adherence to epithelial cell surface [271]. Thus, the renewal of the mucus layer is necessary to protect the organism against bacterial adhesion and invasion of epithelial cells.

We also found interaction between miR-194 and miR-200a with FZD4, a member of Frizzled family genes encoding for an integral membrane protein that functions in multiple signal transduction pathways. Frizzled proteins are receptors for secreted Wnt proteins (Wnt/β-catenin pathway), as well as other ligands, and also play a critical role in the regulation of cell polarity, cell development, inflammatory response among others [272-274]. Activation of Wnt/β-catenin pathway in mouse microglial cells leads to the expression of the inflammatory interleukins IL6, IL12 and IFNγ [275]. In contrast, anti-inflammatory role of Wnt/β-catenin pathway has been demonstrated in mouse colon epithelial stem cells and macrophages infected with *Salmonella* or *Mycobacterium*, where activation of the Wnt/β-catenin pathway (via FDZ4 up regulation) down regulates the pro-inflammatory responses to certain bacterial infections [276-278]. On the other hand, the activation of Wnt/β-catenin pathway by FZD receptors lead to inhibition of GSK3β, increasing SNAIL stability and thus promoting EMT, characteristic of down regulation of miR-194 and miR-200 family, which undergo a feedback regulation by p53 [279, 280]. EMT is characterized by over expression

of SNAI2, ZEB1/2, TGFB1, TGFBR1/2, VIM and CDH2 and down regulation of miR-200 family [226], as we confirm in our study. The over expression of NRP2 that we find in ileum at 2dpi has been also observed during EMT in lung cancer [281]; we found an interaction of NRP2 with miR-200 family , which was experimentally verified in epithelial cells IPEC-J2 after miRNA mimic transfection.

Within Wnt/β-catenin pathway, we found another target of miR-200a. KIF3A, member of the kinesin family of motor proteins, has been described as agonist of β-catenin, activating the transcription downstream of cyclin D1 [282] and increasing the cellular proliferation. The over expression of cyclin D1 in *S.* Typhimurium infection due to down regulation of miR-15 family maintains the cells in a cell cycle stage more favorable for bacterial replication [155].

In our study of gene expression in ileum at 2dpi, we observed high inflammatory response (over expression of IL6, MCP-1 and STAT3) and disruption of epithelial barrier after S. Typhimurium infection [189]. So, we investigated the impact of miR-200 on inflammatory markers as TLR4, IL6, IL8 and IL1A, using a miR-200a mimic in the epithelial cell line IPEC-J2, where we observed the decrease of these inflammatory markers. The down regulation of miR-200 family is associated to inflammatory response in many studies [233, 283-285]. Suppression of miR-200 leads to over expression of c-Jun N-terminal kinase 2 (JNK2) and NF-κβ, which over expresses IL-6 [286]. Also, miR-200 regulates the expression of proinflammatory cytokine IL8 by inhibition of IKBKB expression, resulting in decreased p65 transcriptional activity in IL8 promoter [283]. Inflammation has been associated with down regulation of miR-200 family and induction of EMT [287-289], which is in agreement with our present results of miR-200 and miR-194 repression and subsequent transcriptional regulation of specific target genes that control nuclear factor-κβ signaling pathway, inflammation, cell cycle and migration.

2.3. miR-146 and miR-223 avoids massive inflammation in the intestinal epithelium during Salmonella infection.

The most representative overexpressed miRNAs of all miRNAs were miR-146a/b and miR-223 (the latter is not available in miRNA pig annotation). Such over expression has been shown to regulate innate immunity and inflammatory responses [290], but mechanisms involved are not clear.

Previous studies have reported the induction of miR-146 in macrophage and monocyte response to microbial PAMPs, including S. Typhimurium [135, 139, 161, 291]. Induction of this miRNA depends on NFκB [139, 292], producing a negative feedback control of TLR-TRAF6-IRAK1 signaling that protects against excessive inflammation [293, 294]. miR-146 knockout mice mount an exaggerated inflammatory response to injected LPS, when compared to wild type animals [295]. In our results, miR-223 was significantly over expressed in ileum at 2dpi, which also occurs in neutrophils infiltrated in the infected mucosa [296, 297]. The over expression of this miRNA has been associated with induction of the inflammatory response [230]. miR-223 regulates the production of NLRP3 (component of inflammasome complex induced by pathogens) and IL1β during TLR-activated inflammatory response [298]. Over expression of miR-223 is necessary to prevent the accumulation of NLRP3 and inhibit IL1β production by the inflammasome [299], which disagrees with the high IL1β levels found in S. Typhimurium infection [300].

Although the relevance of miR-223 in pathological infections and inflammatory response has been shown, there is limited information regarding its role in *S*. typhimurium infection; so, we used a miR-223 mimic to investigate the impact of its over expression on inflammatory response and confirm the miRNA targets origin from integrative analysis, using a porcine epithelial cell line. Our results show that up-regulation of miR-223 down regulates TLR4, IL1β, IL8, IL1A, TNFα,

IL6, NLRP3 and CASP1 , evidencing its role on inflammatory response. In bacterial infections over expression of inflammatory genes and over expression of miR-223 is usually present. This is due to a negative feedback mechanism in order to control excessive inflammation. Dysregulations of miR-223 expression have been observed in many inflammatory disorders such as rheumatoid arthritis, inflammatory bowel disease, osteoarthritis and Crohn´s disease, among others [230], where tissue often undergoes excessive inflammation.

We also confirmed repression of selected miR-223 target genes after mimic transfection: ABCB1, RAB10, STAT3 and GLUL. Direct interaction of miR-223 with the 3´UTR of ABCB1 has been documented [301]. ABCB1 down-regulation (as in the present study) is associated with inflammatory reaction (TNF activation) in the gut in response to Salmonella infections, increasing the bacterial invasion [222-224, 302]. On the other hand, over expression of ABCB1 is frequently associated with an increase in intracellular pH, which is beneficial to intracellular survival of *Salmonella* [225]. Similarly, STAT3 plays a central role in *S.* Typhimurium infection, regulating the transcriptional response. Salmonella activates STAT3 through a non-canonical pathway involved in vesicular trafficking between cellular compartments and the *Salmonella* containing vacuoles (SCVs), contributing to their maturation [303]. In inflammatory processes, the over expression of STAT3 is in relationship with the expression of cytokines IL6, IL1β IL11, IL-10 and IFNs [304], promoting and repressing the inflammatory response in different stages of infection [305]. Over expression of IL6 and IL1β decreases miR-223 production, which allows STAT3 expression, stimulating the IL6 and IL1β over expression in response to LPS in a macrophage cell line [304]. In our study, we observed the over expression of miR-223, STAT3, IL6 and IL1 β in ileum at 2dpi, confirming that the over expression of miR-223 affects many targets in inflammatory pathways, including STAT3.

RAB10, another miR-223 target overexpressed in our study, is involved in phagosome maturation [306, 307]. Expression of RAB10 is inducible by LPS that induces the expression of inflammatory cytokines and interferon's upon bacterial LPS stimulation via TLR4 activation [308, 309].

Down regulation of GLUL was confirmed in our study by miR-223 up-regulation in porcine intestinal epithelial cells. The GLUL gene is a glutamine synthetase that catalyzes the conversion of ammonia and glutamate to glutamine [310]. Glutamine is an essential component of proteins and is precursor of amino acids and nucleic acids. Furthermore, glutamine provides energy to enterocytes in the intestinal epithelium [311]. Deficiencies in glutamine may increase membrane permeability, which can be rescued by glutamine supplementation [310]. The over expression of GLUL has been associated with anti-inflammatory response characterized by attenuation of NF-κβ, decreasing IκBα degradation, and inducing heat shock proteins [312]. Over expression of heat shock proteins such as HSPA9B, HSPCA, HSPA1B, HSAPA4 and HSPB1 have been reported previously. [53, 189].

Over expression of PPARγ (as in our study) is responsible for the protective effects of glutamine in reducing intestinal inflammation [313]. With these results, we suggest that miR-223 has a regulatory role on GLUL expression and subsequently with the protective effect on anti-inflammatory response and restoring intestinal permeability. Over expression of PPARγ and heat shock proteins are a consequence of GLUL activation, which is induced by Wnt signaling pathway (that is involved too in cyclin D1 expression) [314].

Besides of determination of the miRNA-target interaction, we determined the regulatory effect of miRNA on their targets genes during in vitro epithelial cell line infection, demonstrating a negative regulation. Target genes and genes involved in inflammatory response decreased their level expression in presence

of S. Typhimurium and miRNA mimics, compared to infection without miRNA mimics. Therefore, we observe the negative control by miRNAs in target genes that are usually over expressed in infection, confirming the miRNA role in the regulation of gene expression.

2.4 miR194, miR-200a and miR-223 dysregulation modulate bacterial invasion

We demonstrate an increase of bacterial invasion after mimic transfection of miR194, miR-200a and miR-223. The over expression of these miRNAs likely affected the expression of their target genes, which could activate mechanisms that increase the bacterial number inside the cell. We identified target genes that could be implicated in the increment of bacterial infection. Down regulation of ABCB1 was previously shown necessary in other studies for increasing adhesion and bacterial invasion [222-224], accompanied with a decrease in intracellular pH, which is necessary to intracellular survival of Salmonella [225].

As mentioned above, dysregulation of miR-194 and miR-200 affect FZD4 expression, which is involved in the activation of Wnt signaling pathway [315]. Over expression of Wnt genes are involved in the protection of the host intestinal cells by blocking the invasion of pathogenic bacteria [316]. In this study, we found FZD4 down regulated by miR-194 and miR-200 over expression, increasing the bacterial invasion by repression of Wnt pathway, which is involved in the activation of approximately 400 genes related with cell growth, apoptosis and inflammatory response. Thus, dysregulation of miR-200 and miR-194 directly related with bacterial invasion through FZD4 repression.

CHAPTER III

Regulatory role of miRNAs in mesenteric lymph nodes after *S.* Typhimurium infection

1. RESULTS.

1.1. Next generation sequencing and mapping of small RNA.

We generated expression profiles of miRNA in porcine MLN samples by high throughput sequencing analysis. RNA-seq of the small RNA fraction produced a total of 30.7 million transcript reads. Most of the sequencing reads were 19 to 25 nucleotides long, which confirms the reliability of the library construction and the sequencing, given that miRNAs fall within that range. From all reads obtained, 14.5 million reads (47.5%) passed quality control thresholds (described in materials and methods), and were mapped to the human and porcine reference genomes (Table 12). The number of annotated miRNAs in the latest version of the porcine miRBase database is still much smaller than in other organisms, featuring 1881 entries for human, 1193 for mouse and only 382 for the pig [93]. Subsequently, as observed in table 12, mapping to human genome resulted more efficient (an average of 34.1% of reads) than mapping to the porcine genome (23.7% of reads). Thus, we used the human miRNA annotation for further analysis based on the assumption that the majority of miRNA sequences are conserved among species [192, 238].

Table 12. Summary of mapped reads to human and pig genomes.

Sample	Input reads	Clean reads	Mature miRNA Human (reads)	Mature miRNA Pig (reads)
Infected MLN 1	6938273	2743029	981186	647903
Infected MLN 2	7690779	3853932	1408080	938818
Control MLN 1	8049942	4000000	1291594	935200
Control MLN 2	8049942	4000000	1299953	940000
TOTAL	**30728936**	**14596961**	**4980813**	**3461921**

To better characterize the small RNA sequences obtained, we classified them into RNA types by performing BLAST searches against the Rfam database. We identified 59.1% of the sequences entered (i.e. clean reads). We found that most of the identified sequences were miRNAs (34.1% of the non-coding reads; Table 13), followed by other non-coding RNA types such as the snoRNA, rRNA and tRNA groups, which accounted for 11.8%, 4.8% and 3.3% of the input reads, respectively (Table 13).

Table 13. Clean small RNA sequencing reads obtained from mesenteric lymph nodes at 2 dpi were classified into non-coding RNA types. Percentage was calculated from total number of clean reads.

Classification	Number of reads	Percentage of reads (%)
miRNA	4980813	34.1
snoRNA	1719157	11.8
rRNA	695180	4.8
tRNA	475861	3.3
Other RNA types	586798	4.0
Unclassified	5969427	40.9

1.2. miRNA profile (miRNAome) of the porcine mesenteric lymph node.

We used mapped miRNAs to decode the miRNA expression profile (miRNAome) of MLN in the two experimental conditions of our study (i.e. infected and control). For that, we considered only miRNAs with at least one mapped read in each library (control or infected groups), thus determining the miRNAome of infected and control MLN. When mapping reads to the human genome, we obtained 492 miRNAs in non-infected control pigs, and 503 miRNAs in infected pigs, all of which are available in Additional file 12A. The number of identified miRNAs decreased to 267 in controls and 276 in infected pigs when mapping to the porcine reference (Additional File 12B).

We evaluated which miRNAs are common to humans and pigs, and which ones are exclusively annotated in any of those genomes (Additional File 12C). We found that most of the miRNAs detected were annotated in both human and pig genomes. We found 53 miRNAs annotated exclusively in human genome, such as the miR-200 family, miR-223, miR-93, miR-25 and miR-147. On the other hand, we found only 13 miRNAs annotated exclusively in the pig, which include miR-7134, miR-2320, miR-4334 and miR-1839 among others (Additional file 12C). Based on the higher number of mapped reads to the human genome, and the high homology between the two genomes (very few miRNAs exclusive of pigs), we used the miRNAs mapped to human databases from now on, for a better characterization of the obtained data.

The most abundant (top 20) miRNAs in MLN are presented in Table 14. miR-21 has by far the highest number of reads of all miRNAs identified, constituting 17.1% and 24.5% of all reads in control and infected groups, respectively. Other miRNAs such as miR-143, miR-126 and miR-26 were also highly expressed in control and infected porcine MLN.

Table 14. Most abundant miRNAs (top 20) present in mesenteric lymph nodes of control and infected pigs. miRNA percentage (%) was calculated respect to total number of reads obtained in each group.

CONTROL		INFECTED	
Mature miRNA	**%**	**Mature miRNA**	**%**
miR-21-5p	17.1	miR-21-5p	24.5
miR-143-3p	11.4	miR-143-3p	7.8
miR-126-3p	8.3	miR-26a-5p	6.5
miR-26a-5p	6.2	miR-126-3p	5.7
miR-148a-3p	6.1	let-7g-5p	5.3
let-7f-5p	4.1	let-7f-5p	4.2
let-7g-5p	3.7	let-7i-5p	4.2
let-7a-5p	2.8	miR-148a-3p	2.9
let-7i-5p	2.8	miR-99a-5p	2.7
miR-99a-5p	2.6	let-7a-5p	2.6
miR-10a-5p	2.1	miR-150-5p	1.4
miR-100-5p	2.1	miR-191-5p	1.4
miR-199a-3p	1.5	miR-100-5p	1.3
miR-146b-5p	1.3	miR-146b-5p	1.3
miR-191-5p	1.2	miR-199a-3p	1.3
miR-24-3p	1.1	miR-181a-5p	1.3
miR-181a-5p	1.0	miR-10a-5p	1.1
miR-27b-3p	1.0	miR-92a-3p	1.1
miR-30d-5p	1.0	miR-24-3p	1.1
miR-142-5p	0.9	miR-27b-3p	1.0

1.3. miRNA differential expression in MLN after *S*. Typhimurium infection.

Differences in expression of miRNAs in porcine MLN after *S*. Typhimurium infection were calculated using the CAP-miRSeq analysis [176]. We determined that a total of 110 miRNAs were differentially expressed (after P-value correction) between the two experimental groups (infected versus non-infected) (Additional file 13). Of those, 50 were down-regulated (fold change -FC- ranging from -5.41 to -1.34) and 60 up-regulated (FC ranging from 1.38 to 7.72) (Figure 22). From all DE miRNAs, we show in Table 15 the most relevant up- and down-regulated miRNAs (number of reads >300) .

