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Study of host-pathogen-microbiota interactions on a *Salmonella enterica* serovar *Typhimurium* piglet model

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Keep your face always toward the sunshine

and shadows will fall behind you (Walt Whitman)

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ABBREVIATIONS

ACTB: beta-actin

ALB: albumin

AMP: antimicrobial peptides

ANXA2: annexin A2

ANXA5: annexin A5

APC: antigen-presenting cells

ATP5B: mitochondrial ATP synthase H^+ transporting F1 complex beta subunit

BPW: buffered peptone water

Caco-2: colon epithelial cells

cCbb: colloidal Comassie Brilliant Blue

CFU: colony forming unit

COL6A3: collagen alpha-3(VI) chain

CRP: C-reactive protein

CKB: creatin kinase

DAVID: Database for Annotation Visualization and Integrated Discovery

DC: dendritic cells

DTT: dithiothreitol

FAE: follicle-associated epithelium

FCS: fetal calf serum

GALT: gut-associated lymphoid tissue

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GO: gene ontology

GOTERM_CC: gene ontology term cellular component

GOTERM_BP: gene ontology term biological process

GOTERM_MF: gene ontology term molecular function

GSN: gelsolin

HARS: histidyl-tRNA synthetase

HEK-293: human endothelial kidney

HSPB1: heat shock protein beta-1

INF-γ: interferon gamma

IL1-α: interleukin 1 alpha

IL1-β: interleukin 1 beta

IL-10: interleukin 10

IPEC-J2: porcine intestinal columnar epithelial cells

KEGG: Kyoto Encyclopedia of Genes and Genomes

LC-MS/MS: liquid chromatography-tandem mass spectrometry

LPS: lipopolysaccharide

MEM: minimum essential medium

MHC: major histocompatibility complex

MYD88: myeloid differentiation primary response gene 88

MYL9: myosin regulatory light chain

MS: mass spectrometry

MSRV: modified semisolid Rappaport-Vassiliadis

NK: natural killer cells

NLR: NOD-like receptors

NTS: nontyphoidal salmonellae

OUT: operational taxonomic unit

PAMP: pathogen associated molecular patterns

PBMC: peripheral blood mononuclear cells

PBS: phosphate buffer solution

PCA1: principal coordinate 1

PCA2: principal coordinate 2

PCoA: principal component analysis

PP: Peyer's patches

PRR: pattern recognition receptors

RNS: reactive nitrogen species

ROS: reactive oxygen species

RPL-32: ribosomal protein L32

SCFA: shorty chain fatty acid

SCV: *Salmonella*-containing vacuole

SDS: sodium dodecyl sulfate

SOD: Cu,Zn-superoxide dismutase

SPI: *Salmonella* Pathogenicity Island

SPPIR: Swiss-Prot and Protein Information Resource

STM^{wt}: wild-type strain *S*. Typhimurium ATCC 14028

$STM^{\Delta znuABC}$: attenuated znuABC mutant strain of *S*. Typhimurium

STRING: Search Tool for the Retrieval of Interacting Genes/Protein

T3SS: type III secretion system

TGF-β: transforming growth factor beta

TJ: tight junction

TLR: toll like receptor

TNF-α: tumor necrosis factor alpha

TPI1: triosephosphate isomerase 1

TRIF: TIR-domain-containing adapter-inducing interferon-beta

UQCRC1: cytochrome b-c1 complex subunit 1

2DE: two-dimensional gel electrophoresis

SUMMARY

Salmonella enterica serovar Typhimurium is a Gram negative gastrointestinal pathogen responsible for food poisoning worldwide. Upon entry into the host, *Salmonella* must overcome the robust line of defense provided by the innate immune system and by microbiota-mediated colonization resistance. It is a fascinating example of pathogen able not only to evade immune response, but also to exploit multiple aspects of it to colonize intestine. *S*. Typhimurium has evolved strategies allowing to thrive in the inflamed gut at the expense of the resident microbiota, taking advantage of the dramatic environmental conditions induced by inflammation. To understand the complex dynamics of interaction among *S*. Typhimurium-host-microbiota, is extremely important for the possible implications in the development strategies resulting in the reduction of colonization and/or frequency of salmonellosis. This thesis aimed at investigating the contribution of virulence, inflammation and microbiota to *S*. Typhimurium infection using piglet as model for salmonellosis. Firstly, we evaluated the capability of *S*. Typhimurium to exploit inflammation to promote its own colonization *in vivo* and *in vitro*. Our findings have shown that lipopolysaccharide (LPS) treatment-induced inflammation enhances the progression of *S.* Typhimurium, making the pathogen more capable to colonize at higher numbers both piglets and porcine enterocytes (IPEC-J2) and monocytes/macrophages cells. Secondly, we investigated the impact of *Salmonella* virulence on the porcine intestinal microbiota. To this end, we compared the effects on the microbiota induced by two different strains of *Salmonella*, a wild type strain and its isogenic attenuated mutant. We observed the existence of a link among virulence, inflammation and microbiota composition. *S*. Typhimurium wild type induces a strong inflammation which results in the reduction of some members of microbiota (i.e. SCFA-producing bacteria), normally involved in the maintaining of

intestinal homeostasis and in the inhibition of pathogen growth. On the contrary, *S*. Typhimurium attenuated strain determines a milder inflammation and is correlated to an increase of protective species of microbiota which could shift the competition between the pathogen and microbiota in favour of the latter explaining the reduced ability of the attenuated strain to colonize host.

Finally, we focused on the physiological alterations of porcine cecum caused by wild type and attenuated *S*. Typhimurium strains. Our results suggest that host could adopt a "nutriprive mechanism" in which deprives environment of nutrients and energy sources, creating intestinal conditions that are detrimental for *Salmonella* growth. Moreover, the minor reduction of metabolic and energetic status of the host upon infection with *Salmonella* wild type in comparison with the attenuated strain could indicate that *Salmonella* wild type is more capable to oppose to the nutriprive mechanism.

Overall, by investigating the interaction dynamics among *S*. Typhimurium-host-microbiota, we have provided insights that the three factors are strictly interrelated and multiple aspects of each of them contribute to determine the outcome of infection.

RIASSUNTO

Salmonella enterica serovar Typhimurium è un batterio Gram negativo, patogeno enterico responsabile di una tossinfezione alimentare a diffusione mondiale. In seguito alla penetrazione nell'ospite, *Salmonella* deve superare la robusta linea di difesa rappresentata dal sistema dell'immunità innata e dalla resistenza alla colonizzazione mediata dal microbiota. *Salmonella* è un affascinante esempio di patogeno capace oltre che di eludere la risposta immunitaria, anche di sfruttarne i molteplici aspetti per colonizzare l'intestino. Ha sviluppato, infatti, strategie di virulenza che gli consentono di prosperare nell'intestino infiammato a discapito del microbiota residente, traendo vantaggio dalle condizioni ambientali fortemente alterate indotte dall'infiammazione.

Comprendere le complesse dinamiche di interazione tra *S*. Typhimurium-ospite-microbiota, è estremamente importante per le possibili implicazioni nello sviluppo di strategie vòlte a ridurre la colonizzazione e/o la frequenza della salmonellosi. Scopo di questa tesi è stato caratterizzare il contributo della virulenza, dell'infiammazione e del microbiota all'infezione da *Salmonella*, utilizzando il suino come modello sperimentale per la salmonellosi.

In primo luogo, abbiamo valutato l'abilità di *S*. Typhimurium di trarre vantaggio dall'infiammazione per favorire la propria colonizzazione, in vivo e in vitro. I nostri risultati hanno mostrato che l'infiammazione indotta dal trattamento con lipopolisaccaride (LPS) migliora la progressione dell'infezione, rendendo il patogeno capace di colonizzare con una carica più elevata sia i suinetti sia gli enterociti (IPEC-J2) e i monociti/macrofagi di origine suina.

Successivamente, abbiamo valutato l'impatto che la virulenza del patogeno ha sul microbiota intestinale suino. A tale scopo, abbiamo comparato gli effetti causati da due differenti ceppi di *Salmonella*, un ceppo wild type e il suo mutante isogenico

attenuato, sulla composizione del microbiota. Abbiamo osservato l'esistenza di un collegamento diretto tra virulenza, infiammazione e composizione del microbiota. *S*. Typhimurium wild type induce una forte risposta infiammatoria che determina la riduzione di membri del microbiota (ad es. i batteri che producono SCFA) normalmente implicati nel mantenimento dell'omeostasi intestinale e nell'inibizione della crescita dei patogeni. Al contrario, il ceppo attenuato di *S*. Typhimurium causa una debole infiammazione che è invece associata ad un aumento di specie protettive del microbiota e ciò potrebbe spostare la competizione tra patogeno e microbiota in favore di quest' ultimo, spiegando così la ridotta abilità di questo ceppo a colonizzare l'ospite.

Infine, la nostra attenzione si è focalizzata sulla risposta dell'ospite ed in particolare sulle modificazioni pato-fisiologiche che verificano a livello del cieco in seguito ad infezione con i due ceppi di *S*. Typhimurium, wild type e attenuato. I nostri risultati suggeriscono l'induzione di un "meccanismo nutriprivo" attraverso il quale l'ospite riduce la disponibilità di nutrienti e di fonti di energia per creare condizioni intestinali che risultano svantaggiose per la crescita di *Salmonella*. Inoltre, è stata osservata una minore riduzione dello stato metabolico ed energetico dell'ospite dopo infezione con *S*. Typhimurium wild type rispetto a quella indotta dal ceppo attenuato che potrebbe indicare una maggiore capacità del ceppo wild type a contrastare il meccanismo nutriprivo.

In conclusione, studiando le dinamiche di interazione tra *S*. Typhimurium-ospite-microbiota, abbiamo fornito ulteriori evidenze del fatto che i tre fattori sono strettamente correlati e che l'esito dell'infezione è il risultato del contributo fornito dai molti aspetti che li caratterizzano.

Chapter 1

INTRODUCTION

1.1 Salmonella

Salmonella was discovered in 1884 by Theobald Smith and Daniel Elmer Salmon who first isolated *Salmonella choleraesuis* from pigs with hog cholera (Salmon & Smith, 1886). Salmonellae are Gram negative, road-shaped (0.7-1.5 x 2.0-5.0 μm), flagellated, facultative anaerobic bacteria belonging to the *Enterobacteriaceae* family. The genus *Salmonella* consists of only two species: *S. enterica* and *S. bongori*. *S. enterica* is further divided into six subspecies and more than 2600 serovars differentiated according to somatic, flagellar and virulence (O, H and Vi, respectively) antigens (Cooke et al., 2007). According to the ability to infect a different range of hosts, *Salmonella* serovars can be classified into three groups (Singh, 2013):

- **host-restricted serovars**, that include *S*. Typhi and *S*. Paratyphi A, B and C in humans, and S. Gallinarum and S. Pullorum in poultry, are responsible for a systemic infection which often results to be fatal;
- **host-adapted serovars**, such as *S*. Choleraesuis and *S*. Dublin, that cause highly severe systemic infection in pigs and cattle respectively; however, they also may accidentally cause disease in other hosts;
- **broad host range serovars** that rarely produce systemic infections but are able to cause a gastrointestinal disease in a wide range of animals. These serovars are collectively called non typhoidal *Salmonella* (NTS) and include some of the most common strains such as *S*. Enteriditis and *S*. Typhimurium. They represent an

important zoonotic hazard because of developing strategies to invade different hosts without any greater resistance

This dissertation is focused on NTS and in particular on *S. enterica* serovar *Typhimurium*.

1.2 The Infectious Cycle of Salmonella

Salmonella infections are basically acquired by ingestion of contaminated food and water. Following ingestion, bacteria encounter the low pH of the stomach to which a reduced number of them survives exploiting an acid tolerance response. The remaining bacteria, gain access to the small intestine and invade the intestine wall, spreading to mesenteric lymph nodes and then to the systemic circulation. *Salmonella* has different pathways to invade the intestinal mucosa (Fig. 1) (Khan, 2014).

Salmonella invasion preferentially occurs via microfold (M) cells located in the follicle-associated epithelium (FAE), sorrounding the Peyer's patches (PP). The M cells are specialized phagocytic cells able to target luminal antigens and to transport them to the basolateral side, where they can interact with lymphoid cells (macrophages, dendritic cells (DC), neutrophils) in the gut-associated lymphoid tissues (Broz et al., 2012). *Salmonella* can also invade non-phagocytic epithelial cells, promoting its own internalization trough the type III secretion system (T3SS-1) encoded by the *Salmonella* Pathogenicity Island 1 (SPI-1). This process involves secretion of SPI-1-encoded virulence factors, which induce large-scale cytoskeletal rearrangements, leading to ruffling and extension of the enterocyte membrane and hence allowing bacteria to be engulfed and dragged inside the host cell (Brawn et al., 2007). An additional route of invasion might be via $CX3CR1^{+}$ macrophages and DCs. DCs are professional antigen-presenting cells (APC) that protrude

prolongations between adjacent enterocytes to capture luminal antigens and to present them, in association with major histocompatibility complex (MHC) molecules, to T cells (Niess et al., 2005). However, following phagocytosis, *Salmonella* develops around it a membrane-bound vacuole called the *Salmonella*-containing vacuole (SCV), and promotes its replication by secreting additional virulence factors encoded by SPI-2 (Brawn et al., 2007). The pathogen is able to spread systematically throughout the reticuloendothelial system. However, in healthy individuals, *Salmonella* infection is limited to the intestine, leading a diarrhea with a significant neutrophil influx into intestinal lumen. In these patients, gastroenteritis is self-limiting and pathogen is eliminated within 14 days (Khan, 2014).

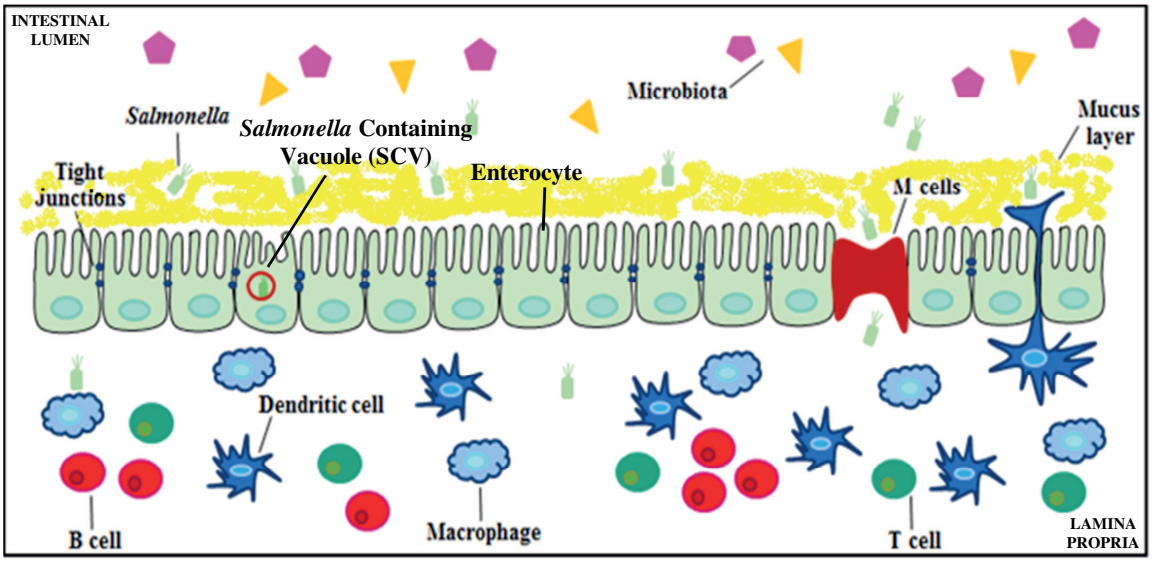

Fig. 1. Schematic representation illustrating the routes of invasion of *S.* Typhimurium. *S.* Typhimurium can cross the intestinal epithelial by invading enterocytes, M cells or dendritic cells protruding prolongations to sample the intestinal lumen.

1.3 Epidemiology

Human infections caused by *Salmonella* spp. are a significant public health concern. The epidemiology of infections is influenced by the type of *Salmonella* serovar involved. Host-restricted serovars, such as *S*. Typhi and *S*. Paratyphi, are responsible for enteric fever, an acute and life-threatening febrile illness primarily connected with low-income countries, which globally causes 22 millions of cases per year and 200,000 deaths (Crump et al., 2004).

The enteric fever is transmitted via the fecal-oral route and it is facilitated by inadequate sanitation and poor hygienic conditions. In fact, high incidence estimates are reported in the developing world, particularly in south-central Asia and south-east Asia (more than 100 cases per 100,000 inhabitants per year), while in Europe, North America, Australia and New Zealand is registered a low incidence (less than 10 cases per 100,000 inhabitants per year) and it is mainly a disease related to travelers visiting endemic areas. Countries with medium incidence are observed in Africa, South America and the rest of Asia (10-100 cases per 100,000 inhabitants per year). The emergence and spread of antibiotic-resistant *S*. Typhi and *S*. Paratyphi strains in developing countries, increases the risk of complications and death (Sanchez-Vargas et al., 2011).

Conversely, NTS are a leading cause of acute food-borne zoonosis worldwide. Transmission to humans generally occurs by either ingestion of contaminated food and water or by direct contact, person-to-person or animal-to-person, via the fecal-oral route. The rapid dissemination of pathogens due to the food production and distribution systems, and the problem of multidrug resistant NTS strains pose an important public health concern (Sanchez-Vargas et al., 2011). NTS cause a self-limiting enterocolitis which rarely requires intervention; however, disease can evolve to septicemia in less than 10% of patients. Approximately 93.8

million human cases of gastroenteritis and 155,000 deaths occur globally each year (Majowicz et al., 2010). Moreover, in sub-Saharan Africa, NTS are also an important cause of invasive bloodstream infection with an estimated mortality rate of 20-25%, particularly in children with malnutrition, malaria and/or HIV infection (Wick, 2011; Feasey et al., 2012).

1.4 Non typhoidal Salmonellae in food-producing animals

Most NTS serovars are important foodborne pathogens of zoonotic origin. They can infect a wide range of farm and wild animal. Poultry, pigs, and cattle are considered the most important farm animal reservoirs for human infections; however, rodents, birds, reptiles, fish and insects can also play an important epidemiological role (Wales et al., 2010).

Salmonella serovars have a widespread geographical and epidemiological distribution; however, they differ in their pathogenic potential between humans and animals as well as in prevalence. For instance, despite NTS are common in reptiles and amphibians, very few human cases of salmonellosis are due to these animals (Hoelzer et al., 2011).

Salmonella colonize the gastrointestinal tract causing an acute enterocolitis that can be followed by a chronic infection, given the ability of the pathogen to establish a persistent colonization. Persistently colonized animals are asymptomatic and indistinguishable from uninfected animals, constituting an important risk factor for humans (Hoelzer et al., 2011). In addition, environmental contamination by manure and waste products from food production represents another significant source of infection, and it is favored by the ability of *Salmonella* to survive for long periods in soil and water (Silva et al., 2014).

The human health risk associated with animals is influenced by multiple factors including age group, herd management system and health status. For example, large-scale intensive farm conditions determine more easily the rapid spread of *Salmonella* among animals and contamination of food products (Hugas and Beloeil, 2014). However, the improvement of good hygienic measures and the implementation of management practices can reduce the risks of infection animal-to-person (Hoelzer et al., 2011).

Nevertheless, ingestion of contaminated food is thought to be the predominant risk factor for salmonellosis. A wide variety of foods, mostly of animal origin, are frequent sources of *Salmonella* infection in humans; they include pork meat, poultry, eggs, raw milk and dairy products. In recent years, the role of food of vegetable origin, as potential vehicles of gastrointestinal infection, has been highlighted. Vegetables and fruits can be contaminated with *Salmonella* in both the pre-harvest (i.e. utilization of organic wastes as fertilizer or contaminated water for irrigation) or post-harvest steps (during storage) of the processing chain (Heaton and Jones, 2008).

The monitoring and surveillance plans are crucial in sustaining food safety standards; moreover, the ability of *Salmonella* to exploit different sources of infection creates public health challenges (Newell et al., 2010).

Chapter 2

SALMONELLA-HOST INTERACTIONS

The intestinal mucosa is the largest surface of mammalian body in direct contact with the external environment, and it is exposed to a wide variety of potentially harmful enteric microbes. The host is protected from the invading pathogens by several types of barriers, including physical, chemical and immunological ones, which exert mechanisms of protection to maintain the integrity of the intestinal epithelium and to limit inflammation-associate damage (Patel & McCormick, 2014).