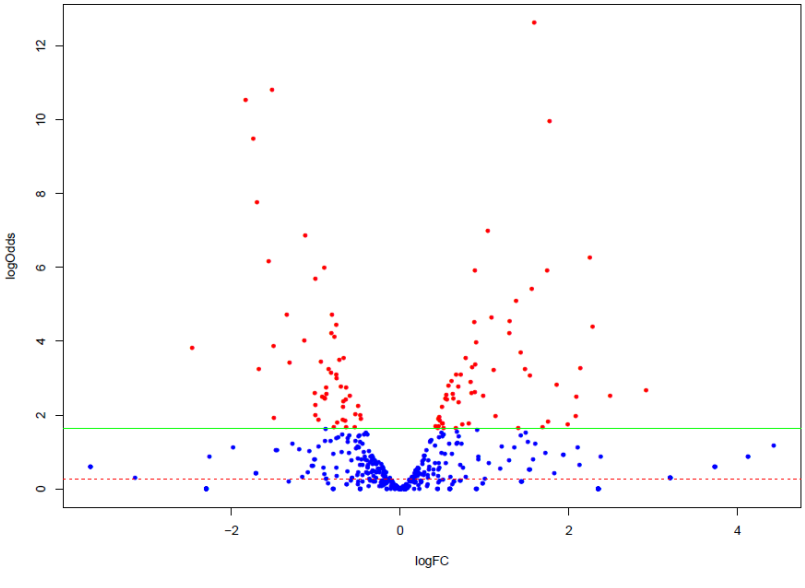


Figure 22. Volcano plot showing differentially expressed miRNAs in MLN compared to non-infected controls, highlighting in red those with a FDR-corrected P-value<0.05.

Results of differential expression were validated by quantitative PCR (qPCR). Although we used the human-mapped miRNAs for analysis, we designed pig-specific primers for qPCR validation given the nature of studied tissue. Based on literature [317] and analysis of stability by Genorm and NormFinder softwares, five miRNAs were selected as reference genes: Let-7a, miR-26a, miR-16-5p, miR-103 and miR-17-5p. We performed qPCR of 15 differentially expressed (DE) miRNAs, selected based on the biological functions where their target genes were implicated (e.g. inflammation, immune response).

Table 15. Differentially expressed miRNAs in *S*. Typhimurium-infected mesenteric lymph nodes compared to controls (corrected P-value <0.05; >300 sequencing reads).

DOWN-REGULATED		UP-REGULATED	
Mature miRNA ID	**FC**	**Mature miRNA ID**	**FC**
hsa-miR-151a-5p	-3.49	hsa-miR-150-5p	2.13
hsa-miR-148a-3p	-2.13	hsa-miR-155-5p	1.93
hsa-miR-10a-5p	-1.99	hsa-miR-23a-3p	1.91
hsa-miR-139-5p	-1.87	hsa-miR-92b-3p	1.64
hsa-miR-128-3p	-1.81	hsa-miR-363-3p	1.61
hsa-miR-204-5p	-1.70	hsa-miR-221-3p	1.60
hsa-miR-32-5p	-1.70	hsa-miR-320a	1.57
hsa-miR-100-5p	-1.65	hsa-miR-30b-5p	1.53
hsa-miR-145-3p	-1.65	hsa-miR-342-3p	1.51
hsa-miR-151a-3p	-1.64	hsa-miR-140-3p	1.51
hsa-miR-218-5p	-1.57	hsa-let-7i-5p	1.50
hsa-miR-199b-5p	-1.55	hsa-miR-7-5p	1.47
hsa-miR-126-3p	-1.53	hsa-miR-181b-5p	1.45
hsa-miR-143-3p	-1.53	hsa-miR-23b-3p	1.42
hsa-miR-1-3p	-1.47	hsa-miR-21-5p	1.42
hsa-miR-148b-3p	-1.41	hsa-miR-20a-5p	1.42
hsa-miR-125a-5p	-1.37	hsa-let-7g-5p	1.41
hsa-miR-125b-5p	-1.35	hsa-miR-378a-3p	1.38
hsa-miR-30d-5p	-1.34		

We successfully validated the results from the small RNA-Seq (Figure 23, Additional file 13), obtaining a positive correlation (R^2= 0.71, p<0.0001) between small RNA sequencing and qPCR using a Pearson correlation analysis, confirming the validation of the sequencing technique (Figure 24).

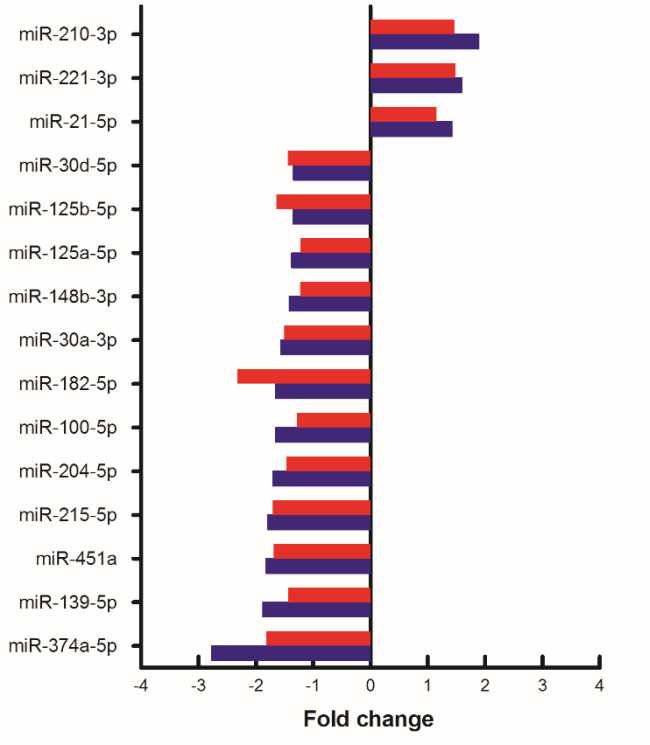


Figure 23. miRNAs differentially expressed in MLN at 2 dpi. Blue: results from small RNA-seq (fold change -FC- values in infected compared to non-infected samples). Red: results from qPCR validation (FC values in infected compared to non-infected samples).

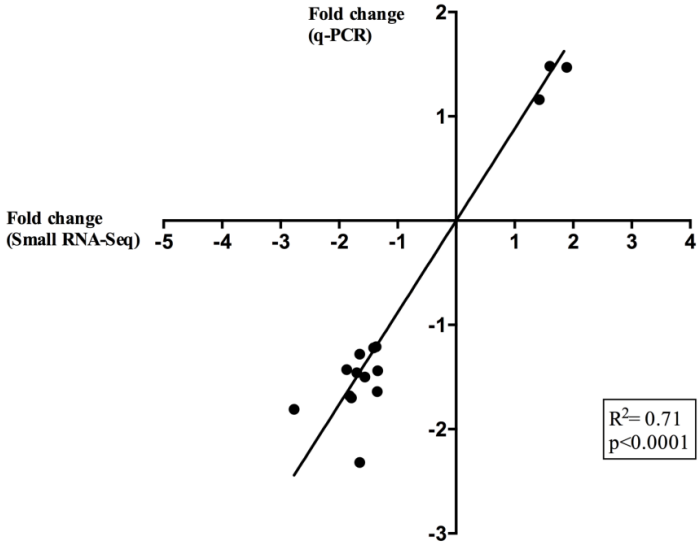


Figure 24. Pearson correlation analysis between small RNA sequencing (small RNA-Seq) and quantitative real time PCR (qPCR) of 15 differentially expressed miRNAs in mesenteric lymph nodes 2 days after infection with *S*. Typhimurium.

1.4. Integrative analysis of miRNA and protein expression in porcine MLN after infection with *S*. Typhimurium

MiRNAs modulate gene expression through mRNA degradation and translational repression mechanisms, being miRNA regulatory networks complex and often predictive. After qPCR validation of small RNA-seq results, we conducted a target gene prediction, and subsequent bioinformatic analysis of the biological functions and integrated networks affected by dysregulation of such target genes, in order to achieve a deeper understanding of the functional alterations induced by differentially expressed miRNAs. We predicted dysregulation of 9,121 target genes based on the differentially expressed miRNAs from the small RNA sequencing results (Additional File 14). Predictions were made using TargetScan and miRTarbase databases. We analyzed target genes together with protein

expression data available from the same biological samples (MLNs from the same experimental infection, microarray analysis previously published by our group [71]), and we selected only target genes encoding proteins included in that historical dataset for further analysis (n=98). Target proteins were matched and paired with DE miRNAs in order to construct a functional miRNA-mRNA regulatory network. The miRNA-mRNA pairing strategy was based on the assumption that in most cases there is a negative correlation between miRNAs and their target mRNA (miRNA up regulated/protein down regulated, and vice versa) [318]. So, miRNA-mRNA pairing resulted in 46 miRNA-protein interactions (Figure 25), indicating that the over expression of 13 miRNAs (miR-210-3p, miR-221-3p, miR-23a-3p, miR-23b-3p, miR-106a-5p, miR-20a-5p, miR-20b-5p, miR-378a-3p, miR-30b-5p, miR-181b-5p, miR-92b-3p, miR-363-3p, and miR-155-5p) could be regulating the down expression of 5 target proteins (STMN1, LASP1, VIM, YWHAZ and ACTR3). On the other hand, down regulation of 17 miRNAs (miR-30d-5p, miR-182-5p, miR-204-5p, miR-128-3p, miR-125a-5p, miR-125b-5p, miR-451a, miR-148a-3p, miR-29b-3p, miR-144-3p, miR-148b-5p, miR-1-3p, miR-143-3p, miR-217, miR-96-5p, miR-130a-3p, and miR-122-5p) could be allowing the over expression of 10 protein targets (PPID, PSMB8, PDIA3, GRB2, HSPA8, SYNCRIP, HSP90B1, PCMT1, FKBP4, and ALDOA).

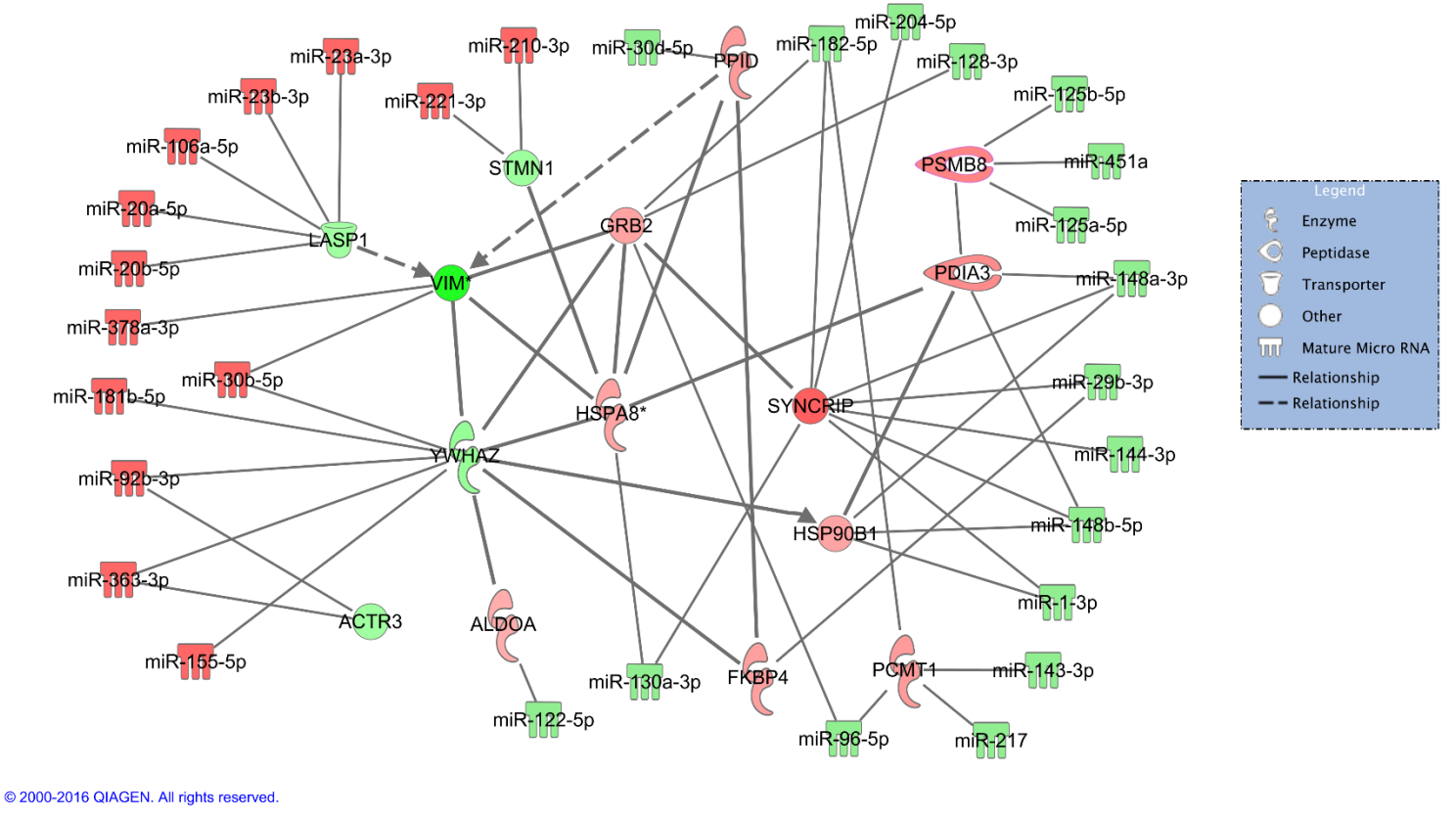


Figure 25. miRNA-protein interaction pathway resulting from the integration of small RNA-seq and proteomic data of mesenteric lymph nodes at 2dpi. Red: molecules over expressed; Green: molecules down expressed.

The biological functions analysis showed that the target proteins of these DE miRNAs were mainly associated with cell death and survival, cellular assembly and organization, inflammatory response and protein degradation (Table 16).

Table 16. Biological functions (P<0.05) affected by differentially expressed proteins in mesenteric lymph node at 2dpi potentially regulated by differentially expressed miRNAs.

Biological functions: Ordered by significance according to IPA results

Category	-log (P-value)	# Molecules	Molecules
Cell Death and Survival	8.4	13	PPID, PDIA3, GRB2, PCMT1, YWHAZ, VIM, PSMB8, HSPA5, STMN1, HSPA8, HSP90B1, FKBP4, ALDOA
Cellular Assembly and Organization	5.7	12	PPID, HSPA8, STMN1, ACTR3, PDIA3, GRB2, YWHAZ, FKBP4, ALDOA, VIM, HSPA5, LASP1
Inflammatory Response	5.5	10	PPID, HSPA8, HSP90B1, GRB2, PDIA3, FKBP4, ALDOA, VIM, PSMB8, HSPA5
Protein Degradation	4.6	4	HSPA8, HSP90B1, PDIA3, HSPA5

We performed expression analysis of selected genes encoding DE proteins that could be regulated by DE miRNAs (Figure 26). In addition to those, we evaluated additional miRNA target genes involved in dysregulated biological functions (e.g. inflammatory response, cell death and survival; previously shown in Table 16). We confirmed over expression of PDIA3, PPID, FKBP4, and PSMB8; and down regulation of STMN1, VIM and YWHAZ. We also observed changes in expression of genes involved in innate inflammatory response (up regulation of TLR4, STAT1, STAT3 and NLRC5), antigen presentation (up regulation of CD40 and CD40L, and down regulation of MDM2), apoptosis inhibition (up regulation of BCL2),

autophagy (up regulation of SQSTM1, and down regulation of LC3) and cell death by pyroptosis (up regulation of IL1B, IFNy and CASP1) (Figure 26).

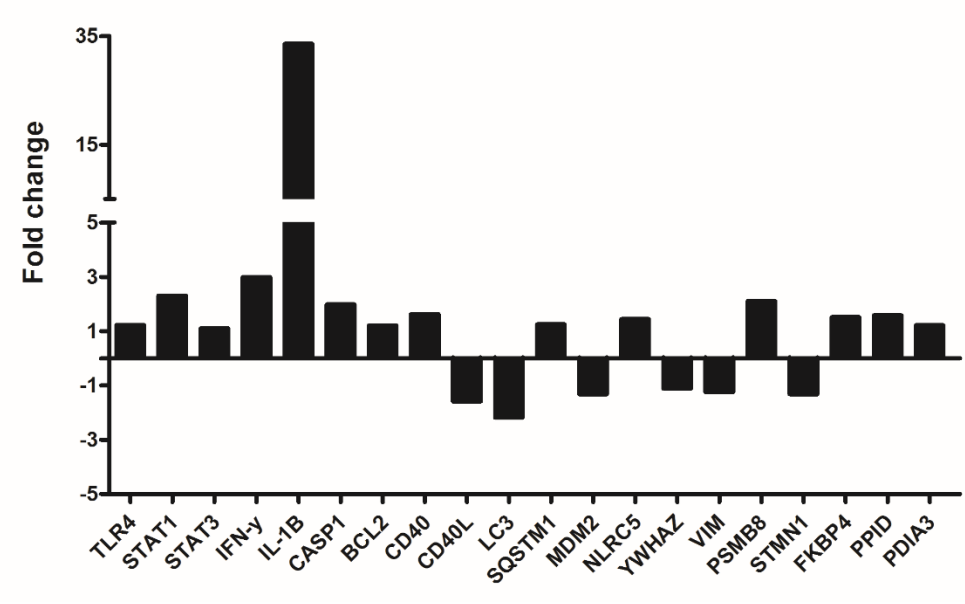


Figure 26. Expression of target genes of differentially expressed miRNAs in MLN samples at 2dpi, as well as selected genes related to biological functions affected by such dysregulation, compared to non-infected controls.