2.1 *Salmonella interaction with the intestinal mucosal epithelia*

A thick layer of mucus that covers the luminal lining of the intestinal epithelium, antimicrobial peptides and immunoglobulins are the first obstacles to *Salmonella* entry (Patel & McCormick, 2014).

- Mucus is primarily composed of mucins. Mucins are highly glycosylated transmembrane proteins secreted by Goblet cells which aggregate to form a gelatinous barrier aiming to constitute a physical barrier against the bacterial invasion as well as to protect intestinal mucosa from dehydration and mechanical damage. (Broz et al., 2012). Nevertheless, mucus is permeable to low molecular weight components allowing the transport of nutrients. The production of mucins can occur constitutively to a low level, or can be modulated in response to microbial component and inflammation. However, the mechanisms regulating their secretion is unknown (Kim & Khan, 2013).
- In addition to mucins, specialized epithelial cells called Paneth cells secrete antimicrobial peptides (AMPs) of ~20-40 amino acids in length. AMPs take part in

the front line of chemical defense against bacteria and function targeting the bacterial membrane and disrupting its integrity (Broz et al., 2012). Four families of AMPs have been identified: defensins, cathelicidins, histatins and lactoferrin (Patel & McCormick, 2014). Defensins are small cationic proteins that directly bind the microbial cell membrane forming large pores that cause the loss of essential ions and nutrients. Cathelicidins are also cationic proteins that act as potent lipopolysaccaride (LPS)- neutralizing factors. Lactoferrin is a multifunctional protein that functions sequestering iron and destabilizing microbial membranes. Histatins are involved in the generation of reactive oxygen species (ROS) and induce apoptosis by inhibiting mitochondrial respiration (Kavanagh & Dowd, 2004). All AMPs are generated as inactive prepropeptides and need to be processed into its active form; their production is upregulated in response to bacteria (Patel & McCormick, 2014).

- Finally, also secretory IgA (SIgA), produced by plasma cells in the in lamina propria and transcytosed in the mucus layer, serve as line of defense against *Salmonella*. Through a process called immune exclusion, SIgA prevent the microbial adhesion to epithelial receptors on the luminal side of the intestinal epithelium, while facilitate the clearence of antigens from the basolateral side (Mantis et al., 2011).

In the lumen outside the mucus layer reside another important mucosal barrier against *Salmonella*: the resident microbiota. Microbiota constitutes a heterogeneous ecosystem containing up ~10^{14} bacteria, which physically block pathogen access to the epithelial layer by competing for nutritional resources and attachment sites. Additionally, microbiota promote resistance to infection through the production of bacteriocins and end-products of metabolism, called shorty chain fatty acids (SCFAs), by individual species of bacteria (Patel & McCormick, 2014). The anaerobic environment of the intestine is dominated by fermentative bacteria which

produce acetate, propionate and butyrate. They are involved in maintenance of the intestinal homeostasis and metabolism. Moreover, recently, mainly butyrate has shown a *Salmonella* invasion-suppressive activity through the down regulation of SPI1 genes. In addition, butyrate can affect *Salmonella* interaction with the intestinal epithelium inducing expression of cathelicidins (Sun & O'Riordan et al., 2013).

Finally, the integrity of the epithelial barrier plays another important protective role against the pathogen. The integrity is guaranteed by the interaction of tight junctions (TJ) with components of cytoskeleton; however, *S.* Typhimurium can alter this barrier interfering with tight junction proteins such as Rho-GTPase and occludin. The resulting modifications contribute to increase the membrane permeability, allowing *Salmonella* to cross the epithelial cell monolayer (Ashida et al., 2012).

2.2 Immune response to Salmonella

Despite the protection provided by the barriers described above, *Salmonella* is capable of penetrating the intestinal epithelium through M cells or enterocytes (Broz et al., 2012). Here, the pathogen encounters the monocyte-derived phagocytic cells of the gut-associated lymphoid tissue (GALT): macrophages and dendritic cells, which act controlling *Salmonella* infection through phagocytosis-mediated killing and the induction of inflammation (Broz et al., 2012).

The direct interaction between *Salmonella* and host cells includes the recognition of components of the pathogen, called pathogen associated molecular patterns (PAMPs), by pattern recognition receptors (PRRs) localized on epithelial cells and inflammatory cells (Santos et al., 2009). Toll-like receptors (TLRs) and NOD-like receptors (NLRs) are examples of PRRs and they can recognize a wide range of PAMPs. TLR4 and TLR5 are involved in *S.* Typhimurium infections; they

detect LPS and flagellin, respectively. The binding of ligands to their respective TLRs or NODs triggers the activation of inflammatory response through the NF-κB signaling pathway, leading to cytokines and chemokines production (Patel & McCormick, 2014).

The secretion of IL18 and IL-23 by mononuclear cells (macrophages and DC), promote a T cell-dependent amplification of inflammatory responses. These cytokines amplify inflammation stimulating T cells to produce INF-γ, IL-22 and IL17 (Broz et al., 2012). In addition to T cells, also natural killer (NK) cells are source of IL-22 in the intestine. Moreover, IL-6 and transforming growth factor (TGF)-β, produced by macrophages, can initiate the differentiation of naïve T cells into Th17 cells (Santos et al., 2009). Macrophages can also produce IL1-β and IL-18 via NLRC4-Caspase-1 axis induced by the recognition flagellin-NLRC4 (Raupach et al., 2006).

Finally, the release of antimicrobial products orchestrated through the IL-23/IL-22 and the influx of neutrophils into the intestinal lumen mediated via the IL-23/IL-17 axis contribute to diarrhea, which limits the presence of nutrients that normally support commensal bacteria growth as well as that of *Salmonella* (Broz et al., 2012).

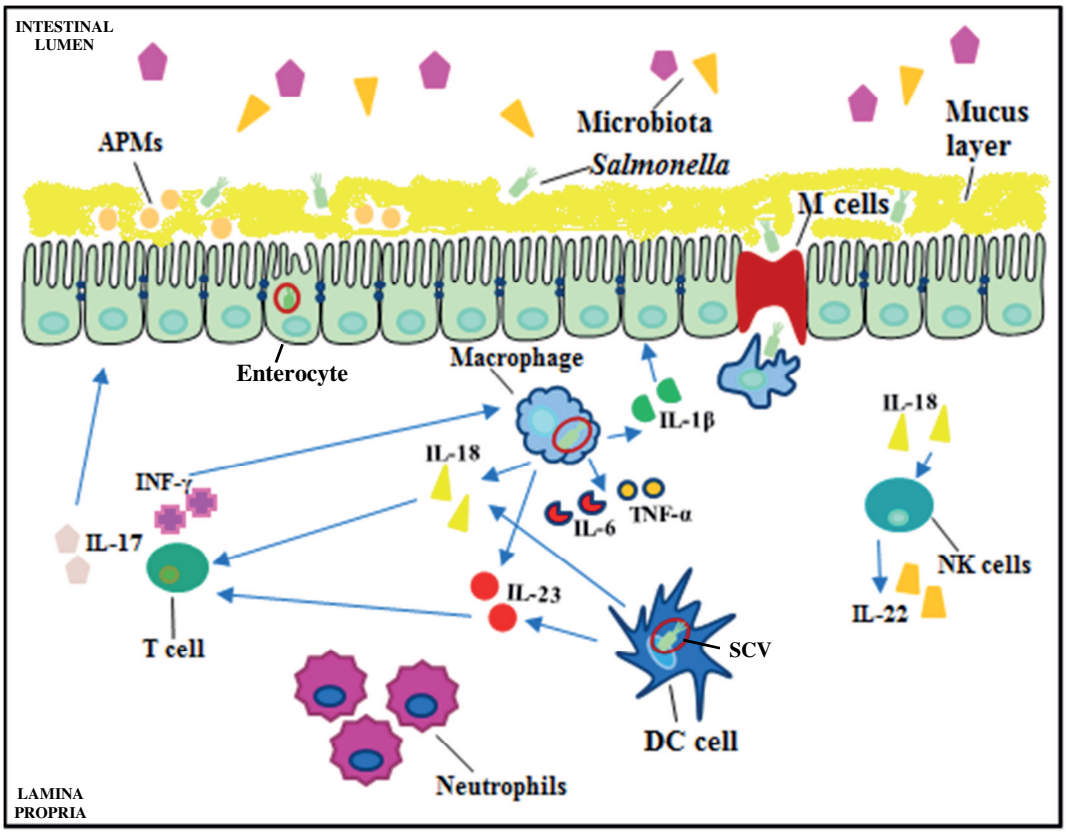

Fig. 2. Schematic representation showing the immune response to *S.* Typhimurium. Upon the invasion, *S.* Typhimurium encounters the monocyte-derived phagocytic cells of the gut-associated lymphoid tissue (GALT): macrophages and dendritic cells, which act controlling *Salmonella* infection through phagocytosis-mediated killing and the induction of inflammation. DC: dendritic cells; NK: Natural Killer cells; AMPs: antimicrobial peptides; SCV: *Salmonella* containing vacuole.

2.3 Salmonella overcomes the intestinal barriers

In spite of several barriers of the mucosal surface and the notable inflammatory response mounted by the host immune system, *S.* Typhimurium is able to thrive in the intestinal lumen increasing its numbers (Santos et al., 2009). It has evolved strategies to interfere with and to manipulate host defensive mechanisms in a

highly sophisticated manner in order to survive and replicate in the inflamed gut (Patel & McCormick, 2014).

Host immune response has a double role in *S*. Typhimurium infections because it limits *Salmonella* colonization and dissemination but, at the same time, *Salmonella* has adapted to take advantage of host immunity at the expense of microbiota (Stecher et al., 2007; Lupp et al., 2007; Behnsen et al., 2015). For this reason, it is not surprising that *Salmonella* actively induces inflammation for its own purposes.

A further confirmation of these evidences came from a relatively recent study conducted by Stecher et al. (2007); it has shown that an avirulent mutant of *S*. Typhimurium, with a defective inflammatory capability, is unable to colonize the murine healthy intestine and is outcompeted by the microbiota. However, in presence of mixed infections with wild-type *S*. Typhimurium, which is able to induce inflammation and therefore to alter microbiota, the defective colonization of the avirulent mutant is reverted (Stecher et al., 2007).

2.4 S. Typhimurium exploits inflammation to compete with intestinal microbiota

The aim of an inflammatory response is to reduce the colonization of pathogens and eventually eliminate them through the release of antimicrobial mediators. The inability of the innate immune response to completely discriminate pathogens from commensal bacteria, implies that inflammation may damage both of them (Stecher, 2015). However, *Salmonella* has developed many strategies to escape host responses and to compete successfully with the microbiota.

Three different hypothesis have been proposed to explain how inflammation could contribute to break colonization resistance (Stecher, 2015):

1. The "**food hypothesis**" suggests the induction of an altered nutrients availability or adhesion receptor sites upon *S.* Typhimurium infection, that can be used by the pathogen at the expense of commensals bacteria (Le Bouquénec & Schouler, 2011; Fischbach et al., 2006; Stecher, 2015). For example, phosphatidylethanolamine is the most abundant phospholipid in the cell membrane of enterocytes. It is metabolized to ethanolamine, a non-fermentable compound which is not utilizable as carbon source by the most of the microbiota that is based on a fermentative metabolism (Behnsen et al., 2015). By contrast, *S.* Typhimurium is able to catabolize ethanolamine by using tetrathionate. During inflammation, thiosulfate is oxidized in tetrathionate by the reactive oxygen species (ROS) produced by the host, and tetrathionate can be used by *S.* Typhimurium as a terminal electron acceptor that support anaerobic respiration of ethanolamine. This ability to utilize inflammation-induced compounds confers a growth advantage for *S.* Typhimurium over the competing microbiota (Thiennimitr et al., 2011). Similarly, *S.* Typhimurium can use high-energy nutrients, such as mucins and galactose-containing glyco-conjugates released as result of inflammation, to outgrow the microbiota (Stecher et al., 2008).
2. The **"differential killing hypothesis":** the release of antibacterial factors induced by *Salmonella* infection may selectively inhibit growth of members of the microbiota, while enteric pathogens would remain unaffected (Stecher et al., 2015). For example, an efficient mechanism of the host immune response is to limiting the access for *Salmonella* to metal ions by releasing the metal-chelating proteins lipocalin-2 and calprotectin. Lipocalin-2, secreted by epithelial cells and neutrophils, blocks bacterial iron acquisition by specifically binding to iron-scavenging siderophore called enterobactin, showing a bacteriostatic effect on those microbes that depend only on enterobactin to acquire iron (Flo et al., 2004). However, *S.* Typhimurium expresses a glycosylated derivative of enterobactin, known as salmochelin, which is not bound

by lipocalin-2 and its production allows to the pathogen to overcome iron starvation (Fischbach et al., 2006). Similarly, calprotectin sequesters zinc in the intestine limiting its availability to microbes. *S.* Typhimurium uses a high-affinity zinc transporter (znuABC) to resist to calprotectin and to acquire zinc overcoming calprotectin-mediated zinc chelation (Liu et al., 2012).

3. The **"oxygen hypothesis"**: the reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by neutrophils in response to *S.* Typhimurium infection, create a highly oxidative environment which is inhospitable for many members of microbiota, but not for *Salmonella*. (Winter et al., 2010; Stecher, 2015). In fact, while in the microbial community, the number of obligate decreases in favor of the facultative anaerobes, *S.* Typhimurium is able to resist to ROS and RNS in different ways. For example, recently, it is demonstrated that *Salmonella* has an efflux pump that is involved in the detoxification of ROS, contributing to the survival and replication of the pathogen (Bogomolnaya et al., 2013). Similarly, *Salmonella* has developed strategies of resistance to nitrosative stress including the protective role of Cu,Zn-superoxide dismutase (SOD) in reducing periplasmic formation of peroxynitrite (De Groote et al., 1997).

Chapter 3

MICROBIOTA

3.1 The mammalian gastrointestinal microbiota

The mammalian gastrointestinal tract is colonized by approximately 10^{14} bacteria forming a complex and dynamic microbial community called microbiota (Isaacson & Kim, 2012). It is mainly populated by anaerobic bacteria, while aerobic and facultative species are present in lower numbers; *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* represent the major phyla among intestinal eubacteria (Sommer & Bäcked, 2013). In contrast to pathogens, commensal bacteria have made the host their one-and only home creating an extraordinary example of symbiotic mutualism in which both, commensals and host, benefit each other (Ahmer & Gunn, 2011; Ivanov & Honda, 2012). Host immune system is likely to distinguish between commensal and pathogenic bacteria, inducing tolerance to microbial epitopes (Srikanth & McCormick, 2008).

Mammals are born sterile. Colonization by microbiota begins immediately after the birth and continues to develop and change during the entire life. Initially, the gastrointestinal tract is populated by bacteria with high multiplication rates; later, microbiota becomes more complex and dominated by specialized species (Falk et al., 1998). Microbiota structure and composition are not constant but differ among and within individuals in relation to internal and environmental factors such as genetic factors, age, diet and exposure to microbes (Ivanov & Honda, 2012).

In recent years, microbiota has begun to receive growing attention since its remarkable role in the health and well-being of humans and animals.

3.2 Role of the intestinal microbiota

The intestinal microbiota affects several aspects of host physiology being involved in protective, biochemical, metabolic, trophic and immunological functions (Littman & Pamer, 2011). Resident bacteria provide an efficient barrier against the invading pathogens; this ability, also known as "colonization resistance", is the result of the direct microbe-microbe interactions such as competition for nutrient availability and for attachment sites. Specific members of microbiota can also stimulate the mucosal immune system and the release of antibacterial peptides (Barman et al., 2008).

The absence or the alteration of microbiota can affect the intestinal environment, also causing modifications of digestive enzyme activity and baseline cytokines production (Srikanth & McCormick, 2008). Moreover, germfree mice show a rudimentary intestinal immune system and are highly susceptible to enteric pathogens (Ahmer & Gunn 2011). All these evidences demonstrate the important role of microbiota in influencing the maturation of the mucosa-associated lymphoid tissue as well as in preventing pathogens invasion (Srikanth & McCormick, 2008).

In addition to the protective and immunological functions, microbiota offers essential metabolic contributions by the production of key vitamins (i.e. vitamin K, biotin and folate) and SCFAs, which have trophic effects on the intestinal epithelium. It is also involved in the fermentation of unused energy substrates (i.e. pectins, cellulose), and in the xenobiotics and bile acids metabolism (Srikanth & McCormick, 2008).

These observations have increased interest in the study of the effects of enteric pathogens on the intestinal flora, and the possible implications in the development of strategies reducing colonization and/or the frequency of infectious disease.

3.3 Animal models for studying Salmonella infections and interactions with the microbiota

The use of animal models to study the interaction between host–pathogen has revealed significant insight into the role played by commensals towards mediating resistance against disease.

Mice are the animal model most commonly used by researchers, for reasons of convenience and cost. Conventional mice are not appropriate as model for *Salmonella* gastroenteritis because they do not get diarrhea upon infection, rather a systemic form that is actually closer to human typhoid fever. To resemble human disease, mice can be treated with antibiotics, most commonly streptomycin (streptomycin-treated mice), in order to eliminate microbiota prior to infection with *Salmonella* (Ahmer & Gunn, 2011).

Gnotobiotic mice have a conditioned microbiota composition. Of those, germ-free mice are completely lacking of microbiota, mono-associated mice are characterized by a single known bacterial species, and poly-associated mice are colonized with several known microbial communities (Falk et al., 1998). However, also these models are not completely suitable for the evaluation of the strategies used by *S.* Typhimurium to survive in the inflamed gastrointestinal environment, because of the lack of an intact microbiota (Elfenbein et al., 2013).

For all these reasons, the use of an animal which is a natural host of *S.* Typhimurium and has an intact microbiota, is optimal to study the interactions among *Salmonella*, microbiota and the vertebrate host during infection. The great similarity between humans and pigs in the gastrointestinal tract and in the disease caused by *S.* Typhimurium, make pig an ideal model for salmonellosis and gastrointestinal research (Zhang et al., 2013).

3.4 Intestinal microbiota in pigs

The microbial community of the porcine gastrointestinal tract has traditionally been studied by culture-dependent microbiological methods; however, in recent years, the advent of molecular techniques has allowed a better characterization of swine microbiota composition (Isaacson & Kim, 2012).

The piglet gastrointestinal tract is without microorganisms at birth. The contact with the skin of the mother as well as with the environment, starts the intestinal bacterial colonization and in a short time, aerobes and facultative anaerobes become the predominant species of the flora (Conway, 1997). However, within 48 h after birth, these bacteria are gradually substituted by obligate anaerobes. At this time and for the whole suckling period, microbiota is dominated by *Lactobacilli* and *Streptococci*, which are able to use lactose as substrate (Swords et al., 1993).

A critical stage in the life cycle of pig is the weaning period. In fact, stressing factors, such as the separation from its mother and the introduction of a solid food, can contribute to alter the microbiota composition as well as the intestinal functionality (Roca et al., 2014). As consequence, immediately after weaning, it is possible to observe a decrease of *Lactobacillus* and an increase of coliforms bacteria. At this moment, pig becomes highly susceptible to overgrowth of pathogens. However, after this intense period of perturbation, microbiota re-stabilizes and develops in the normal adult flora (Hopwood & Hampson, 2003).

In 2011, Kim et al. published a longitudinal study on swine microbiota composition. They showed that two phyla, *Firmicutes* and *Bacteroidetes*, accounted for approximately 90% of all bacteria present in the porcine gastrointestinal tract. Later on, the abundance of *Firmicutes* and bacteria belonging to non-classified group increased, while decreased that of *Bacteroidetes*. The most abundant genus was

Prevotella, a member of *Bacteroidetes*, constituting up 30% of all bacteria present in pigs of 10 weeks of age. However, in pigs of 22 weeks of age, *Prevotella* decreased to 3,5-4% of the bacteria, while *Anaerobacter* (*Firmicute*s) increases. Finally, over time, other abundant species included *Streptococcus*, *Lactobacillus*, *Megasphaera*, *Blautia*, *Oscillibacter*, *Faecalibacterium*, *Pseudobutyrivibrio*, *Roseburia and Butyricoccus* (Kim et al., 2011).

All these data have contributed to demonstrate that microbiota composition varies over time. It is a dynamic process in which different microbial populations succeed each other until reaching a climax community where bacteria are in stable association with the host (Isaacson & Kim, 2012).

3.5 Salmonella-porcine microbiota interactions

It has been known, from studies conducted in mice, that *S.* Typhimurium alters intestinal microbiota (Stecher et al., 2007; Barman et al., 2008). However, there is paucity of information on the effects of *Salmonella* infection on the swine microbiota, because of the majority of investigations have been addressed to characterize the effects of nutritional and dietary additives, antibiotics and production practices on the porcine intestinal microbiota (Isaacson & Kim, 2012).

A recent study has demonstrated that *S.* Typhimurium is able to disrupt the intestinal microbiota of pigs both experimentally and naturally-infected. There was a correlation, as regards the changes in microbiota composition, between the two types of pigs, and the major modifications have been observed in the cecum, colon and feces (Borewicz et al., 2015).

Bearson et al. (2013) have compared the porcine microbiota composition to the *Salmonella*-shedding status of pigs. Profiling microbiota before and after *S. Typhimurium*, the authors have observed the presence of a higher proportion of

Ruminococcaceae family in the "will be" low shedders pigs before infection, suggesting that *Ruminococcaceae* could influence *Salmonella* colonization through the production of SCFA, whose anti-inflammatory properties and the action of inhibition of the pathogens are well known (Tedelind et al., 2007; Bearson et al., 2013). On the contrary, "will be" high shedders pigs have shown an increase of *Phascolarcobacterium* and *Coprobacillus*. After *Salmonella* infection, high-shedders pig showed a decrease in *Prevotella* and a significant increase of *Coriobacteriaceae* family, with the latter that it is known to be involved in inflammatory diseases in mice and humans (Clavel et al., 2010).

Chapter 4

PROTEOMIC STUDY

4.1 Introduction of systems-level analysis to study the host-pathogen interplay

Advances in understanding the molecular mechanisms underlying host-pathogen interactions are important for the development of new diagnostic and therapeutic strategies. The functional interface between pathogenic microbes and their host involves thousands of proteins belonging to both of them (Zhang et al., 2005).

Over the years, the utilization of classical methods (genetic and biochemical) contributed to identify bacterial virulence factors and their host targets, elucidating many aspects of infection biology. However, these approaches alone are not able to completely explain the complexity of host-pathogen interactions; for this reason, it has become important the introduction of systems-level analysis providing an overview of the dynamic host-pathogen interplay (Yang et al., 2015).

Within the last decade, proteomic technologies have provided a useful tool for this purpose, allowing the understanding of both, the *S. Typhimurium* physiology and the host pathophysiological changes, that occur during infections (Arce et al., 2014).

4.2 Protein identification by proteomic analysis.

Proteomes were traditionally investigated using protein microarrays and protein staining with two-dimensional gel electrophoresis (2DE); the latter technique resolves protein mixture into individual protein spots through the coupling of two

perpendicular separation techniques: isoelectric focusing and protein's molecular mass. However, these approaches, in particular 2DE, were limited in sensitivity to characterizing the complex biological samples (Yang et al., 2015).

Over the years, advances in adapting mass spectrometry (MS) to biomolecules have allowed to overcame the intrinsic limitations of these techniques supporting their weaknesses; the utilization of 2DE gel electrophoresis associated with MS characterization of individual protein spots has functioned as pioneer for proteomic studies (Yang et al., 2015).

In the last decade, further progresses have been made in the proteomic field through the development of a MS-based high-sensitivity high-throughput approach. This technology is based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and provides an enzymatic digestion of protein mixture prior to liquid chromatography (Cravatt et al., 2007).

The current proteomic technologies are capable of measuring many samples in a short time allowing us to increase insights on host-pathogen interactions. In fact, today, a large number of studies uses this technique to evaluate the proteome of both pathogen and host (Rodland et al., 2008).

4.3 Analysis of Salmonella-host interaction through proteomics

During infections, bacterial pathogens have to modify their own proteome to survive in the altered host environment. On the other hand, host will modulate its gene and protein expression aiming to oppose to the bacterial infection (Jenner and Young, 2005). For this reason, it is very important to investigate the modifications of host cellular processes that occur in response to infection.

Most of the previous proteomic studies of *Salmonella* have focused on the characterization of virulence factors (Shi et al., 2006; Shi et al., 2009) and on the

detection of changes occurring in *Salmonella* under in vivo mimicking conditions or in the response to growth in presence or absence of oxygen (Sonck et al., 2009; Encheva et al., 2009). On the contrary, little information is available on the host proteome upon bacterial infection, probably because host cells and tissues are much more complex than the bacterial ones, and the few reported studies regarding infections in vitro of isolated cell lines (Yang et al., 2015).

However, recently, several authors have carried out proteomic studies aiming to evaluate the response of different intestinal sections (ileum, colon, lymph nodes) to *Salmonella* using an in vivo porcine model. The results suggested a perturbation of normal host functions after *S. Typhimurium* infection involving pathways related to rearrangements of cytoskeleton, metabolism and inflammation (Collado-Romero et al., 2012; Martins et al., 2012; Arce et al., 2014; Collado-Romero et al., 2015).

In none of these studies it was possible to correlate host proteome modifications directly to *Salmonella* virulence factors; however, it can be inferred that these changes might be caused by the pathogen itself. In fact, the significant alteration of the metabolism of carbohydrates, lipids and vitamins observed after infection, might be explained with the ability of *S. Typhimurium* to utilize nutrients present in the intestinal mucosa for its own growth (Arce et al., 2014).

Chapter 5

Aim of the thesis

S. Typhimurium is a Gram-negative bacteria able to infect a broad range of hosts causing both acute and chronic diseases. It is considered a successful pathogen having acquired evolutionary strategies to cope with most of the host immune defenses and, more importantly, to use the inflammatory response aiming to overcoming intestinal microbiota. Therefore, characterizing the *S.* Typhimurium-host-microbiota interaction is critical to deepen the knowledge about the mechanisms involved in the pathogenicity of this bacteria.

In this work, we used pig as ideal model for salmonellosis. Besides to be a natural host of *S*. Typhimurium, pig is free from the intrinsic limitations of the streptomycin-treated mouse colitis model, namely do not produce naturally gastroenteritis and the lack of an intact microbiota.

Aims of this thesis were to:

Objective 1. evaluate the ability of *S*. Typhimurium to exploit inflammation to favor an active infection in pig;

Objective 2. investigate how *S*. Typhimurium virulence affects porcine intestinal microbiota composition, comparing the effects induced by two different *Salmonella* strains, a wild type and an attenuated strain.

Furthermore, as the amount of knowledge concerning the host physiological changes that occur during *S*. Typhimurium infection is scarce, additional aim of this thesis was to:

Objective 3. describe the pathophysiological alterations observed at intestinal mucosa level upon infection with wild type and attenuated *Salmonella* strains.

Chapter 6

Salmonella Typhimurium exploits inflammation to its own advantage in piglets

Adapted from originally published article:

Chirullo B, Pesciaroli M, Drumo R, Ruggeri J, Razzuoli E, Pistoia C, Petrucci P, Martinelli N, Cucco L, Moscati L, Amadori M, Magistrali CF, Alborali GL and Pasquali P (2015) *Salmonella Typhimurium* exploits inflammation to its own advantage in piglets. *Front. Microbiol.* 6:985. http://dx.doi.org/10.3389/fmicb.2015.00985

Abstract

Salmonella Typhimurium (*S. Typhimurium*) is responsible for foodborne zoonotic infections that, in humans, induce self-limiting gastroenteritis. The aim of this study was to evaluate whether the wild-type strain *S. Typhimurium* (STM14028) is able to exploit inflammation fostering an active infection. Due to the similarity between human and porcine diseases induced by *S. Typhimurium*, we used piglets as a model for salmonellosis and gastrointestinal research. This study showed that STM14028 is able to efficiently colonize in vitro porcine mono-macrophages and intestinal columnar epithelial (IPEC-J2) cells, and that the colonization significantly increases with LPS pre-treatment. This increase was then reversed by inhibiting the LPS stimulation through LPS antagonist, confirming an active role of LPS stimulation in STM14028-intracellular colonization. Moreover, LPS in vivo treatment increased cytokines blood level and body temperature at 4h post infection, which is consistent with an acute inflammatory stimulus, capable to influence the colonization of STM14028 in different organs and tissues. The present study proves for the first time that in acute enteric salmonellosis, *S. Typhimurium* exploits inflammation for its benefit in piglets.

Introduction

Salmonella enterica serovar *Typhimurium* (*S. Typhimurium*) is a pathogenic Gram-negative bacterium of great clinical significance, responsible for foodborne zoonotic infections. The human disease is characterized by self-limiting gastroenteritis that occasionally can cause fever, systemic infection, and severe inflammation of the intestinal mucosal epithelium (Haagsma et al., 2008; Pires et al., 2011).

The architecture of the mucosal epithelium contains several barriers that prevent or block infection by pathogenic bacteria. Mechanisms of protection are exerted by all of these barriers in order to maintain the integrity of the epithelial cell monolayer and limit inflammation-associated damage (Patel and McCormick, 2014). *S. Typhimurium*, however, is able to overcome these barriers and therefore to colonize the intestinal epithelium inducing inflammation and a marked host immune response. The inflammatory response in the gut is induced by the interaction of *S. Typhimurium* with host cells including epithelial cells and antigen-presenting cells (APCs), like macrophages and dendritic cells. The inflammation is characterized by the secretion of several cytokines, including interleukin (IL)-23 and IL-18, which in turn stimulates T cells to produce IL-17, and IL-22 in the gut mucosa (Srinivasan et al., 2007; Godinez et al., 2008, 2009; Raffatellu et al., 2008).

S. Typhimurium acquired an evolutionary adaptation to overcome antimicrobial defenses in the lumen of the inflamed intestine and, more importantly, to exploit inflammation in order to outcompete the intestinal microbiota (Lupp et al., 2007; Stecher et al., 2007; Barman et al., 2008; Lawley et al., 2008; Sassone-Corsi and Ra□atellu, 2015). The capability of *S. Typhimurium* to grow in the inflamed mucosal environment relies upon the acquisition of essential nutrients and anaerobically respired tetrathionate to successfully outgrow the resident microbiota (Ra□atellu et al., 2009; Winter et al., 2010; Liu et al., 2012; Behnsen et al., 2015).

Most of the current studies about *S. Typhimurium* infection have been conducted in mice, which naturally do not develop gastroenteritis, but rather a systemic infection. An experimental mouse model using antibiotic treatment in order to eliminate microflora and to induce colitis, has been recently established (Ahmer and Gunn, 2011). However, this model is based on the lack of an intact microbiota, which limits a comprehensive evaluation of the complex interactions of *S.*

Typhimurium within the gastrointestinal environment (Elfenbein et al., 2013). Here, we utilized pigs as model for gastrointestinal research with the aim of evaluating whether *S. Typhimurium* is able to exploit inflammation favoring an active infection. Our findings provide evidence that the LPS administration induces inflammation that favors a significant increase in colonization of tonsils, cecum, and spleen by *S. Typhimurium*.

Materials and Methods

Salmonella spp. Cultures

A wild-type strain of *Salmonella Typhimurium* ATCC 14028 (STM14028) was used throughout the study. The strain was grown overnight at 37°C in Brain Heart Infusion broth (Oxoid Ltd, UK), harvested by centrifugation at 1500 × g for 10 min and then washed twice in ice-cold (+4°C) phosphate buffer solution (PBS) (Sigma-Aldrich, Italy).

In vitro STM14028 Colonization

Porcine peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll centrifugation and resuspended in complete RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, USA), 2mM L-glutamine, Gentamicin (100µg/ml). Mono-macrophage cells were isolated from porcine PBMCs, by a 4h plastic adherence procedure at 37°C in 5% CO2 atmosphere, followed by extensive washing with PBS (2 times per day for the first 5 days) to eliminate the lymphocyte contamination. After 7–10 days mono-macrophage cells were obtained, the purity of which was ≥90% as determined by FACS (anti-CD14 Mil-2mAb AbCAM cat. 23919-1, and anti-pig macrophages mAb, AbD Serotec, cat MCA2317F). Cells were then collected, resuspended in

complete medium, and transferred into 200µL per wells of 96-well round-bottom microtiter plates. The IPEC-J2 cell line, porcine intestinal columnar epithelial cells established from normal jejunum of a neonatal unsuckled pig (ACC 701), were grown in Minimum Essential Medium (MEM) (Sigma-Aldrich, St. Louis, MO) enriched with Fetal Calf Serum (FCS, Gibco-BRL, USA) (10% v/v), 2mM glutamine, and antibiotics (50µg/mL penicillin, 50µg/mL streptomycin, and 10µg/mL neomycin), at 37°C in 5% CO2 atmosphere. Mono-macrophages and IPECJ2 cell line were employed for in vitro studies. Both types of cells were seeded in 96-well plates at a density of 1×10^5 cells per well and treated overnight with purified lipopolysaccharides (LPS) (1µM/mL; from Escherichia coli 0111:B4, L4391; Sigma-Aldrich) alone or in combination with a natural antagonist of LPS, the RS-LPS (100µM/mL; tlrl-prslps, Invivogen, San Diego, USA).The following day, cell cultures were rinsed and STM14028 was diluted in RPMI-10% FBS, added to the cells at a multiplicity of infection (MOI) of 100:1 and incubated for 1h at 37°C in 5% CO2. After 1h, the cell cultures were rinsed and incubated in a culture medium containing gentamicin sulfate(100µg/ml) to kill extracellular bacteria but not the internal ones, and subsequently incubated for 3 and 24h. Viable intracellular bacteria were recovered by lysing the cells, at both 3 and 24h post treatment time point, in distilled water with 0.1% of Triton X-100 for 10min. The quantification of bacteria was performed by plating serial dilutions on agar triptose plates.

In vivo Studies

Animals

Fourteen commercial hybrid pigs aged ~30 days were utilized in the experiment. All the pigs used throughout the study were the offspring of Salmonella-free sows (negative for *Salmonella* by both serological and bacteriological tests). Before the

onset of the experiment, the piglets were proved to be *Salmonella* free by culture of feces of each animal. Animals were weighed and randomly allocated to two groups of 6 (A and B) and one group (C) of two pigs. Each group was maintained in separate isolation units under natural day–night rhythm with access to feed and water *ad libitum*. Groups A and B were intragastrically administered with 20 ml of sodium bicarbonate bu□er containing 10^9 CFU of *S. Typhimurium* ATCC 14028. At the same time, Group A was intraperitoneally challenged with 12.5µg/kg BW of lipopolysaccharides from *S. enterica* serovar *Typhimurium* (L2262, Sigma Aldrich SRL, Milan, Italy). Group C received only sterile sodium bicarbonate buffer and served as naïve control group. Collection of individual fecal samples (0, 1 day after challenge), blood sampling and registration of rectal temperature (0, 4h, 1 and 2 days after challenge) were performed as well. Pigs were visually monitored by an independent veterinary officer in charge of the study for 6h after the inoculum and then twice a day. Two days after the challenge, pigs were weighed again, and then euthanized using a captive bolt pistol and exsanguination. Samples of tonsils, liver, spleen, mesenteric lymph nodes, ileum, cecum, and colon were collected from each pig for the evaluation of bacterial burden. All the experiments were authorized by national authority and were conducted according to the Italian national regulations enforced at the time of this study (Italian legislative Decree 116/92).

Microbiology

The microbiological analysis of fecal and organ samples were conducted according to the ISO 6579:2002/Amendment 1:2007 protocol. Briefly, samples were weighed and homogenized as 10% suspension in Buffered Peptone Water (BPW) (Oxoid Ltd., UK). This initial solution was then used to perform a decimal dilution series carried out by systematically transferring an aliquot of 1 ml of each successive dilution in 9

ml of BPW. All BPW-diluted samples were incubated at 37°C for 18 ± 3h. Cultures (0.1 ml) were subsequently seeded on Modified Semisolid Rappaport-Vassiliadis (MSRV) agar plates (Oxoid Ltd., UK) and incubated at 41.5°C for 24h for the selective enrichment of Salmonella. A loopful of growth from each MSRV plate was streaked onto Xylose-Lysine-Desoxycholate Agar (Oxoid Ltd., UK) and Brilliant Green Agar (Oxoid Ltd., UK) plates and hence incubated at 37°C overnight. Typical colonies were confirmed serologically as Salmonella by polyvalent antiserum (Salmonella Test Serum; Siemens Healthcare Diagnostics, Italy) and API rapid 20 E (Api Rapid 20E; Biomerieux, Italy). This is a semi-quantitative approach that allow the determination of the concentration of Salmonella in a sample within a tenfold band.

Flow Cytometry of Lymph Nodes Cells

Cell treatment was performed according to an established procedure (Razzuoli et al., 2012), with minor modifications. Briefly, frozen lymph nodes cells were thawed at 38°C and washed with FACS-Buffer (0.1% sodium azide + 2% fetal calf serum in PBS). Then, they were divided into aliquots (106 cells each) and reacted with monoclonal antibody (mAb) CD21 (Southern Biotech, cat. 4530-02), Mil-2 (AbCAM, cat. 239191), PMN (AbD Serotec, cat. MCA2599F), or FACS buffer only (control) for 30min at 4°C, respectively. Cells were washed, and again incubated for 30min at 4°C in FACS buffer containing goat anti-mouse IgG-FITC (Invitrogen, Molecular Probes®, cat: A10683). After washing in FACS buffer, cells were resuspended in 100µL of the same buffer and 1:4 diluted. Samples were analyzed in a GUAVA MILLIPORE flow cytometer (Millipore Software). The typical forward and side scatter gate was set to exclude dead cells from the analysis. The percentage of positive cells beyond the threshold fluorescence channel was assessed in each

sample on 10,000 events and compared between mAb-treated and control cells. For each antibody, results were expressed in terms of net percentage of positive cells.

Analysis of Cytokines

In order to evaluate the serum concentration of IL-1 beta and TNF-alpha ELISA kits were used (cat. N. PLB00B and PTA00 respectively, R&D Systems, Inc. Minneapolis, MN 55413, USA,). These assays employ the quantitative sandwich enzyme immunoassay technique using monoclonal antibodies, specific for porcine IL-1 beta or porcine TNF- alpha, pre-coated onto a microplate. The intensity of the color measured is at 450nm.

Statistical Analysis

For the *in vitro* and *in vivo* assays, the statistical significance of differences between study groups was analyzed using ANOVA and Student's t-test; $p < 0.05$ was chosen as threshold for significance. These symbols were used to indicate the statistical significance: $*P < 0.1$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

Results

Salmonella Infection Induces an Innate Immune Response

In order to verify the involvement of the innate immune response during Salmonella infection in piglets, phenotypic analysis in ileo-cecal lymph nodes was performed in piglets orally infected with STM14028 and euthanized 48h later. As depicted in **Figure 1A**, it was possible to observe a higher increase in the percentage of $CD14^+$ (mainly monocytes) and polymorphonuclear cells with a minor involvement of $CD21^+$ B cells. Moreover, the STM14028-infection was confirmed by the bacterial count in di□erent organs (**Figure 1B**). These findings are consistent with a rapid

recruitment of neutrophils and monocytes/macrophages toward lymph nodes, crucial for the effective response to lipopolysaccharides stimulus or concomitant bacterial infection. These data suggest a prompt involvement of the innate immune response in the regional lymph nodes draining the gut, after an oral infection with STM14028.

LPS Treatment Increases STM14028 Colonization in Isolated Mono-macrophages and IPEC-J2 Cells

In order to assess if STM14028 exploits inflammation in vitro, we used LPS, which is known to stimulate the production of inflammatory molecules either in monocytes/macrophages (Fang et al., 2004) or in IPEC-J2 cells (Razzuoli et al., 2013). Monocytes/macrophages and IPEC-J2 cells were thus primed overnight with purified LPS alone and/or in combination with a natural antagonist of LPS (RS-LPS) capable to inhibit the LPS-stimulation interacting with the TLR-4/MD-2 complex recognized by LPS. Afterwards, the cells were infected with STM14028.

We observed that STM14028 was able to efficiently colonize monocytes/macrophages and IPEC-J2 cells at both 3 and 24h post STM14028-infection (**Figures 2A–D**). A previous treatment with purified LPS significantly increased STM14028 colonization in both cell types at 3 and 24h after infection. When RS-LPS antagonist was used, this markedly inhibited LPS stimulation causing a colonization level similar to the one obtained by STM14028 infection alone (**Figures 2A–D**). Overall, these results suggest that LPS stimulation can create conditions in which STM14028 is more efficiently phagocytized by cultivated cells, within it can find suitable conditions to multiply.