1.5. miRNA- target validation by luciferase assay.

As we have demonstrated by small RNA-seq and qPCR analyses, miR-125 is downregulated in porcine MLNs after *S*. Typhimurium infection. This miRNA is an important regulator of inflammatory response during *Salmonella* infection [140, 319]. As we observed in the interaction network between differentially expressed miRNAs and differentially expressed proteins, miR-125b-5p (down regulated) interacts with PSMB8 (up regulated), which could indicate that this miRNA is a direct regulator of the PSMB8 protein. So, based on such integrative analysis and its biological implication during *S*. Typhimurium infection (PSMB8 is involved in inflammatory response and cell death, Table 16), we designed a luciferase reporter assay to confirm if PSMB8 was target of miR-125b. For that, we performed a hybridization prediction analysis of miRNA with 3' UTR of target gene using RNAhybrid software, and used the most probable prediction (highest score) of the miRNA-target binding (Table 17).

Table 17. Prediction of target sequence and structure in miR-125b/PSMB8 binding. The highest score obtained from RNAhybrid prediction (-15 kcal/mol, scoring criteria) with the possible structure of the miRNA-MRE interaction was used for the design of the luciferase reporter assay.

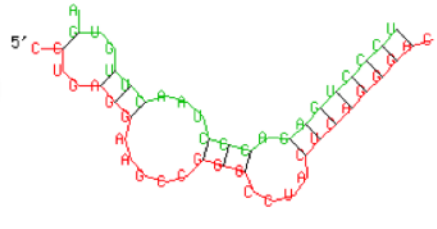
RNAhybrid results

```
dataset: 1
Target: PSMB8
length: 247
MiRNA: miR-125b-5p
length: 22

mfe: -26.4 kcal/mol
p-value: 1.000000e+00

Position: 30
target 5' C UG   AAGCC   CCUA        C 3'
           C  AGG     GGG    CUCAGGGA
           G  UUC     CCC    GAGUCCCU
miRNA  3' A UG   AAU     A            5'
```

Structure miRNA-3´UTR target



Briefly, the miRNA-target interaction was tested by transfecting CHO cells with the miR-125b-5p miRNA mimic, which were then co-transfected with a vector construction containing the interacting 3'UTR of PSMB8. Luciferase activity values obtained indicated a 31% down regulation of the luciferase activity when the miR-125b-5p mimic was present ($P<0.01$; Figure 27). Hence, these results confirm the direct interaction between miR-125b and the 3´UTR of the PSMB8 target gene, validating previous bioinformatic predictions of the interaction between the sequences.

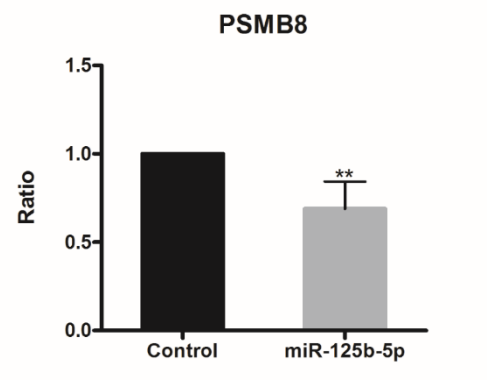


Figure 27. Luciferase reporter assay results. Firefly luciferase activity was measured and normalized by the Renilla luciferase activity. Results are represented as mean ratio +/- SEM from four independent transfection experiments. Asterisks (**) indicate $P<0.01$.

2. Discussion.

The main function of the mesenteric lymph nodes (MLN) is to act as antigen sentinels of the gastrointestinal tract. There, immune response is initiated when antigens are captured by antigen presenting cells (APC) and presented to specific B and T lymphocytes [320]. Salmonella can either travel to the MLN via the draining lymph as free extracellular bacteria, or can be transported by APCs. MLN are considered important niches for *S*. Typhimurium during pig infections. In fact, a previous work from our group showed that *S*. Typhimurium is present and able to replicate in MLN, and the most prominent transcriptomic and proteomic changes occur at 2 days post infection (mainly inflammatory response) [71, 80]. These changes are likely regulated by miRNAs, so investigation of their regulatory role is critical for understanding the host response against *S.* Typhimurium infection. In this study, we provide novel information of biological functions associated with regulatory interactions between miRNAs and their potential target proteins in infected MLN from pigs, using an integrative analysis of small RNA-seq and proteomics.

Using high throughput sequencing we evidenced the miRNAs expressed in MLN of pigs (miRNAome), finding a number of abundant miRNAs in this tissue in our two experimental situations (control and *S*. Typhimurium-infected samples). Previous studies of small RNA profiles in other tissues have shown that there are just a few different miRNAs constituting the majority of the sequencing reads [181]. Our study also found that highly abundant miRNAs such as miR-21 and miR-143 were shared between the two experimental conditions investigated, suggesting that these miRNAs are abundantly expressed in porcine mesenteric lymph nodes. In all samples miR-21 had by far the highest number of reads, constituting 17.1% and 24.5% of all miRNA reads in MLNs from control and infected pigs, respectively. This finding agrees with reports describing miR-21 as

one of the most highly expressed members of the small non coding miRNA family in many mammalian tissues [237]. When comparing to non-infected MLNs, we found that miR-21 was up regulated which is in agreement with reports that found miR-21 expression enhanced in inflammatory diseases, and demonstrating a key role of miR-21 in the pro- and anti-inflammatory response, given the broad range of target genes affected by this miRNA [321]. We suggest that the over expression of miR-21 could be mediating Salmonella-triggered inflammatory response in MLN at different stages of the immediate, early, and late inflammatory response, as well as at the resolution phase. The function of miR-21 is determined by various factors such as cell type involved, the inducing signal, and the transcriptomic profile of the cell [321]. The over expression of miR-21 in our *S*. Typhimurium-infected MLNs could be related with repression of IL1β expression, as it has been shown in other studies of inflammatory diseases [322], although the expression levels of IL1β were high in our results. This apparent disagreement is not necessarily contradictive to the nature of miRNA-mediated regulation, given that a single mRNA can be modulated by multiple miRNAs, and this regulation is very complex, multi-factorial and time dependent (e.g. samples taken at different time points of infection would result in slightly different profiles, indirect regulation of miRNAs) [181].

Other differentially expressed miRNAs from our study have also been related with inflammatory processes and bacterial infections [290, 323-325], such as miR-143, miR-155, miR-150, miR-221 and miR-125a/b. miR-155 is important in the regulation of immunity and inflammation. is miR-155. Our results of miR-155 being over expressed under pathogen-triggered inflammation are in agreement with existing literature after bacterial infections [143, 149]. Similarly to miR-21 and miR-155, down regulation of miR-125a/b has been associated to an increase of inflammatory response after Salmonella infection [319].

Interestingly for an antigen presentation organ like MLN, we identified miRNAs that could be regulating the expression of protein targets during *S*. Typhimurium infection. Down regulation of miR125a/b and miR-451a (regulating PSMB8); miR-148a/b (regulating PDIA3 and HSP90B1); as well as miR-1 and miR-130a (regulating HSPA8) could be responsible of over expression of proteins involved in MHC-I and MHC-II presentation. From these, we validated that PSMB8 was a direct target of miR-125b. PSMB8, PDIA3 and HSP90B1 are implicated in the protein processing in the immunoproteasome and endoplasmic reticulum in the MHC I class antigen presentation against bacterial infections [326]. Down regulation of miR-125a/b has been associated to inflammatory response against bacterial infection by activating macrophages, which make effector molecules (e.g. reactive oxygen, nitrogen intermediates) and inflammatory cytokines (e.g. IL1β, TNFα and IL6) [290, 327, 328]. Our miRNA-protein integrative analysis highlighted that the down regulation of miR-125a/b regulates three proteasome components: PSMB5, PSMB8 (over expressed in our study) and PSMB9. These molecules share the MHC-I pathway with HSP90B1 and PDIA3, which are regulated by miR-148a/b and miR-1, respectively [326]. PDIA3 is part of the MHC-I peptide loading complex, essential for export from endoplasmic reticulum to cell surface [329]. HSP90B1 is critical for the translocation of antigen across the endosomal membrane into the cytosol [330]. Therefore, in this study we observe that down regulation of miR-125a/b, miR-148a/b and miR-1 leads to over expression of proteins involved in MHC-I antigen presentation pathway. It is known that antigenic peptides presented by MHC-I are produced through cytosolic degradation of intracellular proteins by proteasome [331], and presentation of exogenous antigens has been classically attributed to MHC-II [332]. Simultaneously to MHC-I antigen presentation, we observed over expression of HSPA8 (HSC70) protein (target of miR-130a, down regulated in our study), which is involved in MHC-II antigen presentation. This protein plays a

central role in modulating antigen transport within cells to control MHC-II presentation [333]. Van Parys et al. described that some strains of *S.* Typhimurium have the capacity of down regulate MHC-II, increasing the bacterial persistence in tonsils and MLN [334]. However, we have demonstrated in a previous study that *S*. Typhimurium up regulated MHC-II by HSPA8 over expression, which was further confirmed by immunohistochemistry [54]. Although we observed over expressed antigen presentation MHC-I and MHC-II molecules, *S*. Typhimurium evades cell mediated immunity and is able to persist in MLN up to thirty days post infection [204, 334, 335]. Additionally, we observed over expression of miR-181a and miR-20a, which are inductors of T cell signaling that increase T cell activation through T cell receptor induction [336]. Thus, the miRNA-protein regulation results obtained in our study indicate that adaptive immunity is induced by MHC-I, which could be explained by the antigen cross presentation phenomenon, which is the ability of APCs to process exogenous antigens from microbial origin, and present them via MHC-I molecules [337]. Since Salmonella is inside host cell phagosomes, its antigens are theoretically protected from cytosolic degradation by proteasome, unless the phagosome is able to cross present antigens [78]. In this alternative scenario, phagocytosed proteins reach cytosol by chaperone retrotranslocation mechanisms, and are subsequently degraded by proteasomes, binding MHC-I molecules and activating CD8+ T-cells [78, 332].

Other mechanisms that control Salmonella spread into the host include cell death by apoptosis, autophagy and/or pyroptosis [54, 259, 338]. Over expression of miR-21 and down regulation of miR-125a (as in our results) lead to inhibition of apoptosis by BAX repression, and subsequent BCL2 induction (which is also over expressed in our study) [339-341] and direct interaction, respectively [342]. BCL2 is a major regulator of apoptosis [343], thus in *S*. Typhimurium infected MNL, the

apoptotic processes are inhibited by miR-21 and BCL2 over expression. On the other hand, we found in our miRNA differential expression data set some miRNAs involved in autophagy [344], such as miR-30a, miR-204, miR-20a/b, miR-106 and miR-363 (miR-17 family members). So, we decided explore at the gene expression level three members of the autophagy pathway: BECN1, LC3 and SQSTM1. We found a down regulation of LC3, over expression of SQTM1 and we did not see any changes in BECN1. Hence, we did not perceive induction of autophagy in MLN after *S.* Typhimurium infection, given that BECN1 and LC3 are potent autophagy inductor genes, and SQSTM1 interferes with autophagy by binding to the LC3 [344, 345]. This result agrees with the down regulation of ACTR3, YWHAZ, VIM and STMN1, genes implicated in cytoskeletal rearrangement during the autophagic process [346, 347]. Our integrative analysis showed that over expression of miR-363 and miR-92b might be down regulating ACTR3; miR-155, miR-363, miR-92b, miR-181b and miR-30b might be down regulating YWHAZ; miR-378a and miR-30b could be down regulating VIM; and miR-221 and miR-210 are likely implicated in down regulation of STMN1. Stahmin (STMN1) is a protein involved in cytoskeletal rearrangements that functions by binding to tubulin $\alpha\beta$ heterodimers (microtubules), sequestering them and preventing them from being assemble into microtubules. It's role on immune cells is contradictive, because its down regulation has been described as fundamental for microtubule stabilization and macrophage activation [348] and affects T cells polarization [349]. In our study we found that over expression of miR-221 and mir-210 could be affecting to down regulation of STMN1. Cytoskeletal rearrangements have been associated to dysregulation of these proteins in *Salmonella* infection. As part of their intracellular lifestyle, *Salmonella* survive and replicate within membrane bound vacuoles that interact with the host cell. After entry into host cells, Salmonella reverses actin cytoskeleton rearrangements (which are required for entry) and reduces inflammation [350]. ACTR3, VIM and YWHAZ are involved

in cytoskeletal rearrangements [351, 352]. Although the over expression of caspases (observed in our study) have been related with decrease of cytoskeletal proteins [353, 354], we observed in our study that the down regulation of these proteins could be caused by the over expression of miR-363, miR-92b (ACTR3); miR-155, miR-363, miR-92b, miR-181b, miR-30b (YWHAZ); miR-378a, and miR-30b (VIM). Previous studies have shown that a high expression of IL1β and CASP1 could indicate the induction of pyroptosis in MLN after *S.* Typhimurium infection [54]. The over expression of these genes were confirmed in our study, but we did not find any miRNA or protein related with this type of cell death mechanism; instead, we found an interaction between miR-30d and PPID, a potent inductor of necroptosis [355-357].

In conclusion, integrative proteomic and miRNA analysis revealed that the miRNA-protein regulatory network is more complex than previously thought, highlighting that a single miRNA can regulate multiple target mRNAs and vice versa. Differential expression of miRNAs could be regulating the expression of proteins involved in networks critical for antigenic presentation, inflammatory response and cytoskeletal rearrangements through various signaling pathways. Our study provides novel evidence for functional molecular networks in MLN after *S*. Typhimurium infection.

CONCLUSIONS

1. Our results revealed that jejunum, ileum and colon respond differently to infection. We observed a mild transcriptional response in jejunum, affected only at 2 dpi. The higher transcriptional response was obtained in ileum.
2. *S.* Typhimurium colonization was higher in ileum at 2 dpi, where we found the strongest inflammatory response, which leaded to an upregulation of cytokines, chemokines and other inflammatory genes.
3. Lipid metabolism and its related pathways (retinoid X receptor (RXR) related pathways such as LPS/IL-1 mediated inhibition of RXR function, LXR/RXR activation, FXR/RXR activation and hepatic cholestasis) were highly dysregulated by *S*. Typhimurium infection at 1 and 2 dpi in ileum, leading to the disruption of the normal lipid/bile acid absorption pathway.
4. The small RNA sequencing of the S. typhimurium infection released an enormous amount of novel data in ileum and mesenteric lymph node, characterizing the host miRNAome more accurately than the expression microarray (better correlation with qPCR validation results).
5. This study showed miRNA expression changes in porcine ileum and mesenteric lymph node induced by *S.* Typhimurium infection, and its possible effects in post-transcriptional regulation of target genes and immune response to bacterial pathogens.
6. miR-194 represses YWHAZ (critical regulator of IL-6, TNFα, CCL5, IFN and CCL3 expression), and TLR4 (one of the most important receptors of inflammatory response to S. Typhimurium). Thus, down regulation of miR-194 may increase the inflammatory response in ileum.

7. Down regulation of the miR-200 family, miR-194, miR-192 and miR-215 induce epithelial mesenchymal transition in ileum after *S*. Typhimurium infection, supported by the up regulation of ZEB1/2 and SNAI1/2, and repression of p53. Additionally, the over expression of FOXA1 (target of miR-194) could be associated to renewal at the intestinal epithelial surface, since is a critical inductor of mucins and goblet cells' proliferation.
8. Over expression of miR-223 induces a buffering effect on inflammatory response, having a negative effect in the expression of its target genes TLR4, STAT3 and RAB10, which subsequently have a significant impact on expression of pro-inflammatory cytokines as IL8, IL6 and IL1A.
9. In mesenteric lymph nodes from *S*. Typhimurium infected pigs we identified several differentially expressed miRNAs such as miR-21, miR-143, miR-155, miR-150, miR-221 and miR-125a/b, involved in inflammatory processes and bacterial infections.
10. Down regulation of miR125a/b, miR-451a miR-148a/b, miR-1 and miR-130a (involved in over expression of PSMB8, PDIA3, HSP90B1 and HSPA8 proteins) increased MHC-I and MHC-II antigen presentation in mesenteric lymph node.
11. miR-21 up regulation and miR-125a downregulation inhibit cell death by apoptosis via BCL2 induction. However, we found that miR-30d down expression and PPID over expression induce cell death by necroptosis.
12. Down regulation of miR-363, miR-92b, miR-155, miR-363, miR-92b, miR-181b, miR-30b, miR-378a and miR-30b allow up regulation of proteins ACTR3, VIM, YWHAZ and STMN1, involved in cytoskeleton rearrangements necessary for phagosome formation and pathogen replication.

BIBLIOGRAPHY

1. Broz P, Ohlson MB, Monack DM: **Innate immune response to Salmonella typhimurium, a model enteric pathogen**. *Gut Microbes* 2012, **3**(2):62-70.
2. Chaplin DD: **Overview of the immune response**. *J Allergy Clin Immunol* 2010, **125**(2 Suppl 2):S3-23.
3. Uthaisangsook S, Day NK, Bahna SL, Good RA, Haraguchi S: **Innate immunity and its role against infections**. *Ann Allergy Asthma Immunol* 2002, **88**(3):253-264; quiz 265-256, 318.
4. Castelo-Branco C, Soveral I: **The immune system and aging: a review**. *Gynecol Endocrinol* 2014, **30**(1):16-22.
5. Parkin J, Cohen B: **An overview of the immune system**. *Lancet* 2001, **357**(9270):1777-1789.
6. Chua WJ, Hansen TH: **Bacteria, mucosal-associated invariant T cells and MR1**. *Immunol Cell Biol* 2010, **88**(8):767-769.
7. Zhu J, Yamane H, Paul WE: **Differentiation of effector CD4 T cell populations (*)**. *Annu Rev Immunol* 2010, **28**:445-489.
8. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT: **Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages**. *Nat Immunol* 2005, **6**(11):1123-1132.
9. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q *et al*: **A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17**. *Nat Immunol* 2005, **6**(11):1133-1141.

10. Wan YY, Flavell RA: **'Yin-Yang' functions of transforming growth factor-beta and T regulatory cells in immune regulation**. *Immunol Rev* 2007, **220**:199-213.

11. Cuvelier C, Demetter P, Mielants H, Veys EM, De Vos M: **Interpretation of ileal biopsies: morphological features in normal and diseased mucosa**. *Histopathology* 2001, **38**(1):1-12.

12. Tyrer P, Foxwell AR, Cripps AW, Apicella MA, Kyd JM: **Microbial pattern recognition receptors mediate M-cell uptake of a gram-negative bacterium**. *Infect Immun* 2006, **74**(1):625-631.

13. Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl JP, Ricciardi-Castagnoli P: **Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria**. *Nat Immunol* 2001, **2**(4):361-367.

14. Hapfelmeier S, Stecher B, Barthel M, Kremer M, Müller AJ, Heikenwalder M, Stallmach T, Hensel M, Pfeffer K, Akira S *et al*: **The Salmonella pathogenicity island (SPI)-2 and SPI-1 type III secretion systems allow Salmonella serovar typhimurium to trigger colitis via MyD88-dependent and MyD88-independent mechanisms**. *J Immunol* 2005, **174**(3):1675-1685.

15. Ruth MR, Field CJ: **The immune modifying effects of amino acids on gut-associated lymphoid tissue**. *J Anim Sci Biotechnol* 2013, **4**(1):27.

16. Wells JM, Loonen LM, Karczewski JM: **The role of innate signaling in the homeostasis of tolerance and immunity in the intestine**. *Int J Med Microbiol* 2010, **300**(1):41-48.

17. Makala LH, Suzuki N, Nagasawa H: **Peyer's patches: organized lymphoid structures for the induction of mucosal immune responses in the intestine**. *Pathobiology* 2002, **70**(2):55-68.

18. Newberry RD, Lorenz RG: **Organizing a mucosal defense**. *Immunol Rev* 2005, **206**:6-21.

19. Rothkötter HJ: **Anatomical particularities of the porcine immune system--a physician's view**. *Dev Comp Immunol* 2009, **33**(3):267-272.

20. Brandtzaeg P: **Mucosal immunity: induction, dissemination, and effector functions**. *Scand J Immunol* 2009, **70**(6):505-515.

21. Wang Y, Qu L, Uthe JJ, Bearson SM, Kuhar D, Lunney JK, Couture OP, Nettleton D, Dekkers JC, Tuggle CK: **Global transcriptional response of porcine mesenteric lymph nodes to Salmonella enterica serovar Typhimurium**. *Genomics* 2007, **90**(1):72-84.

22. Binns RM, Pabst R: **Lymphoid tissue structure and lymphocyte trafficking in the pig**. *Vet Immunol Immunopathol* 1994, **43**(1-3):79-87.

23. Dunkley KD, Callaway TR, Chalova VI, McReynolds JL, Hume ME, Dunkley CS, Kubena LF, Nisbet DJ, Ricke SC: **Foodborne Salmonella ecology in the avian gastrointestinal tract**. *Anaerobe* 2009, **15**(1-2):26-35.

24. Jacobsen A, Hendriksen RS, Aaresturp FM, Ussery DW, Friis C: **The Salmonella enterica pan-genome**. *Microb Ecol* 2011, **62**(3):487-504.

25. Smadi H, Sargeant JM, Shannon HS, Raina P: **Growth and inactivation of Salmonella at low refrigerated storage temperatures and thermal inactivation on raw chicken meat and laboratory media: mixed effect meta-analysis**. *J Epidemiol Glob Health* 2012, **2**(4):165-179.

26. Chen HM, Wang Y, Su LH, Chiu CH: **Nontyphoid salmonella infection: microbiology, clinical features, and antimicrobial therapy**. *Pediatr Neonatol* 2013, **54**(3):147-152.

27. Control EFSAaECfDPa: **The European Union summary report on trends and sources of**

zoonoses, zoonotic agents and food-borne outbreaks in 2014. In., vol. 13. EFSA Journal; 2015: 4329.

28. Oliveira CJ, Carvalho LF, Garcia TB: **Experimental airborne transmission of Salmonella Agona and Salmonella Typhimurium in weaned pigs**. *Epidemiol Infect* 2006, **134**(1):199-209.

29. Ibarra JA, Steele-Mortimer O: **Salmonella--the ultimate insider. Salmonella virulence factors that modulate intracellular survival**. *Cell Microbiol* 2009, **11**(11):1579-1586.

30. de Jong HK, Parry CM, van der Poll T, Wiersinga WJ: **Host-pathogen interaction in invasive Salmonellosis**. *PLoS Pathog* 2012, **8**(10):e1002933.

31. Elder JR, Chiok KL, Paul NC, Haldorson G, Guard J, Shah DH: **The Salmonella pathogenicity island 13 contributes to pathogenesis in streptomycin pre-treated mice but not in day-old chickens**. *Gut Pathog* 2016, **8**:16.

32. Kaur J, Jain SK: **Role of antigens and virulence factors of Salmonella enterica serovar Typhi in its pathogenesis**. *Microbiol Res* 2012, **167**(4):199-210.

33. Gunn JS, Marshall JM, Baker S, Dongol S, Charles RC, Ryan ET: **Salmonella chronic carriage: epidemiology, diagnosis, and gallbladder persistence**. *Trends Microbiol* 2014, **22**(11):648-655.

34. Boyen F, Haesebrouck F, Maes D, Van Immerseel F, Ducatelle R, Pasmans F: **Non-typhoidal Salmonella infections in pigs: a closer look at epidemiology, pathogenesis and control**. *Vet Microbiol* 2008, **130**(1-2):1-19.

35. Knodler LA: **Salmonella enterica: living a double life in epithelial cells**. *Curr Opin Microbiol* 2015, **23**:23-31.

36. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A, Hoekstra RM, Studies ICoEDBoI: **The global burden of nontyphoidal Salmonella gastroenteritis**. *Clin Infect Dis* 2010, **50**(6):882-889.

37. Herikstad H, Motarjemi Y, Tauxe RV: **Salmonella surveillance: a global survey of public health serotyping**. *Epidemiol Infect* 2002, **129**(1):1-8.

38. Heyndrickx M, Pasmans F, Ducatelle R, Decostere A, Haesebrouck F: **Recent changes in Salmonella nomenclature: the need for clarification**. *Vet J* 2005, **170**(3):275-277.

39. Tindall BJ, Grimont PA, Garrity GM, Euzéby JP: **Nomenclature and taxonomy of the genus Salmonella**. *Int J Syst Evol Microbiol* 2005, **55**(Pt 1):521-524.

40. Moffatt CR, Musto J, Pingault N, Miller M, Stafford R, Gregory J, Polkinghorne BG, Kirk MD: **Salmonella Typhimurium and Outbreaks of Egg-Associated Disease in Australia, 2001 to 2011**. *Foodborne Pathog Dis* 2016, **13**(7):379-385.

41. Pires SM, Vigre H, Makela P, Hald T: **Using outbreak data for source attribution of human salmonellosis and campylobacteriosis in Europe**. *Foodborne Pathog Dis* 2010, **7**(11):1351-1361.

42. Wales AD, Davies RH: **Salmonella Vaccination in Pigs: A Review**. *Zoonoses Public Health* 2016.

43. DE Knegt LV, Pires SM, Hald T: **Attributing foodborne salmonellosis in humans to animal reservoirs in the European Union using a multi-country stochastic model**. *Epidemiol Infect* 2015, **143**(6):1175-1186.

44. Evangelopoulou G, Kritas S, Christodoulopoulos G, Burriel AR: **The commercial impact of pig Salmonella spp. infections in border-free markets during an economic recession**. *Vet World* 2015, **8**(3):257-272.

45. Zhang Z, Liu Z: **Paneth cells: the hub for sensing and regulating intestinal flora**. *Sci China Life Sci* 2016, **59**(5):463-467.

46. Haraga A, Ohlson MB, Miller SI: **Salmonellae interplay with host cells**. *Nat Rev Microbiol* 2008, **6**(1):53-66.

47. Medzhitov R: **Toll-like receptors and innate immunity**. *Nat Rev Immunol* 2001, **1**(2):135-145.

48. Broz P, Monack DM: **Molecular mechanisms of inflammasome activation during microbial infections**. *Immunol Rev* 2011, **243**(1):174-190.

49. Triantafilou M, Hughes TR, Morgan BP, Triantafilou K: **Complementing the inflammasome**. *Immunology* 2016, **147**(2):152-164.

50. Bergsbaken T, Fink SL, Cookson BT: **Pyroptosis: host cell death and inflammation**. *Nat Rev Microbiol* 2009, **7**(2):99-109.

51. Miao EA, Leaf IA, Treuting PM, Mao DP, Dors M, Sarkar A, Warren SE, Wewers MD, Aderem A: **Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria**. *Nat Immunol* 2010, **11**(12):1136-1142.

52. Pham OH, McSorley SJ: **Protective host immune responses to Salmonella infection**. *Future Microbiol* 2015, **10**(1):101-110.

53. Collado-Romero M, Martins RP, Arce C, Moreno Á, Lucena C, Carvajal A, Garrido JJ: **An in vivo proteomic study of the interaction between Salmonella Typhimurium and porcine ileum mucosa**. *J Proteomics* 2012, **75**(7):2015-2026.

54. Martins RP, Aguilar C, Graham JE, Carvajal A, Bautista R, Claros MG, Garrido JJ: **Pyroptosis and adaptive immunity mechanisms are promptly engendered in mesenteric lymph-nodes during pig infections with Salmonella enterica serovar Typhimurium**. *Vet Res* 2013, **44**:120.

55. Srinivasan A, Salazar-Gonzalez RM, Jarcho M, Sandau MM, Lefrancois L, McSorley SJ: **Innate immune activation of CD4 T cells in salmonella-**

infected mice is dependent on IL-18. *J Immunol* 2007, **178**(10):6342-6349.

56. Winter SE, Thiennimitr P, Nuccio SP, Haneda T, Winter MG, Wilson RP, Russell JM, Henry T, Tran QT, Lawhon SD *et al*: **Contribution of flagellin pattern recognition to intestinal inflammation during Salmonella enterica serotype typhimurium infection**. *Infect Immun* 2009, **77**(5):1904-1916.

57. Spits H, Di Santo JP: **The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling**. *Nat Immunol* 2011, **12**(1):21-27.

58. Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, Doherty JM, Mills JC, Colonna M: **A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity**. *Nature* 2009, **457**(7230):722-725.

59. Tumanov AV, Koroleva EP, Guo X, Wang Y, Kruglov A, Nedospasov S, Fu YX: **Lymphotoxin controls the IL-22 protection pathway in gut innate lymphoid cells during mucosal pathogen challenge**. *Cell Host Microbe* 2011, **10**(1):44-53.

60. Goto M, Murakawa M, Kadoshima-Yamaoka K, Tanaka Y, Nagahira K, Fukuda Y, Nishimura T: **Murine NKT cells produce Th17 cytokine interleukin-22**. *Cell Immunol* 2009, **254**(2):81-84.

61. Ivanov II, Frutos ReL, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, Finlay BB, Littman DR: **Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine**. *Cell Host Microbe* 2008, **4**(4):337-349.

62. Godinez I, Raffatellu M, Chu H, Paixão TA, Haneda T, Santos RL, Bevins CL, Tsolis RM, Bäumler AJ: **Interleukin-23 orchestrates mucosal responses to**

Salmonella enterica serotype Typhimurium in the intestine. *Infect Immun* 2009, **77**(1):387-398.

63. Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, Oliver P, Huang W, Zhang P, Zhang J *et al*: **Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense**. *J Exp Med* 2001, **194**(4):519-527.

64. Noriega LM, Van der Auwera P, Daneau D, Meunier F, Aoun M: **Salmonella infections in a cancer center**. *Support Care Cancer* 1994, **2**(2):116-122.

65. Aujla SJ, Dubin PJ, Kolls JK: **Th17 cells and mucosal host defense**. *Semin Immunol* 2007, **19**(6):377-382.

66. Conlan JW: **Neutrophils prevent extracellular colonization of the liver microvasculature by Salmonella typhimurium**. *Infect Immun* 1996, **64**(3):1043-1047.

67. Tsolis RM, Adams LG, Ficht TA, Bäumler AJ: **Contribution of Salmonella typhimurium virulence factors to diarrheal disease in calves**. *Infect Immun* 1999, **67**(9):4879-4885.

68. Wick MJ: **Innate immune control of Salmonella enterica serovar Typhimurium: mechanisms contributing to combating systemic Salmonella infection**. *J Innate Immun* 2011, **3**(6):543-549.

69. Griffin AJ, McSorley SJ: **Development of protective immunity to Salmonella, a mucosal pathogen with a systemic agenda**. *Mucosal Immunol* 2011, **4**(4):371-382.

70. Monack DM, Bouley DM, Falkow S: **Salmonella typhimurium persists within macrophages in the mesenteric lymph nodes of chronically infected Nramp1+/+ mice and can be reactivated by IFNgamma neutralization**. *J Exp Med* 2004, **199**(2):231-241.

71. Martins RP, Collado-Romero M, Martínez-Gomáriz M, Carvajal A, Gil C, Lucena C, Moreno A, Garrido JJ: **Proteomic analysis of porcine mesenteric lymph-nodes after Salmonella typhimurium infection**. *J Proteomics* 2012, **75**(14):4457-4470.

72. Dickson JaH, H: **Salmonella in the Pork Production Chain**. In., vol. 800-456-7675. Pork Safety Fact Sheet: National Pork Board.

73. Martins RP, Lorenzi V, Arce C, Lucena C, Carvajal A, Garrido JJ: **Innate and adaptive immune mechanisms are effectively induced in ileal Peyer's patches of Salmonella typhimurium infected pigs**. *Dev Comp Immunol* 2013, **41**(1):100-104.

74. Behnsen J, Perez-Lopez A, Nuccio SP, Raffatellu M: **Exploiting host immunity: the Salmonella paradigm**. *Trends Immunol* 2015, **36**(2):112-120.

75. Ravindran R, Foley J, Stoklasek T, Glimcher LH, McSorley SJ: **Expression of T-bet by CD4 T cells is essential for resistance to Salmonella infection**. *J Immunol* 2005, **175**(7):4603-4610.

76. Harris DP, Haynes L, Sayles PC, Duso DK, Eaton SM, Lepak NM, Johnson LL, Swain SL, Lund FE: **Reciprocal regulation of polarized cytokine production by effector B and T cells**. *Nat Immunol* 2000, **1**(6):475-482.