LPS Induces Inflammation in Piglets, Which in Turn Favors STM14028 Colonization

To evaluate the influence of inflammation during Salmonella infection in vivo, we established a protocol of inflammation, injecting parenterally LPS in group A and STM14028 by oral route in groups A and B. Piglets in group C were kept untreated and served as control animals. Then, an assessment was made of whether inflammation induced by LPS had favored the progression of *Salmonella* infection.

LPS was able to induce a rise in body temperature in piglets of group A already at 4h post STM14028-infection (**Figure 3**) compared to the control (C) and the STM14028-infected group (B), reaching body temperature similar to those of group A only at 24 and 48h post infection. No significant differences in body weight were measured among the three groups throughout the 48h of analysis (data not shown).

Moreover, a remarkable increase of circulating pro-inflammatory cytokines has been found (**Figure 4**). TNF-alpha was in fact detected in the blood of all animals but it was produced in a higher amount in group A compared to the groups B and C, at 4h post STM14028-infection, without significant differences within groups at 24 and 48h post infection. IL-1 beta blood level was higher in group A than in groups B and C at 4h post STM14028-infection. At 24h post infection we detected a reduction of the IL-1 beta blood concentration in group A with a concomitant slightly increased level in group B. Finally, the IL-1 beta level completely reversed its trend in groups A and B at 48h post infection, with higher concentration of IL1-b in group B compared to group A (**Figure 4**).

These results confirm the induction of a pro-inflammatory status mediated by LPS immediately after its administration. Noteworthy, in group B the level of pro-

inflammatory cytokines required 48h after infection to reach a concentration similar to that measured in group A.

Piglets of the three groups were euthanized 48h after the treatments, and STM14028 infection was assessed in different organs and tissues in order to evaluate the capability of colonizing either locally in the gut milieu, or systemically. As depicted in **Figure 5**, piglets treated with LPS and infected with STM14028 showed a significant increase in colonization of tonsils, cecum, and spleen, whereas in mesenteric lymph nodes, colon, ileum and liver no significant difference in STM14028 colonization was observed (**Figure 5**).

On the whole, these findings provide substantial evidence that LPS is able to induce an inflammatory response, which favors STM14028 survival and colonization in the intestinal and systemic compartments.

Discussion

Intestinal inflammation, induced by both chemical treatments and infectious agents, is known to be associated with a profound dysbiosis of the colonic microbial community structure (Lupp et al., 2007). Many pathogens use inflammation and the accompanying dysbiosis for their advantage in order to overcome colonization resistance (Stecher et al., 2007). In this context, several studies based on the use of the streptomycin treated mouse colitis model, which is characterized by absence of resident microbiota (Ahmer and Gunn, 2011), highlighted the emerging concept that inflammation of the mucosal epithelium plays a role in environmental fitness of *S. Typhimurium*. It has been shown, indeed, that unlike avirulent strains, wildtype *S. Typhimurium* is capable of out-competing commensal microbiota in re-colonization experiments after treatment with antibiotics. Furthermore, *S. Typhimurium* exploits inflammation to promote its own colonization, out-competing the resident microbiota

(Stecher et al., 2007). This model however, despite the important contribution to the study of the pathogens microbiota interaction in an inflammatory environment, presents several limitations. In particular, the inability of the *Salmonella* mouse model to reproduce gastroenteritis and, even more importantly, the elimination of the competing flora represent crucial differences with respect to natural *Salmonella* infection in humans. In this work, an experimental model was used based on piglets infected with a wild type strain of *S. Typhimurium*, STM14028, representing an ideal animal model potentially capable to overcome the intrinsic limitations of the current streptomycin-treated mouse colitis model.

It was initially observed that, after oral infection with STM14028, the immune response is rapidly activated, involving the innate compartment with a marked increase of polymorphonuclear and mono-macrophage populations in ileo-cecal lymph nodes (**Figure 1**). This confirms the involvement of the principal populations engaged in the response to Salmonella infection, already known to be relevant for the response to the LPS stimulation. Moreover, these results extend those of recent studies about the increased expression of pro-inflammatory cytokines (Knetter et al., 2015) and raised lymphocytic infiltration of the gut mucosa after *S. Typhimurium* oral infection of piglets (Gradassi et al., 2013).

It was investigated whether the induction of inflammation by LPS pre-treatment of mono-macrophages and porcine intestinal epithelial IPEC-J2 cells makes these cells more susceptible to STM14028 infection. The results indicate that these cells, primed with LPS, were more prone to the colonization by STM14028 when compared to the LPS untreated control cells. Otherwise, the use of the RS-LPS antagonist, binding the TLR-4/MD-2 complex, inhibits the LPS stimulation. This significantly reduced the STM14028 intracellular colonization down to the values of LPS-untreated cells (**Figure 2**).

The higher colonization of STM14028 in LPS-treated cells could be considered unexpected due to the effect of LPS stimulus. It can however be hypothesized that our results are the consequence of high capability of bacterial uptake. It has been well-established, in fact, that for many facultative intracellular pathogens, as well for *Salmonella*, the key to successful infection lies in the interaction between bacteria and host macrophages. *Salmonella* is able to mount specific strategies to escape killing and survive within phagocytes (Fields et al., 1986; Groisman and Saier, 1990; Gulig et al., 1998; Ruby et al., 2012). Moreover, recent reports have revealed fascinating insights to explain how Salmonella exploits host response. In particular, the internalization of *Salmonella* in macrophages via TLR, able to bind Salmonella LPS, is a crucial factor to favor *Salmonella* virulence in that it facilitates the acidification of the phagosome, which in turn provides a protective niche for *Salmonella* (Arpaia et al., 2011). In addition, in mouse models, it has been observed that LPS present on live *Salmonella* provides an essential signal, via functional TLR-4, for macrophages to produce NO and TNFα (Royle et al., 2003). This may be exploited by Salmonella to modify macrophage functions and promote growth and/or dissemination throughout the host.

Finally, the main effort was to assess whether the inflammation induced LPS-treated piglets was able to influence the colonization of STM14028. Piglets treated with LPS and infected with STM14028 showed a significant increase in body temperature (**Figure 3**) and the production of IL-1beta and TNF-alpha in the blood (**Figure 4**) at 4h post infection. These results indicate that the onset of the LPS-mediated acute inflammation leads to cytokines production and body temperature rise, already at 4h post infection, promoting significant increase of tonsils, cecum, and spleen STM14028-colonization, compared to the control group (**Figure 5**).

These results are in line with studies showing that the parenteral administration of dead Gram-negative bacteria or lipopolysaccharide exacerbated the growth of virulent *S. enterica* in mice (Hormaeche, 1990). It is also possible to envisage that the increased advantage of *Salmonella* in inflamed systemic environment can also be justified by mechanisms other than a compromised barrier integrity or dysbiosis. Moreover, Foster et al. demonstrated that the intravenous administration of an attenuated *Salmonella* strain can exacerbate the growth of virulent strains, which involves IL-10 production and requires TLR-4, and its signaling pathways involving the adaptor molecules, the TIR-domain containing adapter-inducing interferon-β (TRIF), and the Myeloid differentiation primary response gene 88 (MyD88) (Foster et al., 2008). A dysregulated type I IFN response in tissues can affect fundamental regulatory circuits of innate immunity in macrophages, which turns IL-10 into a potent pro-inflammatory cytokine (Sharif et al., 2004). In addition, IL-10 and IFN-γ associated responses may cause a gain of pro-inflammatory activity, as shown in human models of endotoxemia (Lauw et al., 2000).

On the whole, these results, using piglets as model, demonstrate for the first time that in acute enteric salmonellosis, *S.* Typhimurium exploits the inflamed milieu to its own advantage.

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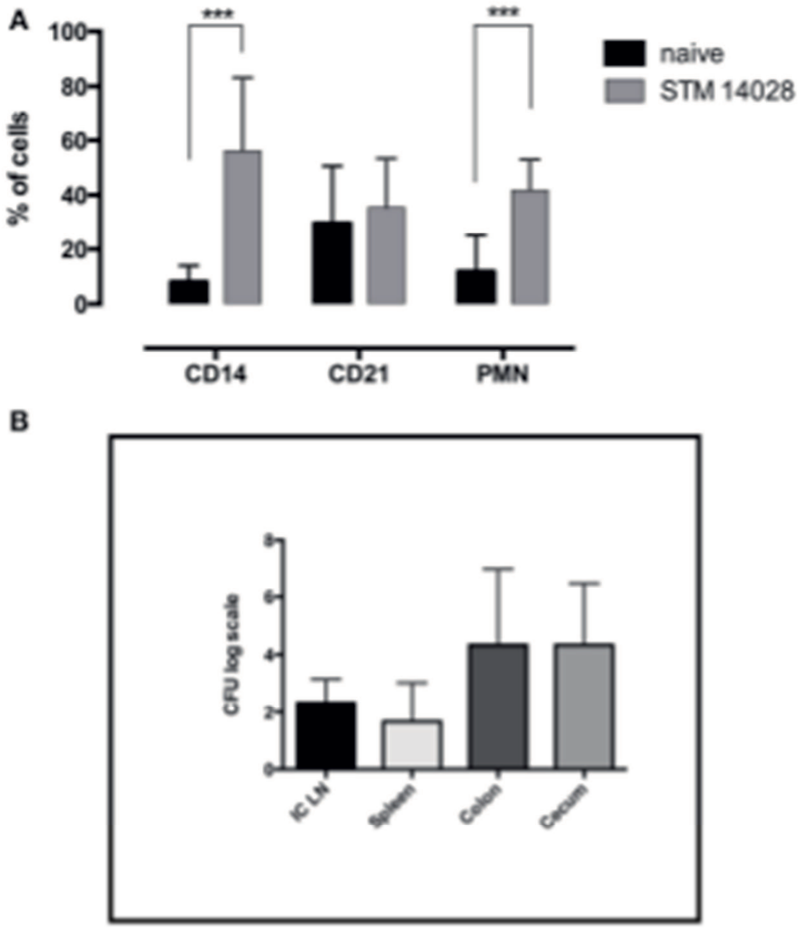


FIGURE 1 | STM14028 infection induces an increase of innate immunity cell compartment and colonizes different organs of piglets orally infected. (A) The prevalence of CD14+, CD21+, and polymorphonuclear (PMN) cells was determined in ileo-cecal lymph nodes, 48h post-infection with STM14028. The differences were statistically significant (***P ≤ 0.001, multiple comparisons t-test). (B) STM14028 count in ileo-cecal lymph nodes (ICLN), spleen, colon, and cecum of infected piglets. Data represent mean with error bars as SEM of six piglets per group.

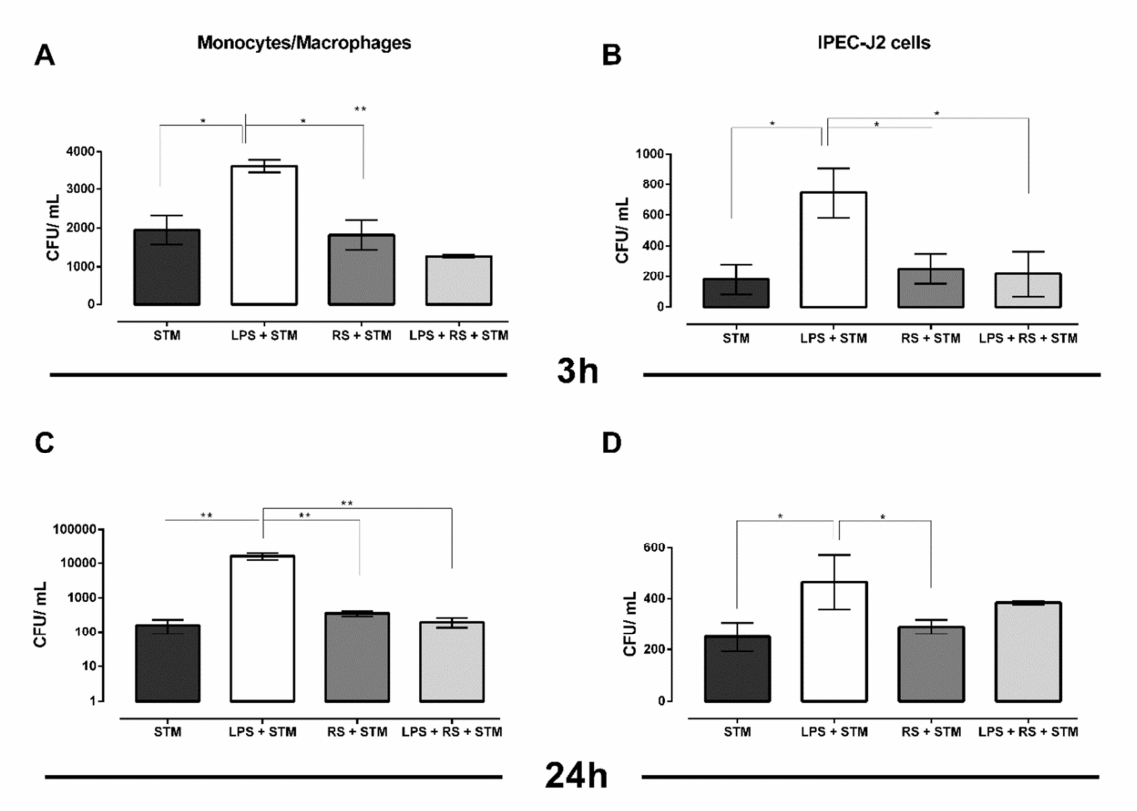
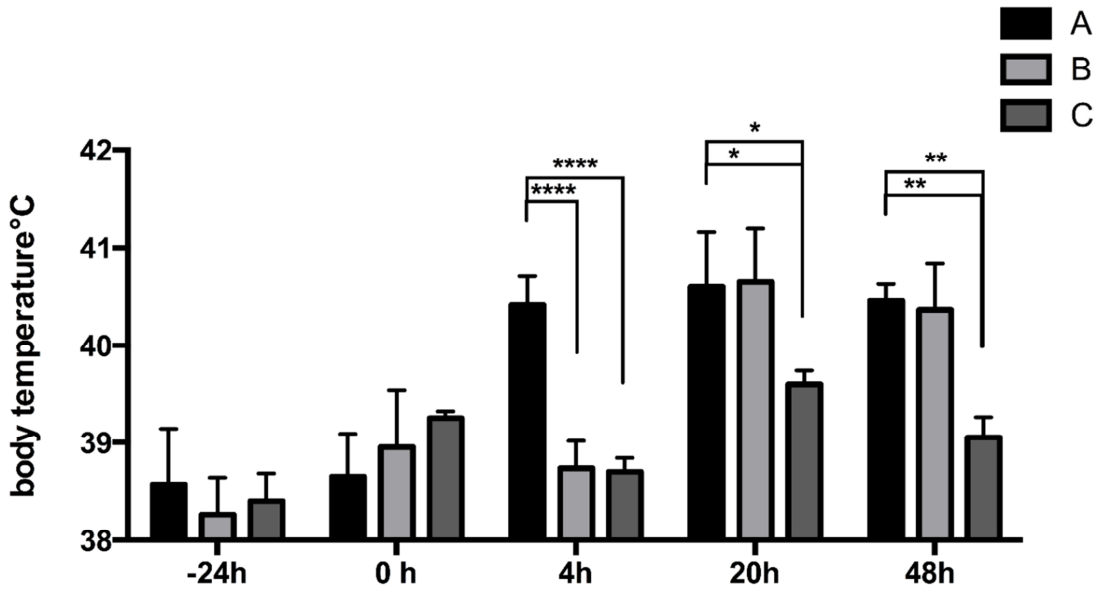


FIGURE 2 | STM14028 colonization of mono-macrophages and IPEC-J2 cells at 3 and 24h post infection (A–D). STM14028 colonization increases with LPS pre-treatment and is reduced by RS-LPS antagonist to the values of LPS-untreated cells (*P ≤ 0.1; **P ≤ 0.01, One-Way Anova Turkey's multiple comparisons test, data from one representative experiment out of three with similar results).



F**IGURE 3 | LPS-treatment of piglets induces a rise in body temperature 4h after infection with STM14028.** The body temperature was measured at different time points on three different groups of piglets: treated with LPS and infected with STM14028 (group A); only STM14028 infected (group B); naïve control group (group C). At 4h post infection, group A showed a significant rise in body temperature compared to the B and C groups. Data refer to one out of two separate experiments performed with comparable results. The differences between groups were statistically significant (****P ≤ 0.0001; *P ≤ 0.1; **P ≤ 0.01 multiple comparisons-Fisher's Least Significant Difference test).

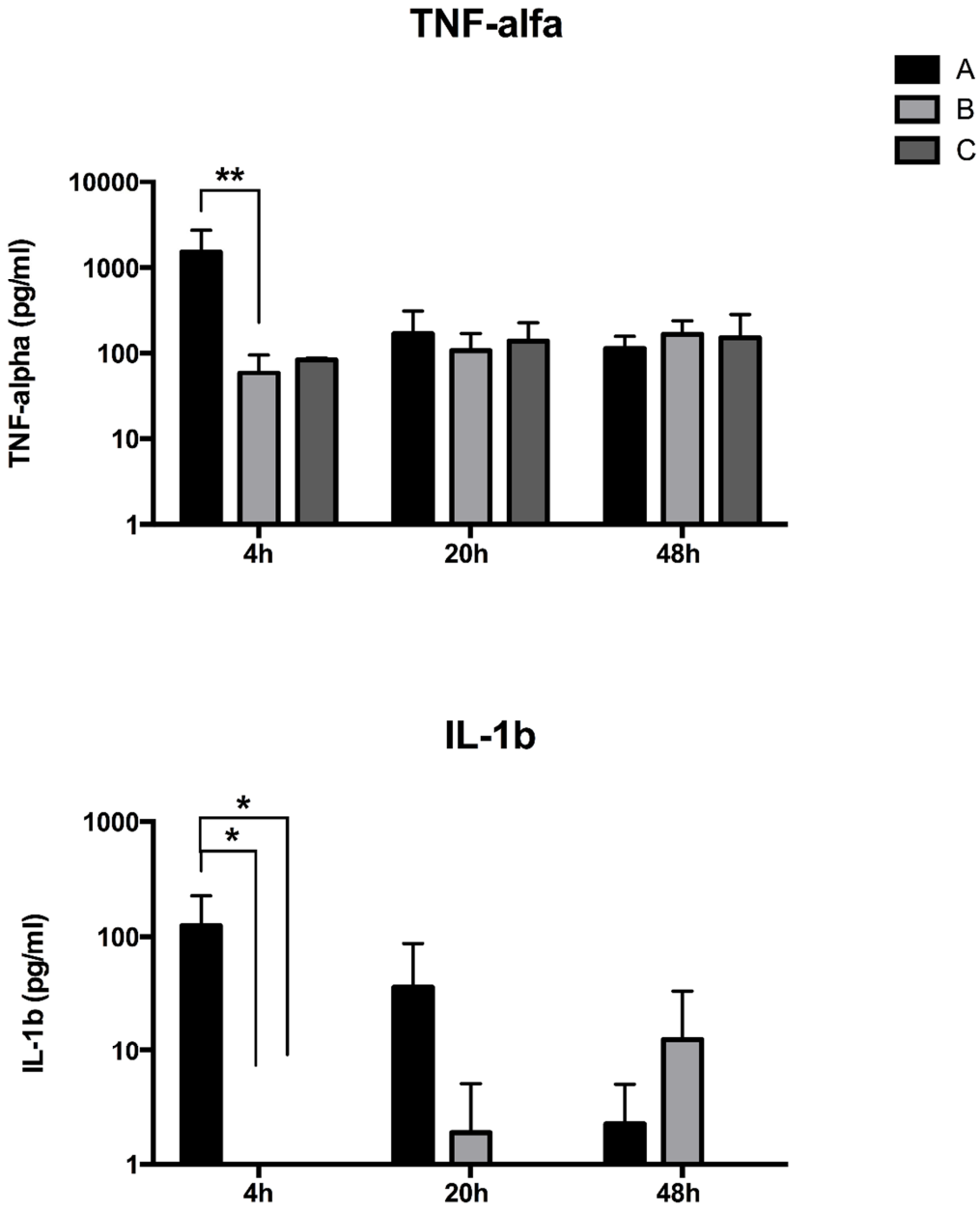


FIGURE 4 | LPS-treated piglets show an increased inflammation 4h post STM14028 infection. IL-1beta and TNF-alpha production was measured at different time points on blood samples from three different groups of piglets: treated with LPS and infected with STM14028 (group A); only STM14028 infected (group B); naïve control group (group C). At 4h post infection, group A showed a significant increase in production of both cytokines compared to the B and C groups. The differences between the groups were statistically significant (*$P \leq 0.1$; **$P \leq 0.01$, multiple comparisons-Fisher's Least Significant Difference test).