77. Ugrinovic S, Brooks CG, Robson J, Blacklaws BA, Hormaeche CE, Robinson JH: **H2-M3 major histocompatibility complex class Ib-restricted CD8 T cells induced by Salmonella enterica serovar Typhimurium infection recognize proteins released by Salmonella serovar Typhimurium**. *Infect Immun* 2005, **73**(12):8002-8008.

78. Houde M, Bertholet S, Gagnon E, Brunet S, Goyette G, Laplante A, Princiotta MF, Thibault P, Sacks D, Desjardins M: **Phagosomes are competent organelles for antigen cross-presentation**. *Nature* 2003, **425**(6956):402-406.

79. Kovacsovics-Bankowski M, Clark K, Benacerraf B, Rock KL: **Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages**. *Proc Natl Acad Sci U S A* 1993, **90**(11):4942-4946.

80. Martins RP, Collado-Romero M, Arce C, Lucena C, Carvajal A, Garrido JJ: **Exploring the immune response of porcine mesenteric lymph nodes to Salmonella enterica serovar Typhimurium: an analysis of transcriptional changes, morphological alterations and pathogen burden**. *Comp Immunol Microbiol Infect Dis* 2013, **36**(2):149-160.

81. Uthe JJ, Royaee A, Lunney JK, Stabel TJ, Zhao SH, Tuggle CK, Bearson SM: **Porcine differential gene expression in response to Salmonella enterica serovars Choleraesuis and Typhimurium**. *Mol Immunol* 2007, **44**(11):2900-2914.

82. Brennan MA, Cookson BT: **Salmonella induces macrophage death by caspase-1-dependent necrosis**. *Mol Microbiol* 2000, **38**(1):31-40.

83. Fink SL, Cookson BT: **Pyroptosis and host cell death responses during Salmonella infection**. *Cell Microbiol* 2007, **9**(11):2562-2570.

84. van der Velden AW, Velasquez M, Starnbach MN: **Salmonella rapidly kill dendritic cells via a caspase-1-dependent mechanism**. *J Immunol* 2003, **171**(12):6742-6749.

85. McSorley SJ: **Immunity to intestinal pathogens: lessons learned from Salmonella**. *Immunol Rev* 2014, **260**(1):168-182.

86. Lee RC, Feinbaum RL, Ambros V: **The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14**. *Cell* 1993, **75**(5):843-854.

87. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G: **The 21-nucleotide let-7 RNA regulates**

developmental timing in Caenorhabditis elegans. *Nature* 2000, **403**(6772):901-906.

88. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T: **Identification of novel genes coding for small expressed RNAs**. *Science* 2001, **294**(5543):853-858.

89. Ambros V, Bartel B, Bartel DP, Burge CB, Carrington JC, Chen X, Dreyfuss G, Eddy SR, Griffiths-Jones S, Marshall M *et al*: **A uniform system for microRNA annotation**. *RNA* 2003, **9**(3):277-279.

90. Lee RC, Ambros V: **An extensive class of small RNAs in Caenorhabditis elegans**. *Science* 2001, **294**(5543):862-864.

91. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ: **miRBase: tools for microRNA genomics**. *Nucleic Acids Res* 2008, **36**(Database issue):D154-158.

92. Berezikov E, Chung WJ, Willis J, Cuppen E, Lai EC: **Mammalian mirtron genes**. *Mol Cell* 2007, **28**(2):328-336.

93. Fromm B, Billipp T, Peck LE, Johansen M, Tarver JE, King BL, Newcomb JM, Sempere LF, Flatmark K, Hovig E *et al*: **A Uniform System for the Annotation of Vertebrate microRNA Genes and the Evolution of the Human microRNAome**. *Annu Rev Genet* 2015, **49**:213-242.

94. Winter J, Jung S, Keller S, Gregory RI, Diederichs S: **Many roads to maturity: microRNA biogenesis pathways and their regulation**. *Nat Cell Biol* 2009, **11**(3):228-234.

95. Ameres SL, Zamore PD: **Diversifying microRNA sequence and function**. *Nat Rev Mol Cell Biol* 2013, **14**(8):475-488.

96. Meister G: **Argonaute proteins: functional insights and emerging roles**. *Nat Rev Genet* 2013, **14**(7):447-459.

97. Vasudevan S, Tong Y, Steitz JA: **Switching from repression to activation: microRNAs can up-regulate translation**. *Science* 2007, **318**(5858):1931-1934.

98. Ritchie W, Rajasekhar M, Flamant S, Rasko JE: **Conserved expression patterns predict microRNA targets**. *PLoS Comput Biol* 2009, **5**(9):e1000513.

99. Lee S, Vasudevan S: **Post-transcriptional stimulation of gene expression by microRNAs**. *Adv Exp Med Biol* 2013, **768**:97-126.

100. Hu W, Coller J: **What comes first: translational repression or mRNA degradation? The deepening mystery of microRNA function**. *Cell Res* 2012, **22**(9):1322-1324.

101. Bazzini AA, Lee MT, Giraldez AJ: **Ribosome profiling shows that miR-430 reduces translation before causing mRNA decay in zebrafish**. *Science* 2012, **336**(6078):233-237.

102. Djuranovic S, Nahvi A, Green R: **miRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay**. *Science* 2012, **336**(6078):237-240.

103. Guo H, Ingolia NT, Weissman JS, Bartel DP: **Mammalian microRNAs predominantly act to decrease target mRNA levels**. *Nature* 2010, **466**(7308):835-840.

104. Wang D, Zhang Z, O'Loughlin E, Lee T, Houel S, O'Carroll D, Tarakhovsky A, Ahn NG, Yi R: **Quantitative functions of Argonaute proteins in mammalian development**. *Genes Dev* 2012, **26**(7):693-704.

105. Bartel DP: **MicroRNAs: target recognition and regulatory functions**. *Cell* 2009, **136**(2):215-233.

106. Landthaler M, Gaidatzis D, Rothballer A, Chen PY, Soll SJ, Dinic L, Ojo T, Hafner M, Zavolan M, Tuschl T: **Molecular characterization of human**

Argonaute-containing ribonucleoprotein complexes and their bound target mRNAs. *RNA* 2008, **14**(12):2580-2596.

107. Hafner M, Landthaler M, Burger L, Khorshid M, Hausser J, Berninger P, Rothballer A, Ascano M, Jungkamp AC, Munschauer M *et al*: **Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP**. *Cell* 2010, **141**(1):129-141.

108. Burroughs AM, Ando Y, de Hoon MJ, Tomaru Y, Suzuki H, Hayashizaki Y, Daub CO: **Deep-sequencing of human Argonaute-associated small RNAs provides insight into miRNA sorting and reveals Argonaute association with RNA fragments of diverse origin**. *RNA Biol* 2011, **8**(1):158-177.

109. Dueck A, Ziegler C, Eichner A, Berezikov E, Meister G: **microRNAs associated with the different human Argonaute proteins**. *Nucleic Acids Res* 2012, **40**(19):9850-9862.

110. Reczko M, Maragkakis M, Alexiou P, Grosse I, Hatzigeorgiou AG: **Functional microRNA targets in protein coding sequences**. *Bioinformatics* 2012, **28**(6):771-776.

111. Marín RM, Sulc M, Vanícek J: **Searching the coding region for microRNA targets**. *RNA* 2013, **19**(4):467-474.

112. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM: **Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs**. *Nature* 2005, **433**(7027):769-773.

113. Lewis BP, Burge CB, Bartel DP: **Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets**. *Cell* 2005, **120**(1):15-20.

114. Hashimoto Y, Akiyama Y, Yuasa Y: **Multiple-to-multiple relationships between microRNAs and target genes in gastric cancer**. *PLoS One* 2013, **8**(5):e62589.

115. Amirkhah R, Farazmand A, Gupta SK, Ahmadi H, Wolkenhauer O, Schmitz U: **Naïve Bayes classifier predicts functional microRNA target interactions in colorectal cancer**. *Mol Biosyst* 2015, **11**(8):2126-2134.

116. Cho S, Jun Y, Lee S, Choi HS, Jung S, Jang Y, Park C, Kim S, Kim W: **miRGator v2.0: an integrated system for functional investigation of microRNAs**. *Nucleic Acids Res* 2011, **39**(Database issue):D158-162.

117. Alexiou P, Maragkakis M, Papadopoulos GL, Reczko M, Hatzigeorgiou AG: **Lost in translation: an assessment and perspective for computational microRNA target identification**. *Bioinformatics* 2009, **25**(23):3049-3055.

118. John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS: **Human MicroRNA targets**. *PLoS Biol* 2004, **2**(11):e363.

119. Lall S, Grün D, Krek A, Chen K, Wang YL, Dewey CN, Sood P, Colombo T, Bray N, Macmenamin P *et al*: **A genome-wide map of conserved microRNA targets in C. elegans**. *Curr Biol* 2006, **16**(5):460-471.

120. Rehmsmeier M, Steffen P, Hochsmann M, Giegerich R: **Fast and effective prediction of microRNA/target duplexes**. *RNA* 2004, **10**(10):1507-1517.

121. Marín RM, Vanícek J: **Efficient use of accessibility in microRNA target prediction**. *Nucleic Acids Res* 2011, **39**(1):19-29.

122. Li Y, Kowdley KV: **MicroRNAs in common human diseases**. *Genomics Proteomics Bioinformatics* 2012, **10**(5):246-253.

123. Gulyaeva LF, Kushlinskiy NE: **Regulatory mechanisms of microRNA expression**. *J Transl Med* 2016, **14**(1):143.

124. Bayoumi AS, Sayed A, Broskova Z, Teoh JP, Wilson J, Su H, Tang YL, Kim IM: **Crosstalk between Long Noncoding RNAs and MicroRNAs in Health and Disease**. *Int J Mol Sci* 2016, **17**(3):356.

125. Wang J, Chen J, Sen S: **MicroRNA as Biomarkers and Diagnostics**. *J Cell Physiol* 2016, **231**(1):25-30.

126. Sayed D, Abdellatif M: **MicroRNAs in development and disease**. *Physiol Rev* 2011, **91**(3):827-887.

127. Navickas R, Gal D, Laucevičius A, Taparauskaitė A, Zdanytė M, Holvoet P: **Identifying circulating microRNAs as biomarkers of cardiovascular disease: a systematic review**. *Cardiovasc Res* 2016.

128. Vijayan M, Reddy PH: **Peripheral biomarkers of stroke: Focus on circulatory microRNAs**. *Biochim Biophys Acta* 2016, **1862**(10):1984-1993.

129. Lu M, Zhang Q, Deng M, Miao J, Guo Y, Gao W, Cui Q: **An analysis of human microRNA and disease associations**. *PLoS One* 2008, **3**(10):e3420.

130. Backes C, Meese E, Keller A: **Specific miRNA Disease Biomarkers in Blood, Serum and Plasma: Challenges and Prospects**. *Mol Diagn Ther* 2016.

131. Chaussabel D, Semnani RT, McDowell MA, Sacks D, Sher A, Nutman TB: **Unique gene expression profiles of human macrophages and dendritic cells to phylogenetically distinct parasites**. *Blood* 2003, **102**(2):672-681.

132. Liu J, Cao X: **Regulatory dendritic cells in autoimmunity: A comprehensive review**. *J Autoimmun* 2015, **63**:1-12.

133. Fairfax BP, Humburg P, Makino S, Naranbhai V, Wong D, Lau E, Jostins L, Plant K, Andrews R, McGee C *et al*: **Innate immune activity conditions the effect of regulatory variants upon monocyte gene expression**. *Science* 2014, **343**(6175):1246949.

134. Gat-Viks I, Chevrier N, Wilentzik R, Eisenhaure T, Raychowdhury R, Steuerman Y, Shalek AK, Hacohen N, Amit I, Regev A: **Deciphering molecular circuits from genetic variation underlying transcriptional responsiveness to stimuli**. *Nat Biotechnol* 2013, **31**(4):342-349.

135. O'Connell RM, Rao DS, Baltimore D: **microRNA regulation of inflammatory responses**. *Annu Rev Immunol* 2012, **30**:295-312.

136. Garo LP, Murugaiyan G: **The Use of MiRNA Antagonists in the Alleviation of Inflammatory Disorders**. *Methods Mol Biol* 2016, **1390**:413-425.

137. Eulalio A, Schulte L, Vogel J: **The mammalian microRNA response to bacterial infections**. *RNA Biol* 2012, **9**(6):742-750.

138. Staff PG: **Correction: Bacterial Infection Drives the Expression Dynamics of microRNAs and Their isomiRs**. *PLoS Genet* 2015, **11**(6):e1005321.

139. Taganov KD, Boldin MP, Chang KJ, Baltimore D: **NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses**. *Proc Natl Acad Sci U S A* 2006, **103**(33):12481-12486.

140. Schulte LN, Westermann AJ, Vogel J: **Differential activation and functional specialization of miR-146 and miR-155 in innate immune sensing**. *Nucleic Acids Res* 2013, **41**(1):542-553.

141. Duerr CU, Hornef MW: **The mammalian intestinal epithelium as integral player in the establishment and maintenance of host-microbial homeostasis**. *Semin Immunol* 2012, **24**(1):25-35.

142. Quinn EM, Wang JH, O'Callaghan G, Redmond HP: **MicroRNA-146a is upregulated by and negatively regulates TLR2 signaling**. *PLoS One* 2013, **8**(4):e62232.

143. O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D: **MicroRNA-155 is induced during the macrophage inflammatory response**. *Proc Natl Acad Sci U S A* 2007, **104**(5):1604-1609.

144. Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B, Fabbri M, Alder H, Liu CG, Calin GA *et al*: **Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock**. *J Immunol* 2007, **179**(8):5082-5089.

145. Elton TS, Selemon H, Elton SM, Parinandi NL: **Regulation of the MIR155 host gene in physiological and pathological processes**. *Gene* 2013, **532**(1):1-12.

146. Lu LF, Thai TH, Calado DP, Chaudhry A, Kubo M, Tanaka K, Loeb GB, Lee H, Yoshimura A, Rajewsky K *et al*: **Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein**. *Immunity* 2009, **30**(1):80-91.

147. Billeter AT, Hellmann J, Roberts H, Druen D, Gardner SA, Sarojini H, Galandiuk S, Chien S, Bhatnagar A, Spite M *et al*: **MicroRNA-155 potentiates the inflammatory response in hypothermia by suppressing IL-10 production**. *FASEB J* 2014, **28**(12):5322-5336.

148. Kurowska-Stolarska M, Alivernini S, Ballantine LE, Asquith DL, Millar NL, Gilchrist DS, Reilly J, Ierna M, Fraser AR, Stolarski B *et al*: **MicroRNA-155 as a proinflammatory regulator in clinical and experimental arthritis**. *Proc Natl Acad Sci U S A* 2011, **108**(27):11193-11198.

149. Imaizumi T, Tanaka H, Tajima A, Yokono Y, Matsumiya T, Yoshida H, Tsuruga K, Aizawa-Yashiro T, Hayakari R, Inoue I *et al*: **IFN-γ and TNF-α synergistically induce microRNA-155 which regulates TAB2/IP-10 expression in human mesangial cells**. *Am J Nephrol* 2010, **32**(5):462-468.

150. Duval M, Cossart P, Lebreton A: **Mammalian microRNAs and long noncoding RNAs in the host-bacterial pathogen crosstalk**. *Semin Cell Dev Biol* 2016.

151. Das K, Garnica O, Dhandayuthapani S: **Modulation of Host miRNAs by Intracellular Bacterial Pathogens**. *Front Cell Infect Microbiol* 2016, **6**:79.

152. Schulte LN, Eulalio A, Mollenkopf HJ, Reinhardt R, Vogel J: **Analysis of the host microRNA response to Salmonella uncovers the control of major cytokines by the let-7 family**. *EMBO J* 2011, **30**(10):1977-1989.

153. Sharbati S, Sharbati J, Hoeke L, Bohmer M, Einspanier R: **Quantification and accurate normalisation of small RNAs through new custom RT-qPCR arrays demonstrates Salmonella-induced microRNAs in human monocytes**. *BMC Genomics* 2012, **13**:23.

154. Liu Y, Chen Q, Song Y, Lai L, Wang J, Yu H, Cao X, Wang Q: **MicroRNA-98 negatively regulates IL-10 production and endotoxin tolerance in macrophages after LPS stimulation**. *FEBS Lett* 2011, **585**(12):1963-1968.

155. Maudet C, Mano M, Sunkavalli U, Sharan M, Giacca M, Förstner KU, Eulalio A: **Functional high-throughput screening identifies the miR-15 microRNA family as cellular restriction factors for Salmonella infection**. *Nat Commun* 2014, **5**:4718.

156. Verma S, Mohapatra G, Ahmad SM, Rana S, Jain S, Khalsa JK, Srikanth CV: **Salmonella Engages Host MicroRNAs To Modulate SUMOylation: a New Arsenal for Intracellular Survival**. *Mol Cell Biol* 2015, **35**(17):2932-2946.

157. Hoeke L, Sharbati J, Pawar K, Keller A, Einspanier R, Sharbati S: **Intestinal Salmonella typhimurium infection leads to miR-29a induced caveolin 2 regulation**. *PLoS One* 2013, **8**(6):e67300.

158. Maudet C, Mano M, Eulalio A: **MicroRNAs in the interaction between host and bacterial pathogens**. *FEBS Lett* 2014, **588**(22):4140-4147.

159. Bao H, Kommadath A, Liang G, Sun X, Arantes AS, Tuggle CK, Bearson SM, Plastow GS, Stothard P, Guan lL: **Genome-wide whole blood microRNAome and transcriptome analyses reveal miRNA-mRNA regulated host response to foodborne pathogen Salmonella infection in swine**. *Sci Rep* 2015, **5**:12620.

160. Zhang T, Yu J, Zhang Y, Li L, Chen Y, Li D, Liu F, Zhang CY, Gu H, Zen K: **Salmonella enterica serovar enteritidis modulates intestinal epithelial miR-128 levels to decrease macrophage recruitment via macrophage colony-stimulating factor**. *J Infect Dis* 2014, **209**(12):2000-2011.

161. Ordas A, Kanwal Z, Lindenberg V, Rougeot J, Mink M, Spaink HP, Meijer AH: **MicroRNA-146 function in the innate immune transcriptome response of zebrafish embryos to Salmonella typhimurium infection**. *BMC Genomics* 2013, **14**:696.

162. Khovidhunkit W, Kim MS, Memon RA, Shigenaga JK, Moser AH, Feingold KR, Grunfeld C: **Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host**. *J Lipid Res* 2004, **45**(7):1169-1196.

163. Yao M, Gao W, Yang J, Liang X, Luo J, Tinghua H: **The regulation roles of miR-125b, miR-221 and miR-27b in porcine Salmonella infection signaling pathway**. *Biosci Rep* 2016.

164. Yao M, Gao W, Tao H, Yang J, Liu G, Huang T: **Regulation signature of miR-143 and miR-26 in porcine Salmonella infection identified by binding site enrichment analysis**. *Mol Genet Genomics* 2016, **291**(2):789-799.

165. García-Feliz C, Collazos JA, Carvajal A, Vidal AB, Aladueña A, Ramiro R, de la Fuente M, Echeita MA, Rubio P: **Salmonella enterica infections in Spanish swine fattening units**. *Zoonoses Public Health* 2007, **54**(8):294-300.

166. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP: **Exploration, normalization, and summaries of high density oligonucleotide array probe level data**. *Biostatistics* 2003, **4**(2):249-264.

167. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J *et al*: **Bioconductor: open software development for computational biology and bioinformatics**. *Genome Biol* 2004, **5**(10):R80.

168. Smyth GK: **Linear models and empirical bayes methods for assessing differential expression in microarray experiments**. *Stat Appl Genet Mol Biol* 2004, **3**:Article3.

169. Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M: **Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research**. *Bioinformatics* 2005, **21**(18):3674-3676.

170. Ritchie ME, Silver J, Oshlack A, Holmes M, Diyagama D, Holloway A, Smyth GK: **A comparison of background correction methods for two-colour microarrays**. *Bioinformatics* 2007, **23**(20):2700-2707.

171. Pradervand S, Weber J, Thomas J, Bueno M, Wirapati P, Lefort K, Dotto GP, Harshman K: **Impact of normalization on miRNA microarray expression profiling**. *RNA* 2009, **15**(3):493-501.

172. Zhao Y, Wang E, Liu H, Rotunno M, Koshiol J, Marincola FM, Landi MT, McShane LM: **Evaluation of normalization methods for two-channel microRNA microarrays**. *J Transl Med* 2010, **8**:69.

173. Hong F, Breitling R, McEntee CW, Wittner BS, Nemhauser JL, Chory J: **RankProd: a bioconductor package for detecting differentially expressed genes in meta-analysis**. *Bioinformatics* 2006, **22**(22):2825-2827.

174. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I: **Controlling the false discovery rate in behavior genetics research**. *Behav Brain Res* 2001, **125**(1-2):279-284.

175. Falgueras J, Lara AJ, Fernández-Pozo N, Cantón FR, Pérez-Trabado G, Claros MG: **SeqTrim: a high-throughput pipeline for pre-processing any type of sequence read**. *BMC Bioinformatics* 2010, **11**:38.

176. Sun Z, Evans J, Bhagwate A, Middha S, Bockol M, Yan H, Kocher JP: **CAP-miRSeq: a comprehensive analysis pipeline for microRNA sequencing data**. *BMC Genomics* 2014, **15**:423.

177. Langmead B: **Aligning short sequencing reads with Bowtie**. *Curr Protoc Bioinformatics* 2010, **Chapter 11**:Unit 11.17.

178. Friedländer MR, Chen W, Adamidi C, Maaskola J, Einspanier R, Knespel S, Rajewsky N: **Discovering microRNAs from deep sequencing data using miRDeep**. *Nat Biotechnol* 2008, **26**(4):407-415.

179. Robinson MD, McCarthy DJ, Smyth GK: **edgeR: a Bioconductor package for differential expression analysis of digital gene expression data**. *Bioinformatics* 2010, **26**(1):139-140.

180. Balcells I, Cirera S, Busk PK: **Specific and sensitive quantitative RT-PCR of miRNAs with DNA primers**. *BMC Biotechnol* 2011, **11**:70.

181. Podolska A, Anthon C, Bak M, Tommerup N, Skovgaard K, Heegaard PM, Gorodkin J, Cirera S, Fredholm M: **Profiling microRNAs in lung tissue from pigs infected with Actinobacillus pleuropneumoniae**. *BMC Genomics* 2012, **13**:459.

182. Busk PK: **A tool for design of primers for microRNA-specific quantitative RT-qPCR**. *BMC Bioinformatics* 2014, **15**:29.

183. Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F, Vandesompele J: **A novel and universal method for microRNA RT-qPCR data normalization**. *Genome Biol* 2009, **10**(6):R64.

184. Andersen CL, Jensen JL, Ørntoft TF: **Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets**. *Cancer Res* 2004, **64**(15):5245-5250.

185. Timoneda O, Balcells I, Córdoba S, Castelló A, Sánchez A: **Determination of reference microRNAs for relative quantification in porcine tissues**. *PLoS One* 2012, **7**(9):e44413.

186. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method**. *Methods* 2001, **25**(4):402-408.

187. Hsu SD, Lin FM, Wu WY, Liang C, Huang WC, Chan WL, Tsai WT, Chen GZ, Lee CJ, Chiu CM *et al*: **miRTarBase: a database curates experimentally validated microRNA-target interactions**. *Nucleic Acids Res* 2011, **39**(Database issue):D163-169.

188. Agarwal V, Bell GW, Nam JW, Bartel DP: **Predicting effective microRNA target sites in mammalian mRNAs**. *Elife* 2015, **4**.

189. Uribe JH, Collado-Romero M, Zaldívar-López S, Arce C, Bautista R, Carvajal A, Cirera S, Claros MG, Garrido JJ: **Transcriptional analysis of porcine intestinal mucosa infected with Salmonella Typhimurium revealed a massive inflammatory response and disruption of bile acid absorption in ileum**. *Vet Res* 2016, **47**(1):11.

190. Krüger J, Rehmsmeier M: **RNAhybrid: microRNA target prediction easy, fast and flexible**. *Nucleic Acids Res* 2006, **34**(Web Server issue):W451-454.

191. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB: **Prediction of mammalian microRNA targets**. *Cell* 2003, **115**(7):787-798.

192. Friedman RC, Farh KK, Burge CB, Bartel DP: **Most mammalian mRNAs are conserved targets of microRNAs**. *Genome Res* 2009, **19**(1):92-105.

193. McNabb DS, Reed R, Marciniak RA: **Dual luciferase assay system for rapid assessment of gene expression in Saccharomyces cerevisiae**. *Eukaryot Cell* 2005, **4**(9):1539-1549.

194. Edwards AM, Massey RC: **Invasion of human cells by a bacterial pathogen**. *J Vis Exp* 2011(49).

195. Collado-Romero M, Arce C, Ramírez-Boo M, Carvajal A, Garrido JJ: **Quantitative analysis of the immune response upon Salmonella**

typhimurium infection along the porcine intestinal gut. *Vet Res* 2010, **41**(2):23.

196. Meyerholz DK, Stabel TJ, Ackermann MR, Carlson SA, Jones BD, Pohlenz J: **Early epithelial invasion by Salmonella enterica serovar Typhimurium DT104 in the swine ileum**. *Vet Pathol* 2002, **39**(6):712-720.

197. Arpaia N, Godec J, Lau L, Sivick KE, McLaughlin LM, Jones MB, Dracheva T, Peterson SN, Monack DM, Barton GM: **TLR signaling is required for Salmonella typhimurium virulence**. *Cell* 2011, **144**(5):675-688.

198. Vavassori P, Mencarelli A, Renga B, Distrutti E, Fiorucci S: **The bile acid receptor FXR is a modulator of intestinal innate immunity**. *J Immunol* 2009, **183**(10):6251-6261.

199. Eloranta JJ, Kullak-Ublick GA: **The role of FXR in disorders of bile acid homeostasis**. *Physiology (Bethesda)* 2008, **23**:286-295.

200. Guyton AC: **Secretory functions of the alimentary tract**. In: *TEXTBOOK of Medical Physiology.* Saunders, Philadelphia: Elsevier; 2006: 791-807.

201. Matsubara T, Li F, Gonzalez FJ: **FXR signaling in the enterohepatic system**. *Mol Cell Endocrinol* 2013, **368**(1-2):17-29.

202. Romain G, Tremblay S, Arena ET, Antunes LC, Covey S, Chow MT, Finlay BB, Menendez A: **Enterohepatic bacterial infections dysregulate the FGF15-FGFR4 endocrine axis**. *BMC Microbiol* 2013, **13**:238.

203. Neimark E, Chen F, Li X, Magid MS, Alasio TM, Frankenberg T, Sinha J, Dawson PA, Shneider BL: **c-Fos is a critical mediator of inflammatory-mediated repression of the apical sodium-dependent bile acid transporter**. *Gastroenterology* 2006, **131**(2):554-567.

204. Van Parys A, Boyen F, Verbrugghe E, Leyman B, Bram F, Haesebrouck F, Pasmans F: **Salmonella Typhimurium induces SPI-1 and SPI-2 regulated**

and strain dependent downregulation of MHC II expression on porcine alveolar macrophages. *Vet Res* 2012, **43**:52.

205. Szydłowski M, Jabłońska E, Juszczyński P: **FOXO1 transcription factor: a critical effector of the PI3K-AKT axis in B-cell development**. *Int Rev Immunol* 2014, **33**(2):146-157.

206. Hasegawa M, Fujimoto M, Poe JC, Steeber DA, Lowell CA, Tedder TF: **A CD19-dependent signaling pathway regulates autoimmunity in Lyn-deficient mice**. *J Immunol* 2001, **167**(5):2469-2478.

207. Minegishi Y, Rohrer J, Coustan-Smith E, Lederman HM, Pappu R, Campana D, Chan AC, Conley ME: **An essential role for BLNK in human B cell development**. *Science* 1999, **286**(5446):1954-1957.

208. Vilagos B, Hoffmann M, Souabni A, Sun Q, Werner B, Medvedovic J, Bilic I, Minnich M, Axelsson E, Jaritz M *et al*: **Essential role of EBF1 in the generation and function of distinct mature B cell types**. *J Exp Med* 2012, **209**(4):775-792.

209. Zhou R, O'Hara SP, Chen XM: **MicroRNA regulation of innate immune responses in epithelial cells**. *Cell Mol Immunol* 2011, **8**(5):371-379.

210. Liu X, Lu R, Xia Y, Sun J: **Global analysis of the eukaryotic pathways and networks regulated by Salmonella typhimurium in mouse intestinal infection in vivo**. *BMC Genomics* 2010, **11**:722.

211. Pitsouli C, Apidianakis Y, Perrimon N: **Homeostasis in infected epithelia: stem cells take the lead**. *Cell Host Microbe* 2009, **6**(4):301-307.

212. Kim M, Ashida H, Ogawa M, Yoshikawa Y, Mimuro H, Sasakawa C: **Bacterial interactions with the host epithelium**. *Cell Host Microbe* 2010, **8**(1):20-35.

213. Buchon N, Broderick NA, Poidevin M, Pradervand S, Lemaitre B: **Drosophila intestinal response to bacterial infection: activation of host**

defense and stem cell proliferation. *Cell Host Microbe* 2009, **5**(2):200-211.

214. Kim M, Ogawa M, Fujita Y, Yoshikawa Y, Nagai T, Koyama T, Nagai S, Lange A, Fässler R, Sasakawa C: **Bacteria hijack integrin-linked kinase to stabilize focal adhesions and block cell detachment**. *Nature* 2009, **459**(7246):578-582.

215. Boyle EC, Brown NF, Finlay BB: **Salmonella enterica serovar Typhimurium effectors SopB, SopE, SopE2 and SipA disrupt tight junction structure and function**. *Cell Microbiol* 2006, **8**(12):1946-1957.

216. Teng GG, Wang WH, Dai Y, Wang SJ, Chu YX, Li J: **Let-7b is involved in the inflammation and immune responses associated with Helicobacter pylori infection by targeting Toll-like receptor 4**. *PLoS One* 2013, **8**(2):e56709.

217. Pan S, Zheng Y, Zhao R, Yang X: **miRNA-374 regulates dexamethasone-induced differentiation of primary cultures of porcine adipocytes**. *Horm Metab Res* 2013, **45**(7):518-525.

218. Huang TH, Uthe JJ, Bearson SM, Demirkale CY, Nettleton D, Knetter S, Christian C, Ramer-Tait AE, Wannemuehler MJ, Tuggle CK: **Distinct peripheral blood RNA responses to Salmonella in pigs differing in Salmonella shedding levels: intersection of IFNG, TLR and miRNA pathways**. *PLoS One* 2011, **6**(12):e28768.

219. Zhu H, Wu H, Liu X, Evans BR, Medina DJ, Liu CG, Yang JM: **Role of MicroRNA miR-27a and miR-451 in the regulation of MDR1/P-glycoprotein expression in human cancer cells**. *Biochem Pharmacol* 2008, **76**(5):582-588.

220. Kovalchuk O, Filkowski J, Meservy J, Ilnytskyy Y, Tryndyak VP, Chekhun VF, Pogribny IP: **Involvement of microRNA-451 in resistance of the MCF-**

7 breast cancer cells to chemotherapeutic drug doxorubicin. *Mol Cancer Ther* 2008, **7**(7):2152-2159.

221. Le May C, Berger JM, Lespine A, Pillot B, Prieur X, Letessier E, Hussain MM, Collet X, Cariou B, Costet P: **Transintestinal cholesterol excretion is an active metabolic process modulated by PCSK9 and statin involving ABCB1**. *Arterioscler Thromb Vasc Biol* 2013, **33**(7):1484-1493.

222. Siccardi D, Mumy KL, Wall DM, Bien JD, McCormick BA: **Salmonella enterica serovar Typhimurium modulates P-glycoprotein in the intestinal epithelium**. *Am J Physiol Gastrointest Liver Physiol* 2008, **294**(6):G1392-1400.

223. Crowe A: **The role of P-glycoprotein and breast cancer resistance protein (BCRP) in bacterial attachment to human gastrointestinal cells**. *J Crohns Colitis* 2011, **5**(6):531-542.

224. Belliard AM, Lacour B, Farinotti R, Leroy C: **Effect of tumor necrosis factor-alpha and interferon-gamma on intestinal P-glycoprotein expression, activity, and localization in Caco-2 cells**. *J Pharm Sci* 2004, **93**(6):1524-1536.

225. Chakravortty D, Rohde M, Jäger L, Deiwick J, Hensel M: **Formation of a novel surface structure encoded by Salmonella Pathogenicity Island 2**. *EMBO J* 2005, **24**(11):2043-2052.