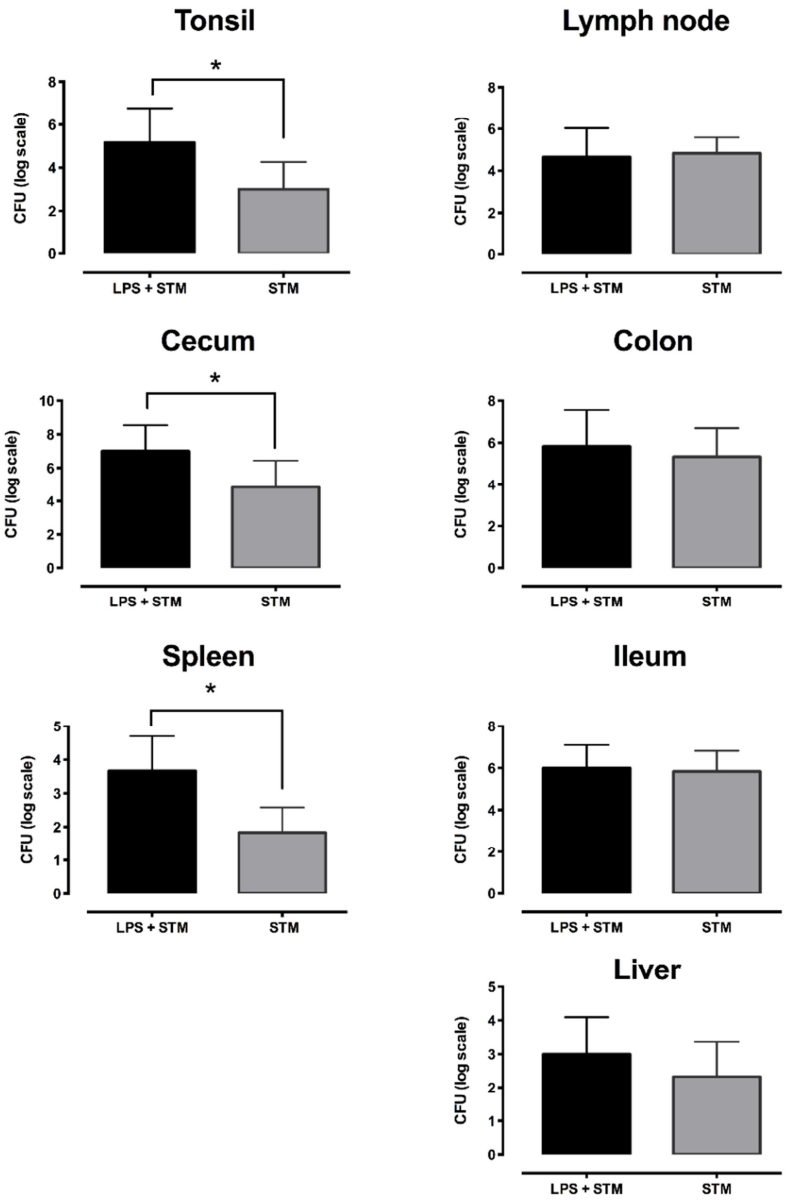


FIGURE 5 | LPS treatment raises STM14028 colonization of tonsils, cecum, and spleen of piglets. Recovery of STM14028 from different organs at 48h post infection of piglets treated with LPS and infected with STM14028 (LPS+STM group) or only infected with STM14028 (STM group). LPS-treatment increases the colonization of tonsils, cecum, and spleen but does not influence the colonization of mesenteric lymph nodes, colon, ileum, and liver of piglets after STM14028 infection. Data refer to one out of two separate experiments performed with comparable results. The differences between the groups were statistically significant (*$P \leq 0.01$, Mann–Whitney unpaired t-test).

Chapter 7

Salmonella enterica serovar *Typhimurium* exploits inflammation to modify swine intestinal microbiota

Adapted from originally published article:

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Abstract

Salmonella enterica serovar Typhimurium is an important zoonotic gastrointestinal pathogen responsible for foodborne disease worldwide. It is a successful enteric pathogen because it has developed virulence strategies allowing it to survive in a highly inflamed intestinal environment exploiting inflammation to overcome colonization resistance provided by intestinal microbiota. In this study, we used piglets featuring an intact microbiota, which naturally develop gastroenteritis, as model for salmonellosis. We compared the effects on the intestinal microbiota induced by a wild type and an attenuated *S*. Typhimurium in order to evaluate whether the modifications are correlated with the virulence of the strain. This study showed that *Salmonella* alters microbiota in a virulence-dependent manner. We found that the wild type *S*. Typhimurium induced inflammation and a reduction of specific protecting microbiota species (SCFA-producing bacteria) normally involved in providing a barrier against pathogens. Both these effects could contribute to impair colonization resistance, increasing the host susceptibility to wild type *S*. Typhimurium colonization. In contrast, the attenuated *S*. Typhimurium, which is characterized by a reduced ability to colonize the intestine, and by a very mild inflammatory response, was unable to successfully sustain competition with the microbiota.

Introduction

Nontyphoidal salmonellae (NTS) as *Salmonella enterica* serovar Typhimurium are a leading cause of acute food-borne zoonoses worldwide being responsible for hundreds of millions of cases of gastroenteritis and bacteremia annually (Hohmann,2001). Pigs are important reservoir of infection for humans as they are

asymptomatic carriers of broad host-range serovars of *Salmonella* (Funk and Gebreyes, 2004; Pires et al., 2011). The intestine is considered to be the biological niche of Salmonella with the intestinal mucosa having a central role in regulating the immune response to bacteria (Hallstrom and McCormick, 2011). However, *Salmonella* has developed strategies to overcome and cope with most of the immune defenses developed by the host (Behnsen et al., 2015). Examples of the strategies used by *Salmonella* to evade mucosal innate immunity include the ability to resist to the reactive oxygen species generated during inflammation (Bogomolnaya et al., 2013), in order to produce energy by an anaerobic respiration chain which uses an electron acceptor specifically generated in the gut under oxidative stress (Winter et al., 2010) and to resist to the sequestration of essential nutrients such as iron and zinc (Raffatellu et al., 2009; Liu et al., 2012). As a matter of fact, the ability to resist to the antimicrobial host responses characterizing gut inflammation promotes the growth of Salmonella in the intestinal lumen over the competing microbiota. During the past few years, there has been an expanding interest concerning the role played by intestinal microbiota in the susceptibility to enteric pathogens. Microbiota contributes to the digestion of dietary substances and to the synthesis of essential food supplements such as vitamins, and to the development or maintenance of the mucosal immune system (Littman and Pamer, 2011). Moreover, it acts as a barrier against invading bacteria both physically, blocking pathogen access to the epithelial layer, and also by outcompeting for nutrients reducing the survival and invasiveness of enteric pathogens (Hallstrom and McCormick, 2011; Sassone-Corsi and Ra□atellu, 2015). However, it has been known that *S*. Typhimurium requires intestinal inflammation to circumvent “colonization resistance” provided by the intestinal microbiota (Santos et al., 2009). It has been shown that *Salmonella* can alter the normal composition of the gut microbiota, and this influence is associated

with *Salmonella* virulence factors that induce inflammatory mucosal host responses (Barman et al., 2008). Furthermore, animals with disrupted microbiota have an increased susceptibility to infection (Barman et al., 2008; Juricova et al., 2013). Most of the studies examining salmonellosis have been carried out in murine models that naturally do not develop gastroenteritis. To resemble the disease in humans, mice can be subjected to antibiotic treatment in order to eliminate microbiota and to develop colitis (Ahmer and Gunn, 2011). Therefore, due to the lack of an intact microbiota, murine models are not suitable for the comprehension of the mechanisms used by *Salmonella* to thrive in the gastrointestinal environment (Elfenbeinetal.,2013). To circumvent this limitation, we chose the pig as a model for our study. The advantage of the pig lies in the great similarity between humans and pigs in the gastrointestinal tract and in the disease caused by *Salmonella* as well as being a natural host of *Salmonella* (Zhang et al., 2013). We hypothesized that the *Salmonella* virulence degree is a determining factor in influencing the capability of the pathogen to overcome protective microbiota. To explore this, we compared the e□ects on the intestinal microbiota of *S.* Typhimurium wild type to that of an attenuated Salmonella strain lacking the ZnuABC transporter. Our findings provide evidence that the microbiota modifications induced by Salmonella are correlated with the virulence of the strain. Moreover, *Salmonella* could overcome colonization resistance through the reduction of microbiota members normally involved in the intestinal homeostasis and in the inhibition of pathogen growth.

MATERIALS AND METHODS

Salmonella spp. cultures

The wild-type strain S. Typhimurium ATCC 14028 (hereafter STM^{wt}) and its isogenic attenuated znuABC mutant (hereafter $STM^{\Delta znuABC}$; Ammendola et al.,

2007), were used throughout the study. Strains were grown overnight at 37◦C in Brain Heart Infusion broth (Oxoid Ltd., Basingstoke, UK), harvested by centrifugation and washed twice in ice-cold phosphate buffer solution (PBS) (Sigma-Aldrich, Milan, Italy).

Animals and samples collection

Thirty-one post weaned piglets old 28 days, from Salmonella-free sows (routinely monitored with microbiological and serological tests), were used in the experiment. Group A (9 piglets) received sterile sodium bicarbonate buffer and it was used as naïve control group. Groups B and C (11 piglets each) were orally infected with 20ml of sterile 10% sodium bicarbonate buffer containing 2 × 109 CFU of $STM^{\Delta znuABC}$ (Group B) or 2 × 109 CFU of STM^{wt} (Group C). At 0, 1, 2, 7, and 12 days post infection (dpi), rectal temperature was recorded and serum and fecal samples were collected to evaluate TNF-α, IL1-α, haptoglobin, and CRP production and to detect fecal excretion of Salmonella, respectively. Four piglets of group A and 5 for groups B and C were sacrificed at 1 dpi, while 5 piglets of group A and 6 for groups B and C at 12dpi. Portions of spleen, ileum, cecum, colon, ileocecal lymph nodes, and tonsil of the soft palate were taken for microbiological analysis, histology, and for mRNA isolation. Feces and cecal and colonic contents were collected to analyze the microbiota composition. All the experiments were authorized by national authority and conducted according to European Directive (2010/63/EU; approval number 54/2012).

Microbiology

Fecal shedding and organs colonization of STM^{wt} and $STM^{\Delta znuABC}$ were determined according to the ISO 6579: 2002/Amendment 1:2007 protocol. Briefly, samples were

weighed and homogenized in nine parts of Buffered Peptone Water (BPW) (Oxoid Ltd., UK). This initial solution was then used to perform a decimal dilution series carried out by systematically transferring an aliquot of 0.5ml of each successive dilution in 4.5ml of BPW. All BPW-diluted samples were incubated at 37°C for 18 ± 3h. 0.1ml of cultures were subsequently seeded on Modified Semisolid Rappaport Vassiliadis (MSRV) agar plates (Oxoid Ltd., UK) and incubated at 41.5°C for 24h for the selective enrichment of Salmonella. A loopful of growth from each MSRV plate was streaked onto Xylose-Lysine-Desoxycholate Agar (Oxoid Ltd., UK) and Brilliant Green Agar (Oxoid Ltd., UK) plates and hence incubated at 37°C overnight. Suspect Salmonella colonies were subjected to biochemical identification by the BBL Enterotube II (BD Franklin Lakes, USA) and serological identification using Salmonella group-specific antisera (Remel, Lenexa, USA). This is a semi-quantitative approach that allows the quantification of Salmonella in a sample within a tenfold band (detection limit 1 CFU/g feces).

Histology

Tissue samples of cecum were fixed in formalin, embedded in paraffin wax and stained with hematoxylin and eosin according to standard procedures.

Immune mediators production

TNF-α, IL1-α, haptoglobin and C-reactive Protein (CRP) production was measured in serum samples from animals bled at 1 and 12 dpi using a sandwich ELISA (Porcine Quantikine ELISA Kit, R&D System, Mn, USA), according to the producer's instructions.

Gene expression

Total RNA was extracted from sections of the cecum, colon, and ileocecal lymph nodes using the PureLink RNA Mini Kit (Ambion, Life Technologies). Reverse transcription of 1µg of RNA was performed for each individual sample using Tetro cDNA Synthesis Kit (Bioline) and 5µl of cDNA were used for real-time reaction using SensiMix II Probe Kit (Bioline). Primers for cytokines (RPL-32, IL-1α, IL-1β, TNF-α, and IFN-γ) were designed using PrimerQuest Design Tool (Integrated DNA Technologies, IDT; see Supplementary Table 1). Fold changes in gene expression were calculated using the ΔΔCt method in comparison to the results for the reference housekeeping gene RPL32.

Fecal 16S rDNA Metagenomics Next-Generation Sequencing

Bacterial genomic DNA (gDNA) was extracted from feces, cecal, and colonic contents using QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Fifty nanograms of gDNA were used to amplify by PCR the hypervariable V3-V4 regions of the 16S rDNA using bacteria/archeal primers 515F/806R with Illumina overhang adapters (Caporasoetal.,2012). One nanogram of PCR amplicon was used for each sample to prepare the sequencing library according to the Illumina Nextera XT DNA Sample Preparation Kit. During this procedure, using a limited cycle PCR, Illumina sequencing adapters, and dual-index barcodes were added to the amplicon. All the libraries were subsequently normalized and pooled by 24 prior to sequencing according to manufacturer's instructions (Illumina Nextera XT DNA Library Preparation Guide). Each pool of 24 samples was sequenced on Illumina MiSeq using a 2 × 250 paired-end (PE) setting on a standard MiSeq flow cell. Sequencing reads were trimmed and all the reads with a quality score below the Q20 parameters were discarded from the analysis. Then, all the PE reads were joined using the

join_paired_ends scripts of QIIME utilities (Caporaso et al., 2010) to create longer fragments. The Lederhosen pipeline (based on UCLUST software and green genes v 13.5 16S database) was used to create the OTU table for each sample. The OTU tables were provided as input for the MatR package to remove.

Quantitative real-time PCR of 16S rRNA gene sequences

q-PCR was performed using bacterial groups-specific 16S rRNA primers (see Supplementary Table 2) to determine the amount of bacteria in the study groups. However, this method is an approximation of microbial abundance as a great amount of bacteria features many copies of the 16S gene. Therefore, both variation in the abundance of organisms and genomic copy number variation can influence the quantitative prediction of 16S gene abundances. Real time PCRs were carried out on SensiMix SYBR Low-ROX Kit (Bioline). The amplification program started with an initial step at 95°C for 10min, followed by 40 cycles of 15s each at 95°C, 15s at 55°C-63°C (depends on the Tm of primers), and 15s at 72°C. The 16S gene copy numbers per µl of DNA, from each sample, were determined by using standard curves generated from fragments of 16S rRNA genes of reference bacteria specific for each group cloned into plasmid (Promega) as templates. The plasmid was purified by using the Wizard Plus SV Minipreps DNA purification kit (Promega) and its concentration was quantified by using a NanoDrop® ND1000 Spectrophotometer. With the molecular weight data of the plasmid and insert sequences, the copy number (g/molecule) was calculated according to the equation defined by Whelan et al. (2003). For each microbial population, the corresponding plasmid was chosen to create a 10-fold standard curve ranged from 10^8 to 10^2 copies. Copy numbers of 16S rRNA genes per µl of sample (feces, caecal, and colonic contents) were transformed into logarithms to achieve normal distribution, and the mean ± standard deviation

was calculated. To estimate the copy number of *Enterobacteriaceae* other than *Salmonella*, for each sample the Salmonella 16S gene copy number was subtracted from the total *Enterobacteriaceae* 16S gene copy number.

Statistical analysis

Statistical analysis was performed using GraphPad 6.0 software for Windows (GraphPad Software Inc.; San Diego; CA). Microbiota analysis by q-PCR were estimated using one-way analysis of variance (One-way ANOVA). Fecal shedding, organs colonization, and cytokines expression were analyzed using non-parametric Mann–Whitney test. Differences in body temperature and differences between groups in the TNFα, IL1-α, haptoglobin, and CRP production were estimated using non-parametric Dunn's test. Moreover, non-parametric Kruskal–Wallis was used to test the presence of significant differences among the sample groups analyzed for each different taxonomical level considered (Phylum, Family, Genus) and Benjamini-Hochberg FDR was applied to correct multiple testing. A $P \leq 0.05$ was considered statistically significant. Non-parametric Dunn's test was also used to estimate differences in alfa diversity.

RESULTS

Pathogenicity of *Salmonella Typhimurium* is positively correlated to bacterial virulence

Piglets infected with $STM^{\Delta znuABC}$ (group B) and STM^{wt} (group C) had a transient increase in body temperature at 1 dpi compared with naïve controls (group A). At 2 dpi, only the group C (STM^{wt}) continued showing a significantly higher body temperature than group A (**Figure 1A**). Moreover, differences in the levels of Salmonella fecal shedding were observed among the study groups. Animals infected

with STM^{wt} and $STM^{\Delta znuABC}$ started to shed bacteria the day after experimental infection and reached the peak of excretion at 2 dpi. However, unlike group C (STM^{wt}) that continued shedding a similar amount of bacteria throughout the whole period of observation, group B ($STM^{\Delta znuABC}$) showed a sharp decline over time (**Figure 1B**). To further assess the inflammatory response induced by STM^{wt} and $STM^{\Delta znuABC}$, piglets were bled at different time points and haptoglobin, CRP, IL1-α, and TNF-α levels were measured in sera. Group C (STM^{wt}) had an early immune response characterized by a significant increase of haptoglobin and IL1-α at 2 dpi, and TNF-α at 2 and 7 dpi, followed by a late production of CRP which reached a significant level at 12 dpi. Conversely, group B ($STM^{\Delta znuABC}$) did not show any different production of haptoglobin, CRP, IL1-α, and TNF-α when compared with the naïve (group A; **Figure 2**). Piglets of different groups were euthanized at 1 and 12 dpi to assess bacterial colonization of organs. As shown in **Figure 3**, colonization occurred as early as 1 dpi, either in gut or in systemic organs. However, piglets infected with STM^{wt} (group C) showed a significant higher degree of colonization than piglets infected with $STM^{\Delta znuABC}$ (group B) in the gut organs ($p < 0.05$) at 1 dpi (**Figure 3**) and in the colon ($p < 0.05$) at 12 dpi (Supplementary Figure 1). Organs samples taken from naïve animals (group A) were negative. These findings confirm that STM^{wt} and $STM^{\Delta znuABC}$ have a differential colonization efficiency. Moreover, $STM^{\Delta znuABC}$ did not show a significant systemic inflammation. We could infer that these results are a direct consequence of the intrinsic incapability of $STM^{\Delta znuABC}$ to induce an inflammatory response but, in alternative, they could be due to the lower colonization of $STM^{\Delta znuABC}$ which is not sufficient to give rise to a systemic immune response

Histology

We compared the cecum histopathological findings from control, $STM^{\Delta znuABC}$ and STM^{wt}-infected piglets at 1 and 12 dpi. At 1 dpi, sections from control piglets did not show inflammatory infiltrate (**Figure 4A**); conversely, piglets infected with $STM^{\Delta znuABC}$ and STM^{wt} showed neutrophilic infiltrate in the lamina propria and submucosa (**Figures 4B, C**). The neutrophilic infiltrate appeared moderate and multifocal in the $STM^{\Delta znuABC}$ (**Figure 4B**), with crypt abscess formation, whereas marked and diffused in the STM^{wt} infected piglets (**Figure 4C**). On the other hand, the neutrophil inflammation was mild at 12 dpi and present in a multifocal pattern in piglets infected with $STM^{\Delta znuABC}$, while inflammation was mild and diffuse in piglets infected with STM^{wt} (data not shown). Overall, a histological investigation indicated the presence of inflammatory infiltrate only in STM^{wt} and $STM^{\Delta znuABC}$. A higher degree of inflammation was observed in piglets infected with STM^{wt}.