226. Lamouille S, Xu J, Derynck R: **Molecular mechanisms of epithelial-mesenchymal transition**. *Nat Rev Mol Cell Biol* 2014, **15**(3):178-196.

227. Jing J, Xiong S, Li Z, Wu J, Zhou L, Gui JF, Mei J: **A feedback regulatory loop involving p53/miR-200 and growth hormone endocrine axis controls embryo size of zebrafish**. *Sci Rep* 2015, **5**:15906.

228. Pichiorri F, Suh SS, Rocci A, De Luca L, Taccioli C, Santhanam R, Zhou W, Benson DM, Hofmainster C, Alder H *et al*: **Downregulation of p53-inducible microRNAs 192, 194, and 215 impairs the p53/MDM2**

autoregulatory loop in multiple myeloma development. *Cancer Cell* 2010, **18**(4):367-381.

229. Dorhoi A, Iannaccone M, Farinacci M, Faé KC, Schreiber J, Moura-Alves P, Nouailles G, Mollenkopf HJ, Oberbeck-Müller D, Jörg S *et al*: **MicroRNA-223 controls susceptibility to tuberculosis by regulating lung neutrophil recruitment**. *J Clin Invest* 2013, **123**(11):4836-4848.

230. Haneklaus M, Gerlic M, O'Neill LA, Masters SL: **miR-223: infection, inflammation and cancer**. *J Intern Med* 2013, **274**(3):215-226.

231. Zakrzewski SS, Richter JF, Krug SM, Jebautzke B, Lee IF, Rieger J, Sachtleben M, Bondzio A, Schulzke JD, Fromm M *et al*: **Improved cell line IPEC-J2, characterized as a model for porcine jejunal epithelium**. *PLoS One* 2013, **8**(11):e79643.

232. Aziz F: **The emerging role of miR-223 as novel potential diagnostic and therapeutic target for inflammatory disorders**. *Cell Immunol* 2016, **303**:1-6.

233. Chuang TD, Khorram O: **miR-200c regulates IL8 expression by targeting IKBKB: a potential mediator of inflammation in leiomyoma pathogenesis**. *PLoS One* 2014, **9**(4):e95370.

234. Bao C, Li Y, Huan L, Zhang Y, Zhao F, Wang Q, Liang L, Ding J, Liu L, Chen T *et al*: **NF-κB signaling relieves negative regulation by miR-194 in hepatocellular carcinoma by suppressing the transcription factor HNF-1α**. *Sci Signal* 2015, **8**(387):ra75.

235. Vaishnavi V, Manikandan M, Munirajan AK: **Mining the 3'UTR of autism-implicated genes for SNPs perturbing microRNA regulation**. *Genomics Proteomics Bioinformatics* 2014, **12**(2):92-104.

236. Liang G, Malmuthuge N, Guan Y, Ren Y, Griebel PJ, Guan lL: **Altered microRNA expression and pre-mRNA splicing events reveal new**

mechanisms associated with early stage Mycobacterium avium subspecies paratuberculosis infection. *Sci Rep* 2016, **6**:24964.

237. Sharbati S, Friedländer MR, Sharbati J, Hoeke L, Chen W, Keller A, Stähler PF, Rajewsky N, Einspanier R: **Deciphering the porcine intestinal microRNA transcriptome**. *BMC Genomics* 2010, **11**:275.

238. Lim LP, Glasner ME, Yekta S, Burge CB, Bartel DP: **Vertebrate microRNA genes**. *Science* 2003, **299**(5612):1540.

239. Wu F, Zhang S, Dassopoulos T, Harris ML, Bayless TM, Meltzer SJ, Brant SR, Kwon JH: **Identification of microRNAs associated with ileal and colonic Crohn's disease**. *Inflamm Bowel Dis* 2010, **16**(10):1729-1738.

240. Liang G, Malmuthuge N, McFadden TB, Bao H, Griebel PJ, Stothard P, Guan lL: **Potential regulatory role of microRNAs in the development of bovine gastrointestinal tract during early life**. *PLoS One* 2014, **9**(3):e92592.

241. Braun CJ, Zhang X, Savelyeva I, Wolff S, Moll UM, Schepeler T, Ørntoft TF, Andersen CL, Dobbelstein M: **p53-Responsive micrornas 192 and 215 are capable of inducing cell cycle arrest**. *Cancer Res* 2008, **68**(24):10094-10104.

242. Rokavec M, Li H, Jiang L, Hermeking H: **The p53/microRNA connection in gastrointestinal cancer**. *Clin Exp Gastroenterol* 2014, **7**:395-413.

243. Pichiorri F, Suh SS, Rocci A, De Luca L, Taccioli C, Santhanam R, Zhou W, Benson DM, Hofmainster C, Alder H *et al*: **Downregulation of p53-inducible microRNAs 192, 194, and 215 Impairs the p53/MDM2 Autoregulatory Loop in Multiple Myeloma Development**. *Cancer Cell* 2016, **30**(2):349-351.

244. Siegl C, Rudel T: **Modulation of p53 during bacterial infections**. *Nat Rev Microbiol* 2015, **13**(12):741-748.

245. Hsieh CH, Rau CS, Jeng JC, Chen YC, Lu TH, Wu CJ, Wu YC, Tzeng SL, Yang JC: **Whole blood-derived microRNA signatures in mice exposed to lipopolysaccharides**. *J Biomed Sci* 2012, **19**:69.

246. Harris JF, Micheva-Viteva S, Li N, Hong-Geller E: **Small RNA-mediated regulation of host-pathogen interactions**. *Virulence* 2013, **4**(8):785-795.

247. Cadamuro AC, Rossi AF, Maniezzo NM, Silva AE: **Helicobacter pylori infection: host immune response, implications on gene expression and microRNAs**. *World J Gastroenterol* 2014, **20**(6):1424-1437.

248. Baud J, Varon C, Chabas S, Chambonnier L, Darfeuille F, Staedel C: **Helicobacter pylori initiates a mesenchymal transition through ZEB1 in gastric epithelial cells**. *PLoS One* 2013, **8**(4):e60315.

249. Senanayake U, Das S, Vesely P, Alzoughbi W, Fröhlich LF, Chowdhury P, Leuschner I, Hoefler G, Guertl B: **miR-192, miR-194, miR-215, miR-200c and miR-141 are downregulated and their common target ACVR2B is strongly expressed in renal childhood neoplasms**. *Carcinogenesis* 2012, **33**(5):1014-1021.

250. Gaulke CA, Porter M, Han YH, Sankaran-Walters S, Grishina I, George MD, Dang AT, Ding SW, Jiang G, Korf I *et al*: **Intestinal epithelial barrier disruption through altered mucosal microRNA expression in human immunodeficiency virus and simian immunodeficiency virus infections**. *J Virol* 2014, **88**(11):6268-6280.

251. McKenna LB, Schug J, Vourekas A, McKenna JB, Bramswig NC, Friedman JR, Kaestner KH: **MicroRNAs control intestinal epithelial differentiation, architecture, and barrier function**. *Gastroenterology* 2010, **139**(5):1654-1664, 1664.e1651.

252. Cichon C, Sabharwal H, Rüter C, Schmidt MA: **MicroRNAs regulate tight junction proteins and modulate epithelial/endothelial barrier functions**. *Tissue Barriers* 2014, **2**(4):e944446.

253. Khella HW, Bakhet M, Allo G, Jewett MA, Girgis AH, Latif A, Girgis H, Von Both I, Bjarnason GA, Yousef GM: **miR-192, miR-194 and miR-215: a convergent microRNA network suppressing tumor progression in renal cell carcinoma**. *Carcinogenesis* 2013, **34**(10):2231-2239.

254. Wang B, Herman-Edelstein M, Koh P, Burns W, Jandeleit-Dahm K, Watson A, Saleem M, Goodall GJ, Twigg SM, Cooper ME *et al*: **E-cadherin expression is regulated by miR-192/215 by a mechanism that is independent of the profibrotic effects of transforming growth factor-beta**. *Diabetes* 2010, **59**(7):1794-1802.

255. Liu H, Brannon AR, Reddy AR, Alexe G, Seiler MW, Arreola A, Oza JH, Yao M, Juan D, Liou LS *et al*: **Identifying mRNA targets of microRNA dysregulated in cancer: with application to clear cell Renal Cell Carcinoma**. *BMC Syst Biol* 2010, **4**:51.

256. Ashida H, Mimuro H, Ogawa M, Kobayashi T, Sanada T, Kim M, Sasakawa C: **Cell death and infection: a double-edged sword for host and pathogen survival**. *J Cell Biol* 2011, **195**(6):931-942.

257. Knodler LA, Vallance BA, Celli J, Winfree S, Hansen B, Montero M, Steele-Mortimer O: **Dissemination of invasive Salmonella via bacterial-induced extrusion of mucosal epithelia**. *Proc Natl Acad Sci U S A* 2010, **107**(41):17733-17738.

258. Winter SE, Bäumler AJ: **Salmonella exploits suicidal behavior of epithelial cells**. *Front Microbiol* 2011, **2**:48.

259. Valle E, Guiney DG: **Characterization of Salmonella-induced cell death in human macrophage-like THP-1 cells**. *Infect Immun* 2005, **73**(5):2835-2840.

260. Matta A, Bahadur S, Duggal R, Gupta SD, Ralhan R: **Over-expression of 14-3-3zeta is an early event in oral cancer**. *BMC Cancer* 2007, **7**:169.

261. Rosenberger CM, Podyminogin RL, Navarro G, Zhao GW, Askovich PS, Weiss MJ, Aderem A: **miR-451 regulates dendritic cell cytokine responses to influenza infection**. *J Immunol* 2012, **189**(12):5965-5975.

262. Tian H, Liu C, Zou X, Wu W, Zhang C, Yuan D: **MiRNA-194 Regulates Palmitic Acid-Induced Toll-Like Receptor 4 Inflammatory Responses in THP-1 Cells**. *Nutrients* 2015, **7**(5):3483-3496.

263. Zhu X, Li D, Yu F, Jia C, Xie J, Ma Y, Fan S, Cai H, Luo Q, Lv Z *et al*: **miR-194 inhibits the proliferation, invasion, migration, and enhances the chemosensitivity of non-small cell lung cancer cells by targeting forkhead box A1 protein**. *Oncotarget* 2016, **7**(11):13139-13152.

264. Wang L, Qin H, Li L, Feng F, Ji P, Zhang J, Li G, Zhao Z, Gao G: **Forkhead-box A1 transcription factor is a novel adverse prognosis marker in human glioma**. *J Clin Neurosci* 2013, **20**(5):654-658.

265. Bramswig NC, Kaestner KH: **Transcriptional regulation of α-cell differentiation**. *Diabetes Obes Metab* 2011, **13 Suppl 1**:13-20.

266. Bernardo GM, Keri RA: **FOXA1: a transcription factor with parallel functions in development and cancer**. *Biosci Rep* 2012, **32**(2):113-130.

267. Specian RD, Oliver MG: **Functional biology of intestinal goblet cells**. *Am J Physiol* 1991, **260**(2 Pt 1):C183-193.

268. Kim YS, Ho SB: **Intestinal goblet cells and mucins in health and disease: recent insights and progress**. *Curr Gastroenterol Rep* 2010, **12**(5):319-330.

269. Jonckheere N, Vincent A, Perrais M, Ducourouble MP, Male AK, Aubert JP, Pigny P, Carraway KL, Freund JN, Renes IB *et al*: **The human mucin MUC4 is transcriptionally regulated by caudal-related homeobox, hepatocyte nuclear factors, forkhead box A, and GATA endodermal transcription factors in epithelial cancer cells**. *J Biol Chem* 2007, **282**(31):22638-22650.

270. Ye DZ, Kaestner KH: **Foxa1 and Foxa2 control the differentiation of goblet and enteroendocrine L- and D-cells in mice**. *Gastroenterology* 2009, **137**(6):2052-2062.

271. Van der Sluis M, De Koning BA, De Bruijn AC, Velcich A, Meijerink JP, Van Goudoever JB, Büller HA, Dekker J, Van Seuningen I, Renes IB *et al*: **Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection**. *Gastroenterology* 2006, **131**(1):117-129.

272. Huang HC, Klein PS: **The Frizzled family: receptors for multiple signal transduction pathways**. *Genome Biol* 2004, **5**(7):234.

273. MacDonald BT, He X: **Frizzled and LRP5/6 receptors for Wnt/β-catenin signaling**. *Cold Spring Harb Perspect Biol* 2012, **4**(12).

274. Schaale K, Neumann J, Schneider D, Ehlers S, Reiling N: **Wnt signaling in macrophages: augmenting and inhibiting mycobacteria-induced inflammatory responses**. *Eur J Cell Biol* 2011, **90**(6-7):553-559.

275. Halleskog C, Mulder J, Dahlström J, Mackie K, Hortobágyi T, Tanila H, Kumar Puli L, Färber K, Harkany T, Schulte G: **WNT signaling in activated microglia is proinflammatory**. *Glia* 2011, **59**(1):119-131.

276. Silva-García O, Valdez-Alarcón JJ, Baizabal-Aguirre VM: **The Wnt/β-catenin signaling pathway controls the inflammatory response in infections caused by pathogenic bacteria**. *Mediators Inflamm* 2014, **2014**:310183.

277. Liu X, Lu R, Wu S, Zhang YG, Xia Y, Sartor RB, Sun J: **Wnt2 inhibits enteric bacterial-induced inflammation in intestinal epithelial cells**. *Inflamm Bowel Dis* 2012, **18**(3):418-429.

278. Bansal K, Trinath J, Chakravortty D, Patil SA, Balaji KN: **Pathogen-specific TLR2 protein activation programs macrophages to induce Wnt-beta-catenin signaling**. *J Biol Chem* 2011, **286**(42):37032-37044.

279. Tahoun A, Mahajan S, Paxton E, Malterer G, Donaldson DS, Wang D, Tan A, Gillespie TL, O'Shea M, Roe AJ *et al*: **Salmonella transforms follicle-associated epithelial cells into M cells to promote intestinal invasion**. *Cell Host Microbe* 2012, **12**(5):645-656.

280. Gujral TS, Chan M, Peshkin L, Sorger PK, Kirschner MW, MacBeath G: **A noncanonical Frizzled2 pathway regulates epithelial-mesenchymal transition and metastasis**. *Cell* 2014, **159**(4):844-856.

281. Nasarre P, Gemmill RM, Potiron VA, Roche J, Lu X, Barón AE, Korch C, Garrett-Mayer E, Lagana A, Howe PH *et al*: **Neuropilin-2 Is upregulated in lung cancer cells during TGF-β1-induced epithelial-mesenchymal transition**. *Cancer Res* 2013, **73**(23):7111-7121.

282. Liu Z, Rebowe RE, Wang Z, Li Y, DePaolo JS, Guo J, Qian C, Liu W: **KIF3a promotes proliferation and invasion via Wnt signaling in advanced prostate cancer**. *Mol Cancer Res* 2014, **12**(4):491-503.

283. Chuang TD, Ho M, Khorram O: **The regulatory function of miR-200c on inflammatory and cell-cycle associated genes in SK-LMS-1, a leiomyosarcoma cell line**. *Reprod Sci* 2015, **22**(5):563-571.

284. Guo L, Zhang Y, Zhang L, Huang F, Li J, Wang S: **MicroRNAs, TGF-β signaling, and the inflammatory microenvironment in cancer**. *Tumour Biol* 2016, **37**(1):115-125.

285. Rokavec M, Öner MG, Hermeking H: **Inflammation-induced epigenetic switches in cancer**. *Cell Mol Life Sci* 2016, **73**(1):23-39.

286. Rokavec M, Wu W, Luo JL: **IL6-mediated suppression of miR-200c directs constitutive activation of inflammatory signaling circuit driving transformation and tumorigenesis**. *Mol Cell* 2012, **45**(6):777-789.

287. Zhao Y, Xu Y, Li Y, Xu W, Luo F, Wang B, Pang Y, Xiang Q, Zhou J, Wang X *et al*: **NF-κB-mediated inflammation leading to EMT via miR-200c is**

involved in cell transformation induced by cigarette smoke extract. *Toxicol Sci* 2013, **135**(2):265-276.

288. Guo L, Chen C, Shi M, Wang F, Chen X, Diao D, Hu M, Yu M, Qian L, Guo N: **Stat3-coordinated Lin-28-let-7-HMGA2 and miR-200-ZEB1 circuits initiate and maintain oncostatin M-driven epithelial-mesenchymal transition**. *Oncogene* 2013, **32**(45):5272-5282.

289. Zidar N, Boštjančič E, Jerala M, Kojc N, Drobne D, Štabuc B, Glavač D: **Down-regulation of microRNAs of the miR-200 family and up-regulation of Snail and Slug in inflammatory bowel diseases - hallmark of epithelial-mesenchymal transition**. *J Cell Mol Med* 2016.

290. Staedel C, Darfeuille F: **MicroRNAs and bacterial infection**. *Cell Microbiol* 2013, **15**(9):1496-1507.

291. Monk CE, Hutvagner G, Arthur JS: **Regulation of miRNA transcription in macrophages in response to Candida albicans**. *PLoS One* 2010, **5**(10):e13669.

292. Quinn SR, O'Neill LA: **A trio of microRNAs that control Toll-like receptor signalling**. *Int Immunol* 2011, **23**(7):421-425.

293. Meisgen F, Xu Landén N, Wang A, Réthi B, Bouez C, Zuccolo M, Gueniche A, Ståhle M, Sonkoly E, Breton L *et al*: **MiR-146a negatively regulates TLR2-induced inflammatory responses in keratinocytes**. *J Invest Dermatol* 2014, **134**(7):1931-1940.

294. Park H, Huang X, Lu C, Cairo MS, Zhou X: **MicroRNA-146a and microRNA-146b regulate human dendritic cell apoptosis and cytokine production by targeting TRAF6 and IRAK1 proteins**. *J Biol Chem* 2015, **290**(5):2831-2841.

295. Boldin MP, Taganov KD, Rao DS, Yang L, Zhao JL, Kalwani M, Garcia-Flores Y, Luong M, Devrekanli A, Xu J *et al*: **miR-146a is a significant brake on**

autoimmunity, myeloproliferation, and cancer in mice. *J Exp Med* 2011, **208**(6):1189-1201.

296. Szabady RL, McCormick BA: **Control of neutrophil inflammation at mucosal surfaces by secreted epithelial products**. *Front Immunol* 2013, **4**:220.

297. Tyrkalska SD, Candel S, Angosto D, Gómez-Abellán V, Martín-Sánchez F, García-Moreno D, Zapata-Pérez R, Sánchez-Ferrer Á, Sepulcre MP, Pelegrín P *et al*: **Neutrophils mediate Salmonella Typhimurium clearance through the GBP4 inflammasome-dependent production of prostaglandins**. *Nat Commun* 2016, **7**:12077.

298. Taïbi F, Metzinger-Le Meuth V, Massy ZA, Metzinger L: **miR-223: An inflammatory oncomiR enters the cardiovascular field**. *Biochim Biophys Acta* 2014, **1842**(7):1001-1009.

299. Luo X, Ranade K, Talker R, Jallal B, Shen N, Yao Y: **microRNA-mediated regulation of innate immune response in rheumatic diseases**. *Arthritis Res Ther* 2013, **15**(2):210.

300. Netea MG, Simon A, van de Veerdonk F, Kullberg BJ, Van der Meer JW, Joosten LA: **IL-1beta processing in host defense: beyond the inflammasomes**. *PLoS Pathog* 2010, **6**(2):e1000661.

301. Yang T, Zheng ZM, Li XN, Li ZF, Wang Y, Geng YF, Bai L, Zhang XB: **MiR-223 modulates multidrug resistance via downregulation of ABCB1 in hepatocellular carcinoma cells**. *Exp Biol Med (Maywood)* 2013, **238**(9):1024-1032.

302. Mercado-Lubo R, Zhang Y, Zhao L, Rossi K, Wu X, Zou Y, Castillo A, Leonard J, Bortell R, Greiner DL *et al*: **A Salmonella nanoparticle mimic overcomes multidrug resistance in tumours**. *Nat Commun* 2016, **7**:12225.

303. Hannemann S, Gao B, Galán JE: **Salmonella modulation of host cell gene expression promotes its intracellular growth**. *PLoS Pathog* 2013, **9**(10):e1003668.

304. Chen Q, Wang H, Liu Y, Song Y, Lai L, Han Q, Cao X, Wang Q: **Inducible microRNA-223 down-regulation promotes TLR-triggered IL-6 and IL-1β production in macrophages by targeting STAT3**. *PLoS One* 2012, **7**(8):e42971.

305. Lu R, Wu S, Zhang YG, Xia Y, Zhou Z, Kato I, Dong H, Bissonnette M, Sun J: **Salmonella Protein AvrA Activates the STAT3 Signaling Pathway in Colon Cancer**. *Neoplasia* 2016, **18**(5):307-316.

306. Cardoso CM, Jordao L, Vieira OV: **Rab10 regulates phagosome maturation and its overexpression rescues Mycobacterium-containing phagosomes maturation**. *Traffic* 2010, **11**(2):221-235.

307. Smith AC, Heo WD, Braun V, Jiang X, Macrae C, Casanova JE, Scidmore MA, Grinstein S, Meyer T, Brumell JH: **A network of Rab GTPases controls phagosome maturation and is modulated by Salmonella enterica serovar Typhimurium**. *J Cell Biol* 2007, **176**(3):263-268.

308. Wang D, Lou J, Ouyang C, Chen W, Liu Y, Liu X, Cao X, Wang J, Lu L: **Ras-related protein Rab10 facilitates TLR4 signaling by promoting replenishment of TLR4 onto the plasma membrane**. *Proc Natl Acad Sci U S A* 2010, **107**(31):13806-13811.

309. Sender V, Stamme C: **Lung cell-specific modulation of LPS-induced TLR4 receptor and adaptor localization**. *Commun Integr Biol* 2014, **7**:e29053.

310. Zhou Q, Souba WW, Croce CM, Verne GN: **MicroRNA-29a regulates intestinal membrane permeability in patients with irritable bowel syndrome**. *Gut* 2010, **59**(6):775-784.

311. Fasina YO, Bowers JB, Hess JB, McKee SR: **Effect of dietary glutamine supplementation on Salmonella colonization in the ceca of young broiler chicks**. *Poult Sci* 2010, **89**(5):1042-1048.

312. Kim M, Wischmeyer PE: **Glutamine**. *World Rev Nutr Diet* 2013, **105**:90-96.

313. Peng Z, Ban K, Wawrose RA, Gover AG, Kozar RA: **Protection by enteral glutamine is mediated by intestinal epithelial cell peroxisome proliferator-activated receptor-γ during intestinal ischemia/reperfusion**. *Shock* 2015, **43**(4):327-333.

314. Audard V, Cavard C, Richa H, Infante M, Couvelard A, Sauvanet A, Terris B, Paye F, Flejou JF: **Impaired E-cadherin expression and glutamine synthetase overexpression in solid pseudopapillary neoplasm of the pancreas**. *Pancreas* 2008, **36**(1):80-83.

315. George SJ: **Wnt pathway: a new role in regulation of inflammation**. *Arterioscler Thromb Vasc Biol* 2008, **28**(3):400-402.

316. Liu X, Wu S, Xia Y, Li XE, Zhou ZD, Sun J: **Wingless homolog Wnt11 suppresses bacterial invasion and inflammation in intestinal epithelial cells**. *Am J Physiol Gastrointest Liver Physiol* 2011, **301**(6):G992-G1003.

317. Junker Mentzel CM, Skovgaard K, Cordoba S, Uribe JH, Busk PK, Cirera S: **Wet-lab Tested MicroRNA Assays for qPCR Studies with SYBR®Green and DNA Primers in Pig Tissues**. *Microrna* 2014.

318. Zhang G, Yin S, Mao J, Liang F, Zhao C, Li P, Zhou G, Chen S, Tang Z: **Integrated analysis of mRNA-seq and miRNA-seq in the liver of Pelteobagrus vachelli in response to hypoxia**. *Sci Rep* 2016, **6**:22907.

319. Yao M, Gao W, Yang J, Liang X, Luo J, Huang T: **The regulation roles of miR-125b, miR-221 and miR-27b in porcine Salmonella infection signalling pathway**. *Biosci Rep* 2016, **36**(4).

320. von Andrian UH, Mempel TR: **Homing and cellular traffic in lymph nodes**. *Nat Rev Immunol* 2003, **3**(11):867-878.

321. Sheedy FJ: **Turning 21: Induction of miR-21 as a Key Switch in the Inflammatory Response**. *Front Immunol* 2015, **6**:19.

322. Liu PT, Wheelwright M, Teles R, Komisopoulou E, Edfeldt K, Ferguson B, Mehta MD, Vazirnia A, Rea TH, Sarno EN *et al*: **MicroRNA-21 targets the vitamin D-dependent antimicrobial pathway in leprosy**. *Nat Med* 2012, **18**(2):267-273.

323. Yan H, Chen Y, Zhou S, Li C, Gong G, Chen X, Wang T, Chen S, Sha Z: **Expression Profile Analysis of miR-221 and miR-222 in Different Tissues and Head Kidney Cells of Cynoglossus semilaevis, Following Pathogen Infection**. *Mar Biotechnol (NY)* 2016, **18**(1):37-48.

324. How CK, Hou SK, Shih HC, Huang MS, Chiou SH, Lee CH, Juan CC: **Expression profile of MicroRNAs in gram-negative bacterial sepsis**. *Shock* 2015, **43**(2):121-127.

325. Xia X, Li Z, Liu K, Wu Y, Jiang D, Lai Y: **Staphylococcal LTA-Induced miR-143 Inhibits Propionibacterium acnes-Mediated Inflammatory Response in Skin**. *J Invest Dermatol* 2016, **136**(3):621-630.

326. Neefjes J, Jongsma ML, Paul P, Bakke O: **Towards a systems understanding of MHC class I and MHC class II antigen presentation**. *Nat Rev Immunol* 2011, **11**(12):823-836.

327. Zhang Y, Zhang M, Zhong M, Suo Q, Lv K: **Expression profiles of miRNAs in polarized macrophages**. *Int J Mol Med* 2013, **31**(4):797-802.

328. Banerjee S, Cui H, Xie N, Tan Z, Yang S, Icyuz M, Thannickal VJ, Abraham E, Liu G: **miR-125a-5p regulates differential activation of macrophages and inflammation**. *J Biol Chem* 2013, **288**(49):35428-35436.

329. Seo MJ, Kim GR, Son YM, Yang DC, Chu H, Min TS, Jung ID, Park YM, Han SH, Yun CH: **Interactions of dendritic cells with cancer cells and modulation of surface molecules affect functional properties of CD8+ T cells**. *Mol Immunol* 2011, **48**(15-16):1744-1752.

330. Imai T, Kato Y, Kajiwara C, Mizukami S, Ishige I, Ichiyanagi T, Hikida M, Wang JY, Udono H: **Heat shock protein 90 (HSP90) contributes to cytosolic translocation of extracellular antigen for cross-presentation by dendritic cells**. *Proc Natl Acad Sci U S A* 2011, **108**(39):16363-16368.

331. Kloetzel PM, Ossendorp F: **Proteasome and peptidase function in MHC-class-I-mediated antigen presentation**. *Curr Opin Immunol* 2004, **16**(1):76-81.

332. Cresswell P, Ackerman AL, Giodini A, Peaper DR, Wearsch PA: **Mechanisms of MHC class I-restricted antigen processing and cross-presentation**. *Immunol Rev* 2005, **207**:145-157.

333. Deffit SN, Blum JS: **A central role for HSC70 in regulating antigen trafficking and MHC class II presentation**. *Mol Immunol* 2015, **68**(2 Pt A):85-88.

334. Van Parys A, Boyen F, Leyman B, Verbrugghe E, Maes D, Haesebrouck F, Pasmans F: **Induction of seroconversion and persistence of Salmonella Typhimurium in pigs are strain dependent**. *Comp Immunol Microbiol Infect Dis* 2013, **36**(5):465-471.

335. Lopez-Medina M, Perez-Lopez A, Alpuche-Aranda C, Ortiz-Navarrete V: **Salmonella modulates B cell biology to evade CD8(+) T cell-mediated immune responses**. *Front Immunol* 2014, **5**:586.

336. Mehta A, Baltimore D: **MicroRNAs as regulatory elements in immune system logic**. *Nat Rev Immunol* 2016, **16**(5):279-294.

337. Fehres CM, Unger WW, Garcia-Vallejo JJ, van Kooyk Y: **Understanding the biology of antigen cross-presentation for the design of vaccines against cancer**. *Front Immunol* 2014, **5**:149.

338. Owen KA, Casanova JE: **Salmonella Manipulates Autophagy to "Serve and Protect"**. *Cell Host Microbe* 2015, **18**(5):517-519.

339. Dong J, Zhao YP, Zhou L, Zhang TP, Chen G: **Bcl-2 upregulation induced by miR-21 via a direct interaction is associated with apoptosis and chemoresistance in MIA PaCa-2 pancreatic cancer cells**. *Arch Med Res* 2011, **42**(1):8-14.

340. Xu LF, Wu ZP, Chen Y, Zhu QS, Hamidi S, Navab R: **MicroRNA-21 (miR-21) regulates cellular proliferation, invasion, migration, and apoptosis by targeting PTEN, RECK and Bcl-2 in lung squamous carcinoma, Gejiu City, China**. *PLoS One* 2014, **9**(8):e103698.

341. Najafi Z, Sharifi M, Javadi G: **Degradation of miR-21 induces apoptosis and inhibits cell proliferation in human hepatocellular carcinoma**. *Cancer Gene Ther* 2015, **22**(11):530-535.

342. Tong Z, Liu N, Lin L, Guo X, Yang D, Zhang Q: **miR-125a-5p inhibits cell proliferation and induces apoptosis in colon cancer via targeting BCL2, BCL2L12 and MCL1**. *Biomed Pharmacother* 2015, **75**:129-136.

343. Gross A: **BCL-2 family proteins as regulators of mitochondria metabolism**. *Biochim Biophys Acta* 2016, **1857**(8):1243-1246.

344. Xu J, Wang Y, Tan X, Jing H: **MicroRNAs in autophagy and their emerging roles in crosstalk with apoptosis**. *Autophagy* 2012, **8**(6):873-882.

345. Liu B, Wen X, Cheng Y: **Survival or death: disequilibrating the oncogenic and tumor suppressive autophagy in cancer**. *Cell Death Dis* 2013, **4**:e892.

346. Mostowy S: **Multiple roles of the cytoskeleton in bacterial autophagy**. *PLoS Pathog* 2014, **10**(11):e1004409.

347. Frankel LB, Wen J, Lees M, Høyer-Hansen M, Farkas T, Krogh A, Jäättelä M, Lund AH: **microRNA-101 is a potent inhibitor of autophagy**. *EMBO J* 2011, **30**(22):4628-4641.

348. Xu K, Harrison RE: **Down-regulation of Stathmin Is Required for the Phenotypic Changes and Classical Activation of Macrophages**. *J Biol Chem* 2015, **290**(31):19245-19260.

349. Filbert EL, Le Borgne M, Lin J, Heuser JE, Shaw AS: **Stathmin regulates microtubule dynamics and microtubule organizing center polarization in activated T cells**. *J Immunol* 2012, **188**(11):5421-5427.

350. LaRock DL, Chaudhary A, Miller SI: **Salmonellae interactions with host processes**. *Nat Rev Microbiol* 2015, **13**(4):191-205.

351. Guignot J, Servin AL: **Maintenance of the Salmonella-containing vacuole in the juxtanuclear area: a role for intermediate filaments**. *Microb Pathog* 2008, **45**(5-6):415-422.

352. Sluchanko NN, Gusev NB: **14-3-3 proteins and regulation of cytoskeleton**. *Biochemistry (Mosc)* 2010, **75**(13):1528-1546.

353. Byun Y, Chen F, Chang R, Trivedi M, Green KJ, Cryns VL: **Caspase cleavage of vimentin disrupts intermediate filaments and promotes apoptosis**. *Cell Death Differ* 2001, **8**(5):443-450.

354. Sokolowski JD, Gamage KK, Heffron DS, Leblanc AC, Deppmann CD, Mandell JW: **Caspase-mediated cleavage of actin and tubulin is a common feature and sensitive marker of axonal degeneration in neural development and injury**. *Acta Neuropathol Commun* 2014, **2**:16.

355. Marshall KD, Baines CP: **Necroptosis: is there a role for mitochondria?** *Front Physiol* 2014, **5**:323.

356. Vanden Berghe T, Linkermann A, Jouan-Lanhouet S, Walczak H, Vandenabeele P: **Regulated necrosis: the expanding network of non-apoptotic cell death pathways**. *Nat Rev Mol Cell Biol* 2014, **15**(2):135-147.

357. Galluzzi L, Kroemer G: **Necroptosis: a specialized pathway of programmed necrosis**. *Cell* 2008, **135**(7):1161-1163.