Influence of *Salmonella* infection on the expression of pro-inflammatory cytokines

Pro-inflammatory (IL1-α, IL1-β, TNF-α) and regulatory (IFNγ) cytokines were observed so as to evaluate the early immune response in the ileocecal lymph nodes, colon, and cecum at 1 and 12 dpi (**Figures 5A–H**; Supplementary Figures 2A–H, 3A,B). At 1 dpi, we observed a tendency of the pro-inflammatory cytokines to increase in all organs analyzed; however, only the increase of IL1-β ($p < 0.05$) in the cecum and in the colon, and IL1-α ($p < 0.05$) in the lymph nodes of group C (STM^{wt}) were statistically significant (**Figures 5A–C**; Supplementary Figures 2A–C, 3A–C). At 12 dpi, overall expression of IL1-α, IL1-β, and TNF-α returned to baseline levels (**Figures 5E–G**; Supplementary Figures 2E–G, 3E–G). Moreover, TNF-α ($p < 0.01$), IL1-β ($p < 0.01$), and IL1-α ($p < 0.05$) were significantly down-regulated in the colon

of piglets infected with STM1znuABC (group B; Supplementary Figures 2E–G), and IL1-α ($p < 0.05$) also in the lymph nodes of group C (STM^{wt}; Supplementary Figures 3E–G).

S. Typhimurium alters composition of the microbiota in the post-weaned piglets model

Aiming to more specifically analyze the impact of STM^{wt} and $STM^{\Delta znuABC}$ on some of the most representative bacterial members, we used quantitative real time PCR (q-PCR). As depicted in **Figure 6**, consistent changes in the microbiota were present primarily in the cecal contents at 1 day post-*Salmonella* infection, with a significant increase of total 16S rRNA gene copies (representative of total bacterial numbers) in piglets infected with STM^{wt} (group C; $p < 0.05$) compared to naïve animals (group A) and piglets infected with $STM^{\Delta znuABC}$ (group B; $p < 0.05$). Differences in the *Lactobacillus/Lactococcus* group were statistically significant between groups B and C ($p < 0.05$) and very close to significance between groups A (naïve) and C (STM^{wt}) in the cecum (**Figure 6A**). In the feces (Supplementary Figure 4), the *Lactobacillus/Lactococcus* group showed significant differences at 1, 2, 7, and 12 dpi ($p < 0.05$) between groups A and C, and only at 12 dpi between groups B and C ($p < 0.05$; Supplementary Figure 4). A decrease in the *Eubacterium rectale/Clostridium coccoides* group was evident in group C ($p < 0.05$) at 12 dpi in the cecum and at 2 dpi in the feces ($p < 0.01$; **Figure 6B**; Supplementary Figure 4C). No differences among the three experimental groups were observed for *Bacteroides* in any of the samples analyzed. Conversely, at 1 dpi an evident increase in the Bifidobacterium group was observed in all the districts investigated between groups A and C ($p < 0.01$ for cecal content and $p < 0.001$ for colon and feces) and between groups B and C ($p < 0.01$; **Figure 6A**; Supplementary Figures 4B, 5A). At 12 dpi, the

Bifidobacterium group showed a sharp reduction in groups B ($p < 0.001$) and C ($p < 0.001$) in the cecal content and in group B ($p < 0.05$) in the colonic one when compared to group A (naïve; **Figure 6B**; Supplementary Figure 5B). The levels of the *Enterobacteriaceae* other than *Salmonella* decreased significantly in both groups of animals infected with Salmonella strains in the cecal and colonic contents at 12 dpi (**Figure 6B**; Supplementary Figure 5B). A higher level of Salmonella, consistent with the microbiological findings, was observed in group C (STM^{wt}) compared to group B ($STM^{\Delta znuABC}$) in all the intestinal samples, while Salmonella was never detected in group A (naïve; **Figure 6**; Supplementary Figures 4, 5). These results show that *S*. Thyphimurium is able to alter intestinal microbiota in pigs inducing modifications correlated to its virulence.

Bacterial diversity of the fecal microbiota after *Salmonella* infection

Massive parallel sequencing of the 16S rDNA hypervariable V3-V4 region was performed on fecal samples available from the three experimental groups A, B, and C. The sequencing yielded a total of 177198 reads passing quality control (median reads per sample 11030). OTU classification yielded a median of 5742 OTUs per sample. Sequencing reads are available at http://www.ncbi.nlm.nih.gov/bioproject/PRJNA302126 (BioProject accession ID: PRJNA302126). We evaluated the bacterial diversity of the fecal microbiota associated with *Salmonella* strains by estimating alpha- and beta-diversity. Shannon index demonstrated that the fecal microbiota diversity in piglets infected with $STM^{\Delta znuABC}$ (group B) and STM^{wt} (group C) was significantly higher than the naïve animals (group A), at 0 and at 2 dpi respectively ($p < 0.01$). However, group C showed a significant lower alfa diversity at 12 dpi than group A (**Figure 7A**). Using Fisher's alpha, an index not influenced by the sample size and less affected by the abundance of the most common species than

Shannon's index, we found a higher diversity in piglets belonging to group C compared to group B at 2 dpi ($p < 0.05$). At the same time, Fisher's alpha confirmed the significant lower alfa diversity in group C at 12 dpi compared to group A ($p < 0.05$; **Figure 7B**). The beta diversity was calculated using both unweighted Unifrac and Bray-Curtis dissimilarity; principal component analysis (PCoA) was performed. As shown in **Figure 8A**, using Unifrac, four out of five samples belonging to group C (STM^{wt}) clustered separately along the principal coordinate 1 (PCA1) at 12 dpi. In addition, a clear separation of group B ($STM^{\Delta znuABC}$) from the rest of the samples is noticeable along the principal coordinate 2 (PCA2). The PCoA using Bray-Curtis dissimilarity did not allow any clear separation of the groups, although all the five samples belonging to group C (STM^{wt}), at 2 dpi, clustered at the extreme right along the principal coordinate 1 (PCA1; **Figure 8B**). On the light of these data, it can inferred that Shannon and Unifrac results, in which it seem to be differences among groups at time 0, could be biased by small sample size. Therefore, the microbiota composition of the different groups could be considered similar at time 0.

Salmonella strains-associated alterations in fecal microbiota by NGS

In order to compare how the composition of the fecal bacteria differed among treatment groups, the Kruskal–Wallis test and the Benjamini-Hochberg FDR method were used to identify differentially abundant taxa. The distribution of reads into phyla revealed that the bacterial communities of all samples consisted primarily of *Firmicutes* and *Bacteroidetes*. Microbiota analysis showed that 7 phyla, 112 families, 404 genera, and 15 phyla, 143 families, and 719 genera differed across groups A, B, and C, respectively at 2 and 12 dpi. **Figures 9A,B** and Supplementary Figure 6 represent heatmaps showing the genus-level clustering according to frequency within each sample (abundance ratio greater than 0.1) at times 0, 2, and 12 dpi; abundant

genera were color coded red, and white color coding indicated missing genera. The most remarkable difference reported in the piglets infected with STM^{wt} (group C) compared with naïve (group A) is that they showed an abundant presence of lactic acid-producing bacteria and a reduction of short chain fatty acids (SCFAs)-producing bacteria (**Figures 9A,B**). Analysis of data also revealed that piglets infected with STM^{wt} (group C) initially showed a decrease in *Prevotella* at 2 dpi compared to the naïve (group A). In addition, at 12 dpi, a more abundant presence of *Prevotella*, *Phascolarcobacterium*, and *Eubacterium* was evident in group C (STM^{wt}) rather than in groups A and B. Moreover, clustering analysis highlighted the differences in the sample distributions according to the treatment type. At 2 dpi, the most represented genera displayed a perfect clusterization of each single sample into its belonging study group (**Figure 9A**). Similarly, at 12 dpi, each piglet grouped into its belonging treatment group, except 2 samples (5 and 12) clustered in a different study group (**Figure 9B**). Moreover, at 12 dpi, groups A (naïve) and B ($STM^{\Delta znuABC}$) are more similar to each other, while group C (STM^{wt}) featured more relevant effects (**Figure 9B**). No significant differences were detected when each single group was analyzed longitudinally according to the three collection times. These data show that infection with different strains of *S.* Typhimurium is associated with different alterations of fecal microbiota.

DISCUSSION

The importance of pigs as a source of *Salmonella* in the food chain is well-known. However, while *Salmonella* pathogenicity has been widely studied in mice, our knowledge on the modality of interaction of this pathogen with pigs is still limited. It has been known that different and multiple factors can influence the dynamics of *Salmonella* colonization in swine, including pathogen features (virulence

mechanisms, exposure dosage), pig immune responses and the complex interplay between the pathogen and the intestinal microbiota (Bearson et al., 2013). In this study, we used a post-weaned piglet model to compare differences in the host colonization, inflammatory response, and modification of the intestinal microbiota induced by STM^{wt} and $STM^{\Delta znuABC}$ in order to elucidate the interplay among host, pathogen and gut microbiota. $STM^{\Delta znuABC}$ was chosen due to the fact that our previous studies have revealed that this strain is strongly attenuated either in mice or in pigs (Ammendola et al., 2007; Pasquali et al., 2008; Pesciaroli et al., 2013). Moreover, studies carried out in a mouse model showed that ZnuABC-mediated zinc uptake confers resistance to the antimicrobial protein calprotectin and promotes *Salmonella* growth over the competing intestinal microbiota (Liu et al., 2012). Here, we demonstrate that a different organs colonization, intestinal inflammation and modification of porcine microbiota are correlated with the different virulence of *Salmonella* strains. The inflammatory response evaluated through the expression of the immune mediators, and corroborated by histological findings, has shown that STM^{wt} induces a prompt increase of serum markers of inflammation during the early stage of infection (1 dpi). Moreover, at the same time point, the expression of tissue-associated markers showed a tendency to increase even if only IL1-β in cecum and colon ($p < 0.01$) and IL1-α in ileocecal lymph nodes ($p < 0.01$) reach statistical significance. The prompt induction of host response could be due to the rapid and high-level replication of STM^{wt} as showed by our microbiological data. On the contrary, at 1 dpi, the histological and immunological analysis revealed a mild intestinal inflammation and a poor systemic response induced by $STM^{\Delta znuABC}$ confirming characteristics of attenuation in growth and virulence of this strain. As a whole, these observations indicate that the host is able to mount a rapid innate immune response following *Salmonella* infection within a few hours after gut

colonization. The magnitude of the response and the severity of the clinical manifestations provide evidence that the host response and lesions are correlated and dependent to *S*. Typhimurium virulence. It is known that, similarly to what happens in vitro and in murine models of infection (Barthel et al., 2003; Stecher et al., 2007; Barman et al., 2008), *S*. Typhimurium strains induce an acute inflammatory response in the intestinal mucosa also in piglets (Bearson et al., 2013). Several studies have proved how *S*. Typhimurium takes advantages of inflammation to compete with the resident microbiota and to colonize the inflamed gut in mice (Lupp et al., 2007; Stecher et al., 2007; Barman et al., 2008; Winter et al., 2010) and piglets (Chirullo et al., 2015). In our study, we investigated the impact of *S*. Typhimurium on the porcine intestinal microbial communities. We found that *S*. Typhimurium infection modifies either the number or the composition of gut resident bacteria. In particular, these changes were associated with STM^{wt}, while the attenuated $STM^{\Delta znuABC}$ seemed to be less fit to sustain competition with the microbiota. These observations are in agreement with the studies performed in mice, where attenuated *Salmonella* mutants do not colonize intestine as well as wild-type strains as they are not able to trigger an efficient inflammatory response (Stecher et al., 2007; Lawley et al., 2008; Raffatellu et al., 2009; Winter et al., 2010). The major changes in the microbiota composition are mainly related to the significantly more abundant presence of *Lactobacillus/Lactococcus* group after STM^{wt} infection. This observation is in agreement with the results obtained by Videnska et al. (2013), which showed a significant increase of *Lactobacillaceae* in chicken cecal microbiota after S. Enteritidis infection. A possible explanation could be attributable to the microaerophilic growth of *Lactobacilli*, which may allow them to survive under conditions of increased redox potential due to the production of reactive oxygen species by granulocytes infiltrating the site of inflammation as occurs in a highly

inflamed gut (Videnska et al., 2013). Indeed, there is evidence that lactic acid accumulation could contribute to impair the intestinal defense barrier and increase the osmotic load in the intestinal lumen (Ling et al., 2014). The utilization of next-generation high-throughput sequencing allowed a wider description of the intestinal microbiota. In our study, clustering analysis shows that the microbiota composition changed after infection with *Salmonella* strains and the characteristics of the modifications were correlated with the virulence of the strain used. Our analysis reveals a different abundance of the most represented genera in piglets infected with STM^{wt} when compared with $STM^{\Delta znuABC}$ and naïve piglets. In fact, microbiota of piglets infected with STM^{wt} was characterized by an overall reduction of SCFA-producing bacteria (*Ruminococcaceae* including *Faecalibacterium*, *Roseburia*, *Butyrivibrio*, and *Clostridium* genera). SCFAs such as acetate and butyric acid are produced by microbial fermentation of carbohydrates and provide beneficial immunomodulatory and anti-inflammatory properties (Ling et al., 2014). In particular, butyric acid contributes to the inhibition of *Salmonella* in an acidic environment (Bearson et al., 2006), decreases invasion of intestinal cells by down-regulating expression of Pathogenicity island 1 (Gantois et al., 2006) and reduces the Salmonella-induced proinflammatory response of enterocytic cells in vitro (Malago et al., 2005). In line with these observations, previous studies showed that *Faecalibacterium*, which is correlated with butyrate production, also exhibits anti-inflammatory effects counterbalancing intestinal microbiota dysbiosis (Sokol et al., 2008). Hence, the reduced abundance of SCFA-producing bacteria induced by STM^{wt} could explain the enhanced inflammatory status observed in the gastrointestinal tract of piglets treated with this *Salmonella* strain; and it could be of interest to investigate the mechanisms leading to a reduction of this potentially protective component of the intestinal microbiota. Moreover, upon infection with

Salmonella strains, microbiota composition also showed changes in *Prevotella*, *Phascolarcobacterium* and *Eubacterium*. Similarly, to what elsewhere reported (Bearson et al., 2013), we observed a decrease of *Prevotella* in piglets infected with STM^{wt} at 2 dpi. However, the limitation of available information about the biological function of such genera makes difficult to extrapolate any significant meaning to our findings. At the same time, it should be acknowledged that the alpha and beta diversity patterns across the three groups within the three time points analyzed presented several discrepancies that can be attributable to the sensitivity of the next-generation sequencing technology and to the relative small sample size. However, both alpha-diversity indices converge on a significant lower alpha-diversity in group C compared to group A at dpi 12. At the same time, the significant difference found in the whole microbiome composition at time 0 between group A and group B, highlighted by Shannon alpha index and Unifrac beta-diversity PCoA, may raise the possibility that the inability of the mutant strain to colonize the intestine could be related to the composition of the microflora. Although we cannot discard this hypothesis, the present data does not allow any speculation and further studies using a larger sample size and, possibly, a more detailed time-course is warranted. Overall, our data show that the results of the interaction among *Salmonella*, the intestinal microbiota and the gut response are influenced by the specific characteristics of the three factors. The virulence of *Salmonella* and the alteration of microbiota composition is crucial in determining the outcome of the infection.

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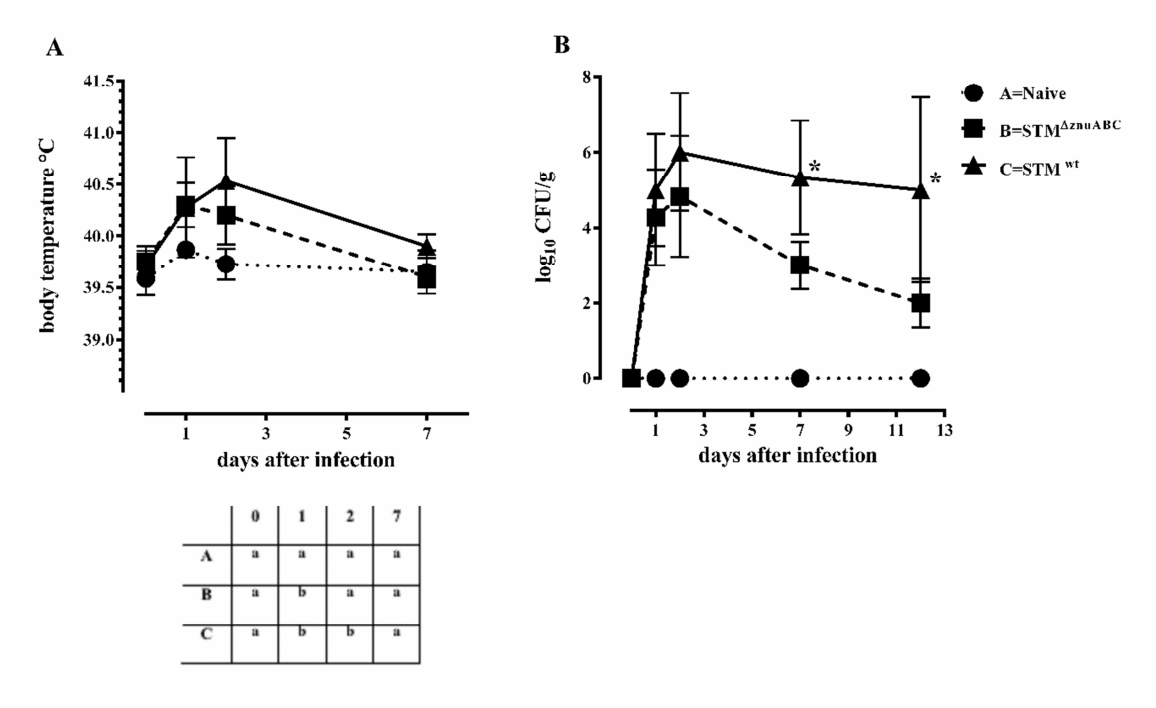


	0	1	2	7
A	a	a	a	a
B	a	b	a	a
C	a	b	b	a

FIGURE 1 | STM1znuABCQ4 (group B) Q5 shows a lower virulence in piglets compared to the STMwt (group C). **(A)** Mean values and standard deviation (SD) bars of body temperature of study groups in different time points. In the table on the bottom the levels of significance were reported among groups at different time points. Different letters at each time point represent significant different results ($P \leq 0.05$, Dunn's test). **(B)** Mean values and SD bars of CFU/g of STM1znuABC and STMwt shed in feces. Results of piglets infected with STM1znuABC were compared to results of STMwt and differences were statistically significant when $P \leq 0.05$, Mann–Whitney test

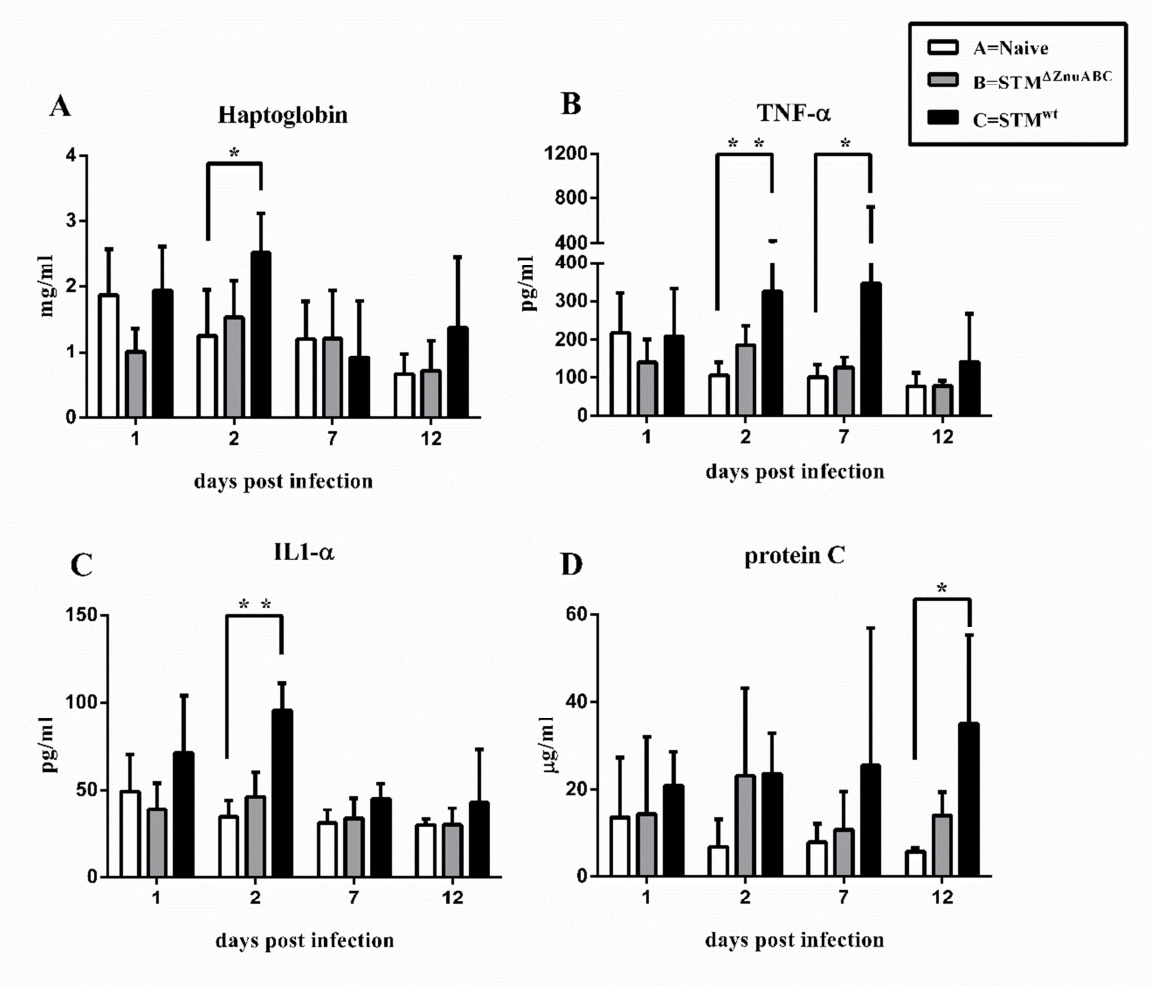


FIGURE 2 | (A–D) S. Typhimurium induces an inflammatory response correlated to the virulence of the bacterial strain. Haptoglobin, TNF-α, IL1-α, and C-reactive protein levels in serum of animals were determined by ELISA. The asterisks indicate statistical significance: *P ≤ 0.05 and **P ≤ 0.01 (multiple comparisons-Dunn's test).

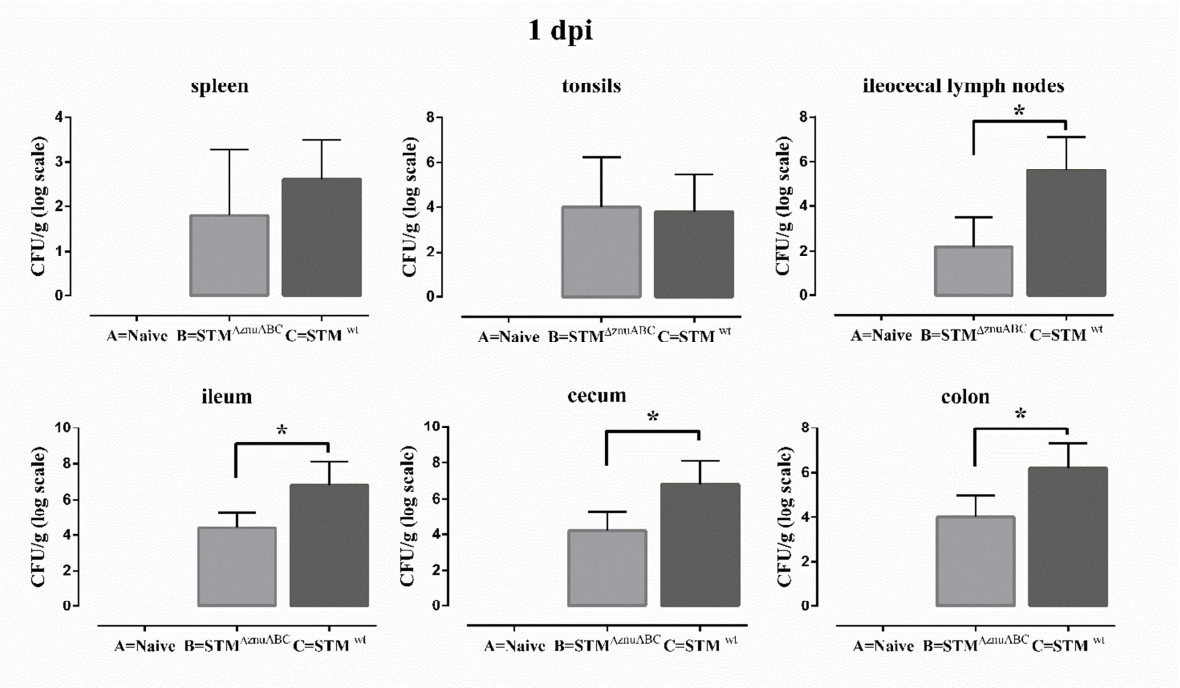


FIGURE 3 | STM^{wt} induces a higher organs colonization than STM$^{\Delta znuABC}$ at 1 dpi. Piglets were orally infected with 2 × 109 CFU of STM$^{\Delta znuABC}$ (group B) or STM^{wt} (group C), and bacterial burdens were determined at 1 dpi. Differences between groups B and C were estimated using non-parametric Mann–Whitney test and were considered significant when $P \leq 0.05$. Organ samples taken from naïve animals (group A) were negative. Error bars represent one SD from the mean.

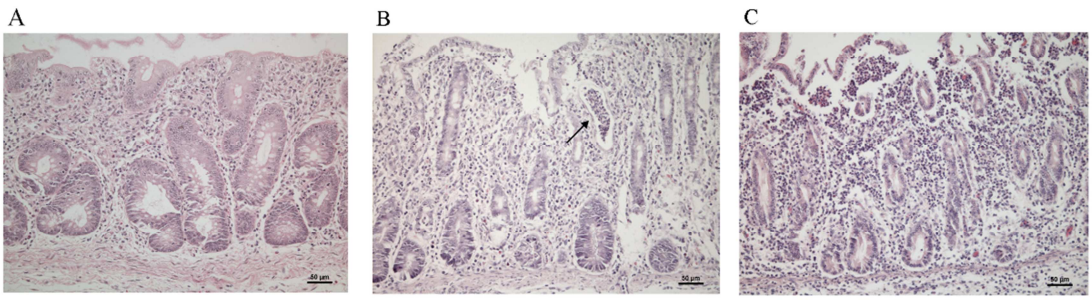


FIGURE 4 | (A–C) Photomicrographies showing histological changes of the cecum. **(A)** Naïve control piglets; **(B)** piglets infected with STM$^{\Delta znuABC}$: multifocal and moderate neutrophilic infiltrate (arrows), crypt abscess formation; **(C)** piglets infected with STM^{wt}: marked and diffuse neutrophilic infiltration.

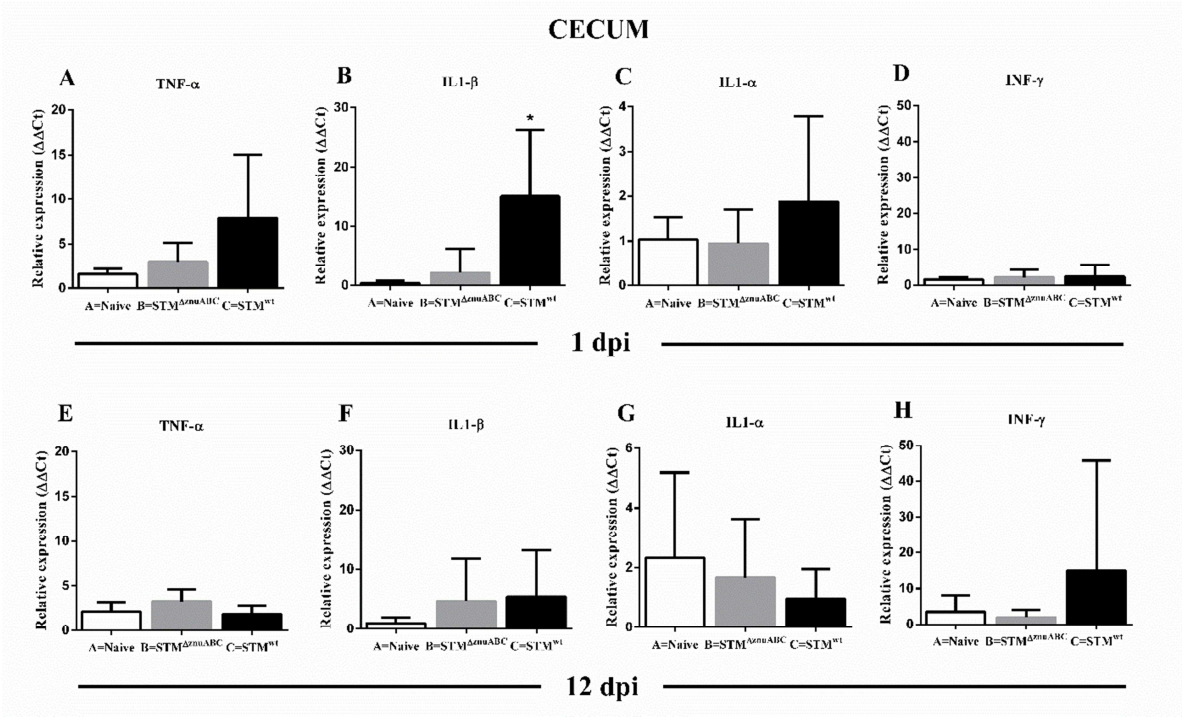


FIGURE 5 | (A–H) Cytokines expression reveals that unlike STM^{wt}, $STM^{\Delta znuABC}$ strain is not able to induce a strong host immune response. TNF-α, IL1-α, IL1-β, and INF-γ expression was measured in the cecum at 1 and 12 dpi by real time RT-PCR. Gray bars and black bars represent $STM^{\Delta znuABC}$-infected (group B) and STM^{wt}-infected piglets (group C), respectively. The asterisk indicates statistical significance $*P \leq 0.05$, Mann–Whitney test.

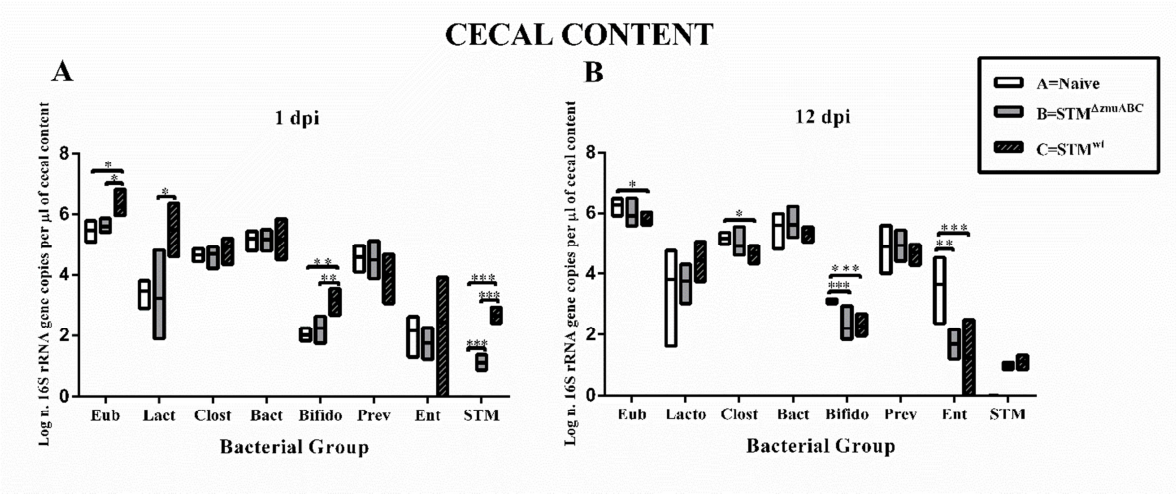


FIGURE 6 | STM^{wt} and STM$^{\Delta znuABC}$ differently modify cecal microbiota of piglets. Piglets were sacrificed at 1 and 12 dpi **(A,B)**. Bacterial genomic DNA was isolated from cecal content and qPCR analysis measured the abundance of specific commensal bacterial groups. White bars represent uninfected controls (group A). Gray and gray-black bars represent STM$^{\Delta znuABC}$- (group B) and STM^{wt}- (group C) infected piglets, respectively. P-values were calculated using One-way ANOVA with Bonferroni's post-test. Significant differences between groups are indicated by *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001. Eub, all bacteria; Lacto, *Lactobacillus/Lactococcus* group; Clost, *Eubacterium rectale/Clostridium coccoides*; Bact, *Bacteroides* sp.; Bifido, *Bifidobacterium*; Prev, *Prevotellaceae*; Ent, *Enterobacteriaceae* other than *Salmonella*; *STM*, *S. Typhimurium.*

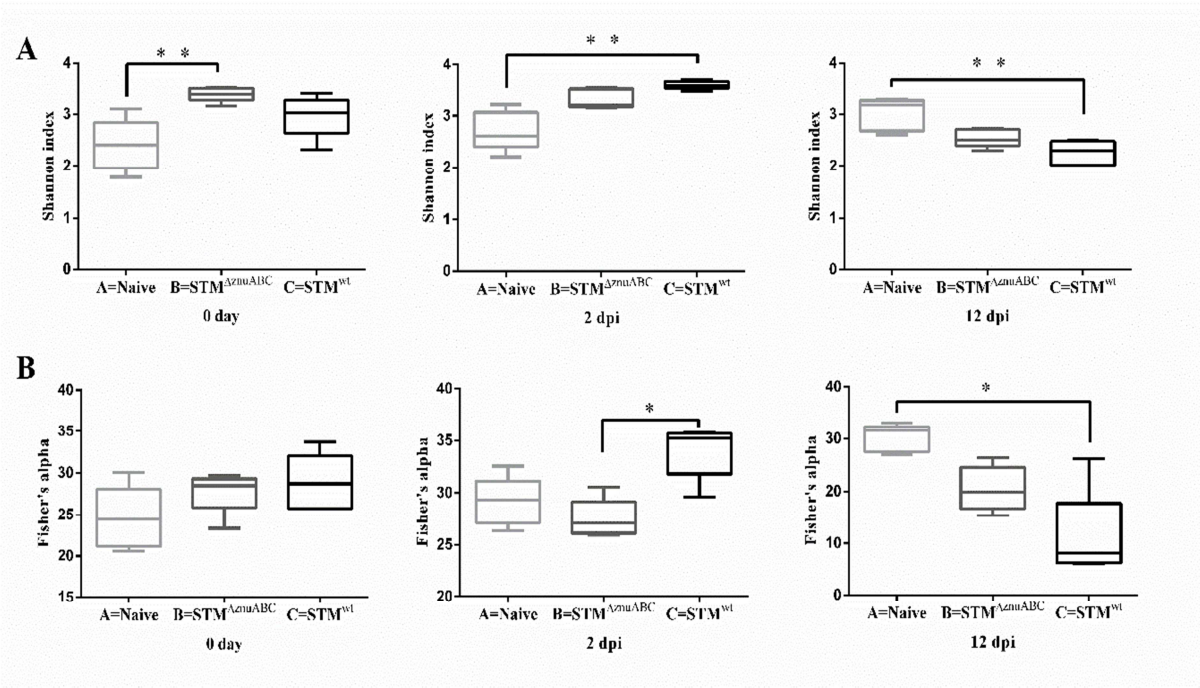
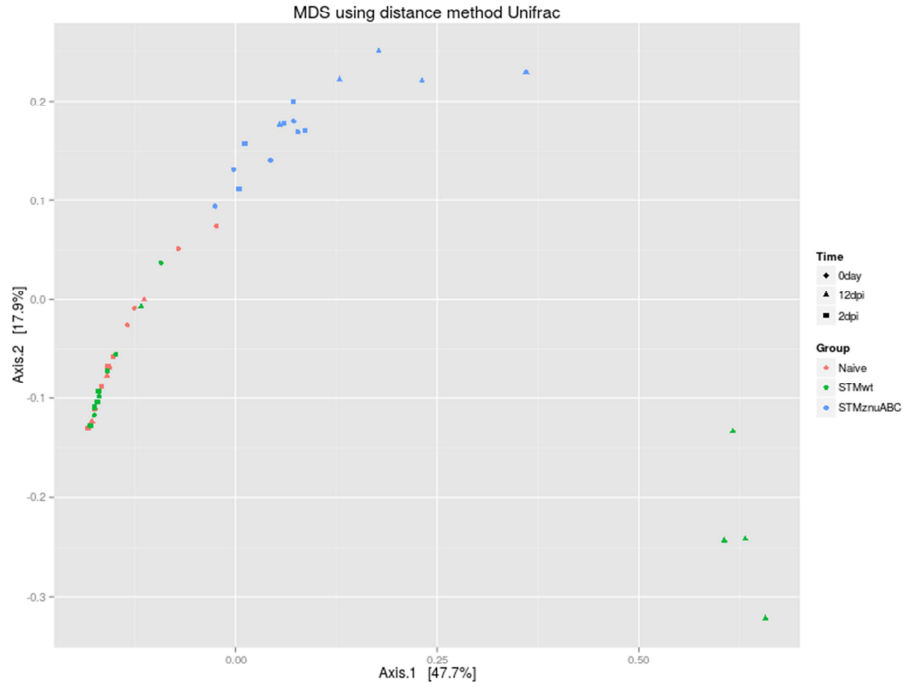


FIGURE 7 | Structural comparison of fecal microbiota among groups A, B, and C. The Shannon index **(A)** and Fisher's alpha **(B)** were used to estimate diversity of the fecal microbiota in naïve animals (group A) and in $STM^{\Delta znuABC}$- (group B) and STM^{wt}- (group C) infected piglets. Boxes represent median, and first and third quartiles; whiskers indicate minimum and maximum values. The asterisks indicate statistical significance *$P \leq 0.05$ and **$P \leq 0.01$, Dunn's test.

A



B

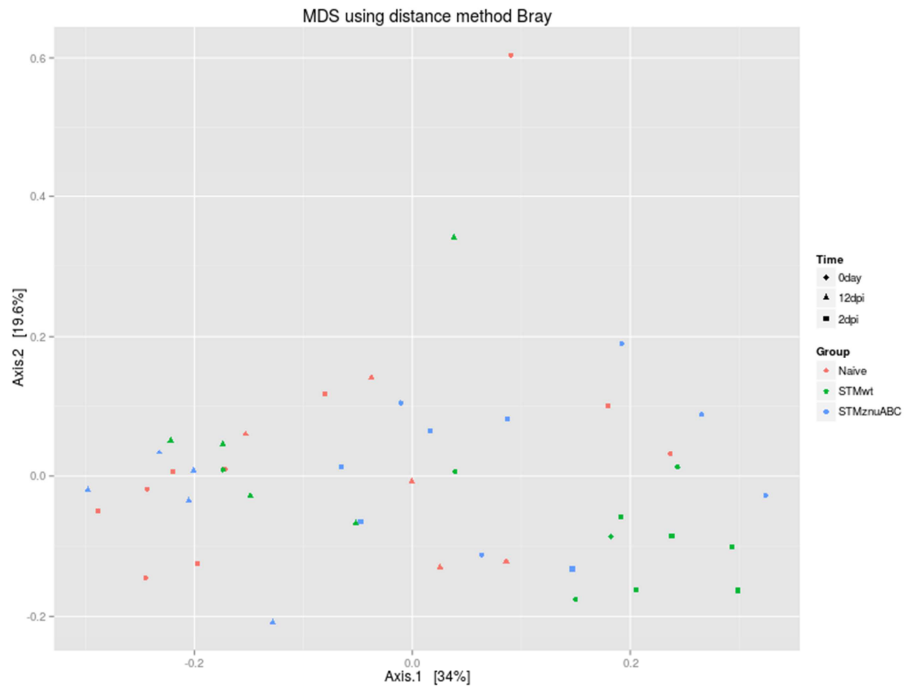


FIGURE 8 | Principal Coordinate analysis plot (PCoA) of unweighted UniFrac distances (A) and Bray-Curtis dissimilarity (B) for the fecal microbiota across the three study groups. PCA, principal coordinate

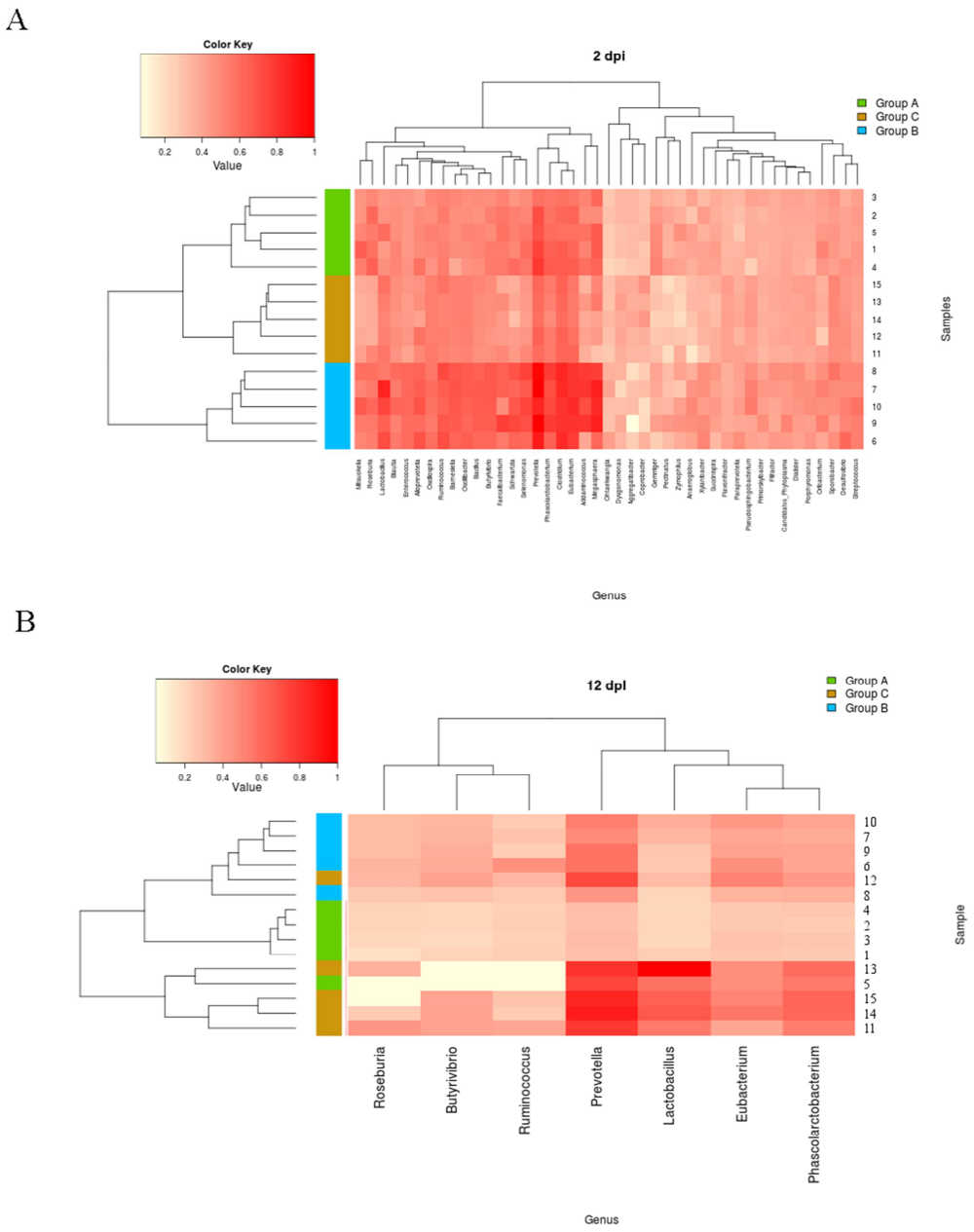


FIGURE 9 | (A,B) Heatmap indicating genus-level changes in the microbiota composition of piglets Naïve (group A), and piglets infected with STM$^{\Delta znuABC}$ (group B) or with STM^{wt} (group C) at 2 and 12 dpi. The relative abundance of the most represented genera is indicated by a gradient of color from white (low abundance) to red (high abundance). The hierarchical clustering analysis of the samples, based on the similarity of the microbiota composition, are displayed on the left. Animals 1–5: group A (Naïve), green; animals 6–10: group B (STM$^{\Delta znuABC}$), blue; piglets 11–15: group C (STM^{wt}), orange.

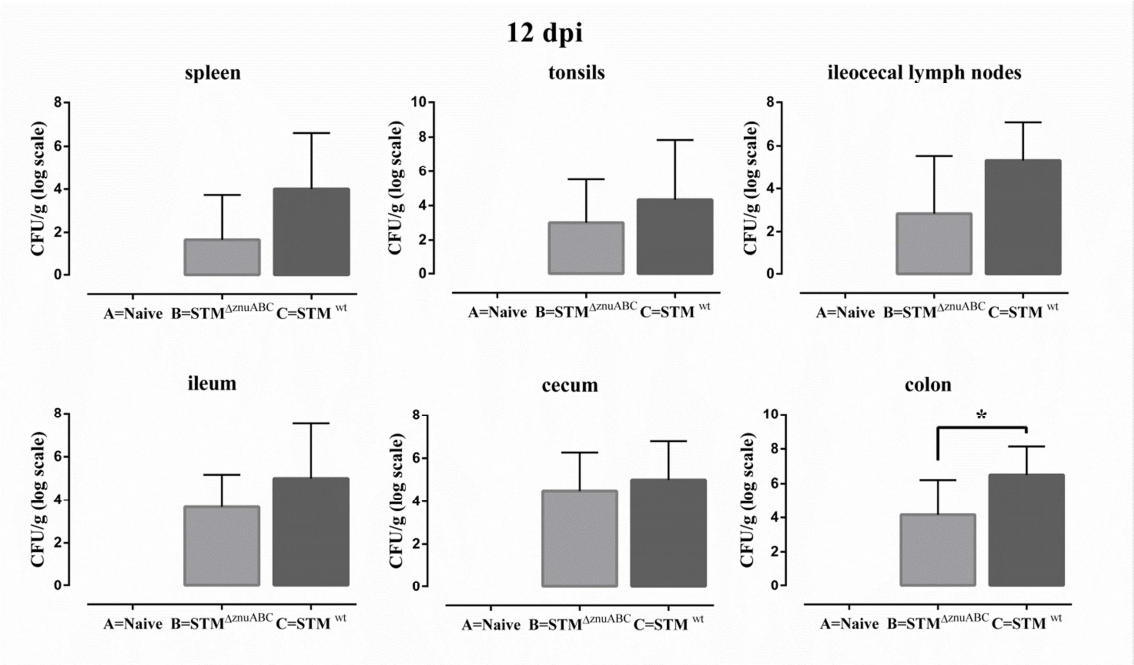
SUPPLEMENTARY MATERIALS

Supplementary Table 1. Oligonucleotide sequences and DNA probes for the detection of porcine cytokines. F and R indicate primers forward and reverse.

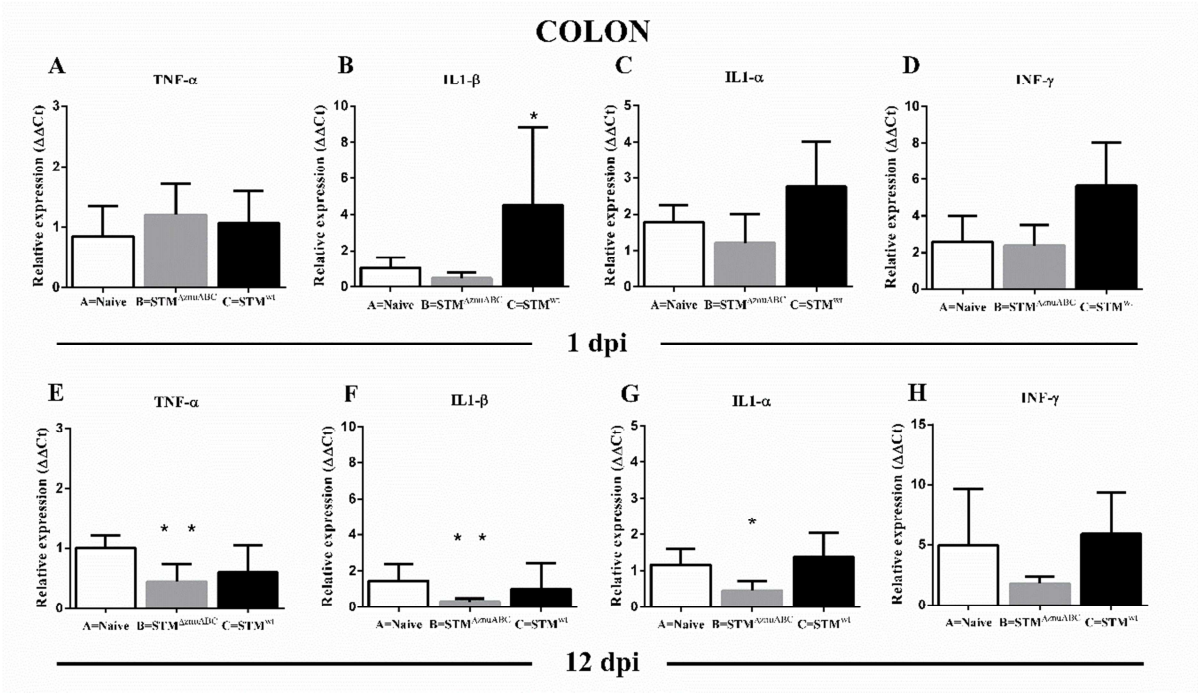
mRNA target	Oligonucleotides (5'-3')
TNF-α	F: CCTACTGCACTTCGAGGTTATC
	R: ACGGGCTTATCTGAGGTTTG
	Probe: 56-FAM/CTGGCCCAA/ZEN/GGACTCAGATCATCG/IBFQ
IL1-α	F: AGACCAGTCTCCTCTTCTTCT
	R: CCTGCCTTGTGGCAATAAAC
	Probe: 56-FAM/TACTTCAAA/ZEN/TCAGCCGCCCATCCA/IBFQ
IL1-β	F: GACCTTAGGGATCAAGGGAAAG
	R: CCATGTCCCTCTTTGGGTATC
	Probe: 56-FAM/TGATGAAAG/ZEN/ATAACACGCCCACCCT/IBFQ
IFN-γ	F: TCAAAGATAACCAGGCCATTCA
	R: CAGTTTCCCAGAGCTACCATTTA
	Probe: 56-FAM/AGGAGCATG/ZEN/GATGTGATCAAGCAAGA/IBFQ
RPL32	F: CTCAGTGAGTTAAGGATCCAGTG
	R: CGAGCCCACTATTCATTTCAAC
	Probe: 56-FAM/TGTGGCAGA/ZEN/TGTGGTTTAGACCCC/IBFQ

Supplementary Table 2. Real-time PCR primers used in this study. The primer sets were tested for sensitivity and specificity against a panel of genomic DNAs and showed minimal or no cross-reactivity.

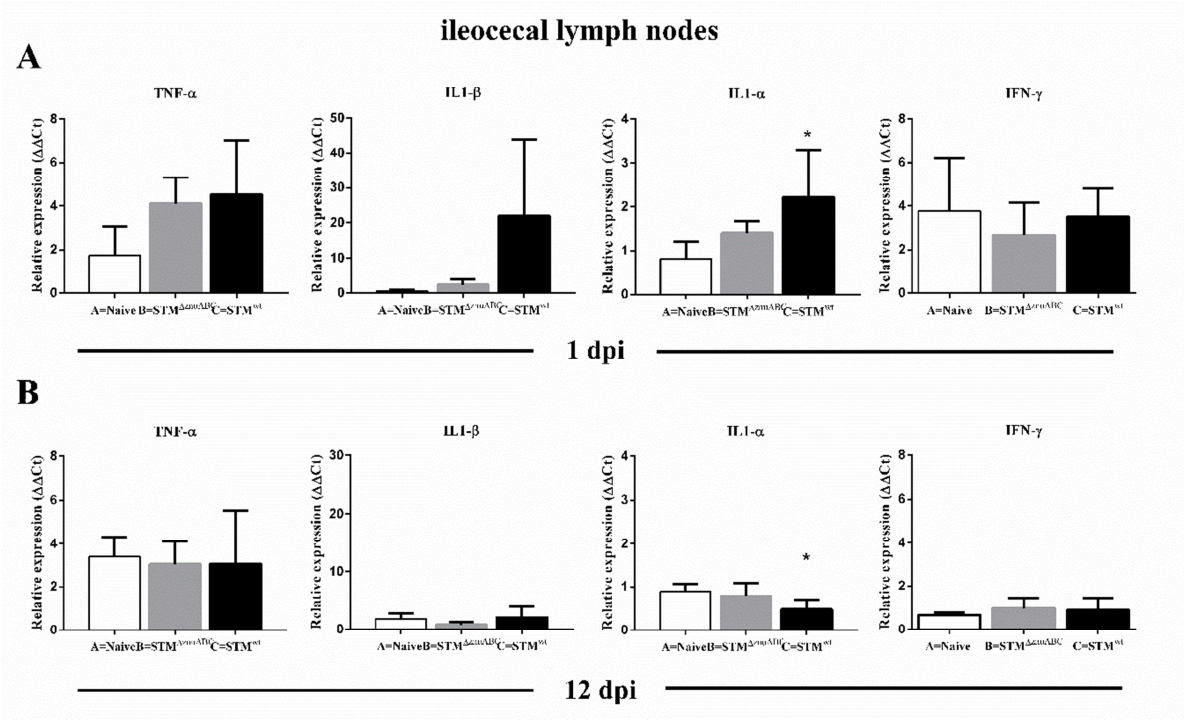
Group	Primer	Sequence (5' to 3')	References
Eubacteria	UniF340 UniR514	ACTCCTACGGGAGGCAGCAGT ATTACCGCGGCTGCTGGC	M. Barman D. Unold, K. Shifley, E. Amir, K. Hung, N. Bos, and N. Salzman et al. Infect Immun. **76**:907–15, 2008.
Lactobacillus/Lactococcus	LabF362 LabR677	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	
Eubacterium rectale/ Clostridium coccoides	UniF338 C.cocR491	ACTCCTACGGGAGGCAGC GCTTCTTAGTCAGGTACCGTC AT	
Bacteroides	BactF285 UniR338	GGTTCTGAGAGGAGGTCCC GCTGCCTCCCGTAGGAGT	
Enterobacteriaceae	UniF515 Ent826R	GTGCCAGCMGCCGCGGTAA GCCTCAAGGGCACAACCTCCA AG	
Salmonella	Sal454 Uni785R	TGTTGTGGTTAATAACCGCA GACTACCAGGGTATCTAATCC	
Bifidobacteria	F-Bifid 09c R-Bifid 06	CGGGTGAGTAATGCGTGACC TGATAGGACGCGACCCCA	J. P. Furet, O. Firmesse, M. Gourmelon, C. Bridonneau, J. Tap, S. Mondot, J. Doré, and G. Corthier, FEMS Microbiol Ecol. **68**:351-62, 2009.
Prevotella	PrevF PrevR	CGGGTTGTAAACTGCTTTTAT GAAG TTTATTGGGTTTAAAGGGAGC G	S. M. D. Bearson, H. K. Allen, B. L. Bearson, T. Looft, B. W. Brunelle et al, Infect Genet Evol. **16**:330-40, 2013.



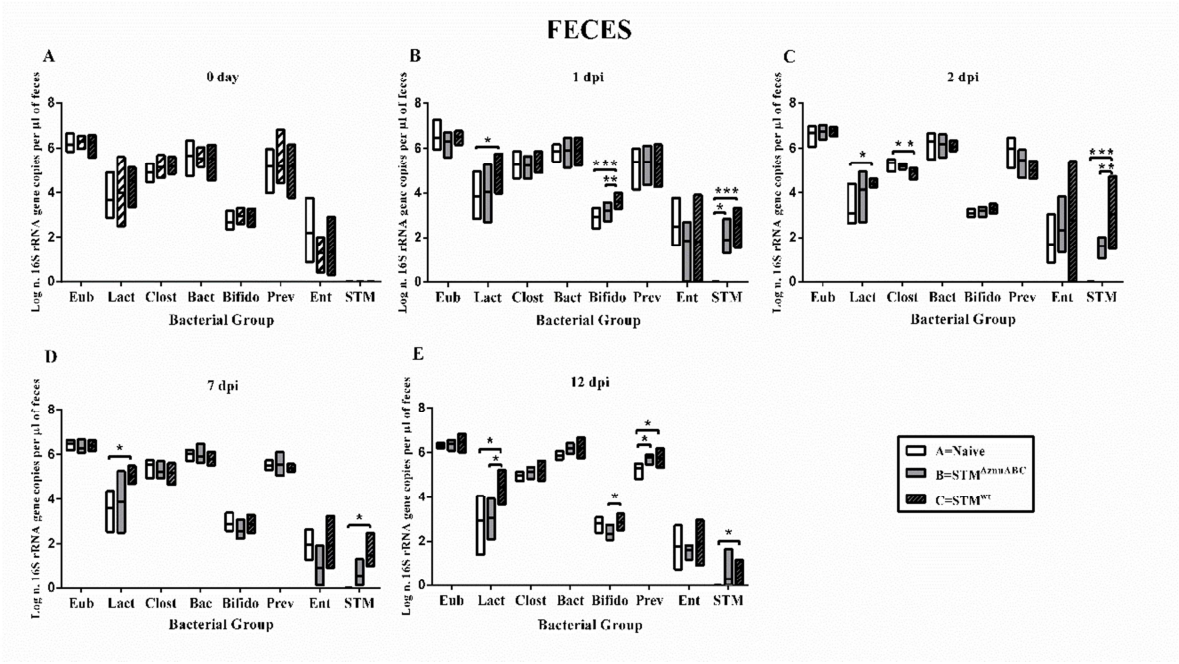
Supplementary Figure 1. STM^{wt} induces a higher colonization than STM$^{\Delta znuABC}$ at 12 dpi. Piglets were orally infected with 2×10^9 CFU of STM$^{\Delta znuABC}$ (group B) or STM^{wt} (group C) and bacterial burdens were determined at 12 dpi. Differences between groups B and C were considered significant when $P\leq0.05$ (*), Mann Whitney test. Error bars indicate one SD from the mean.



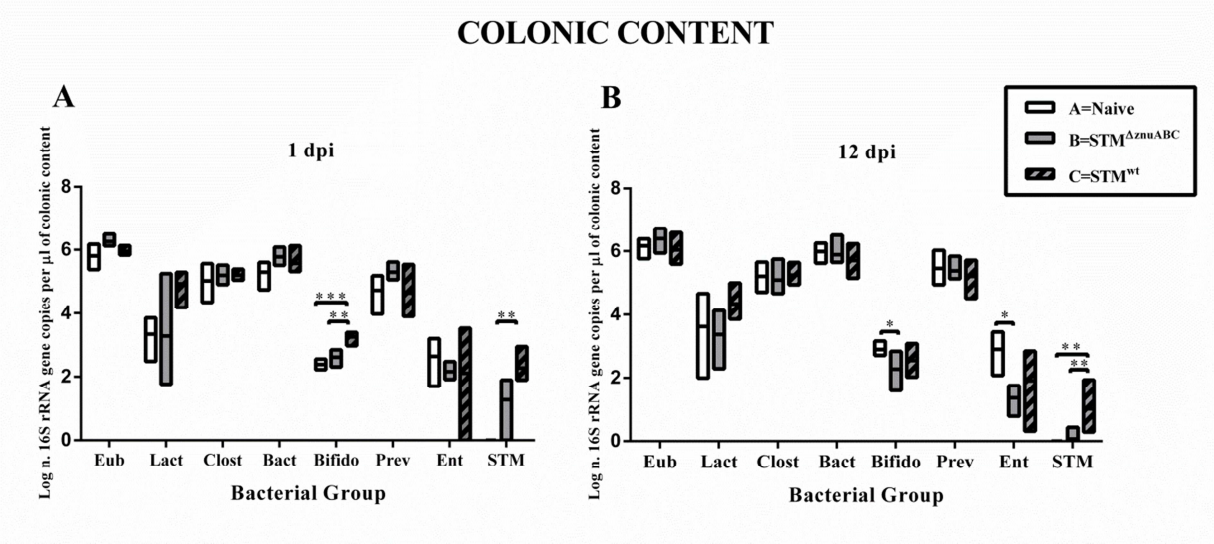
Supplementary Figure 2 (A-H). TNF-α, IL1-α, IL1-β and INF-γ expression was measured in the colon at 1 and 12 dpi, by real time RT-PCR. Grey bars and black bars represent $STM^{\Delta znuABC}$- (group B) and STM^{wt}-infected piglets (group C), respectively. The asterisks indicate statistical significance *$P \leq 0.05$ and **$P \leq 0.01$), Mann-Whitney test.



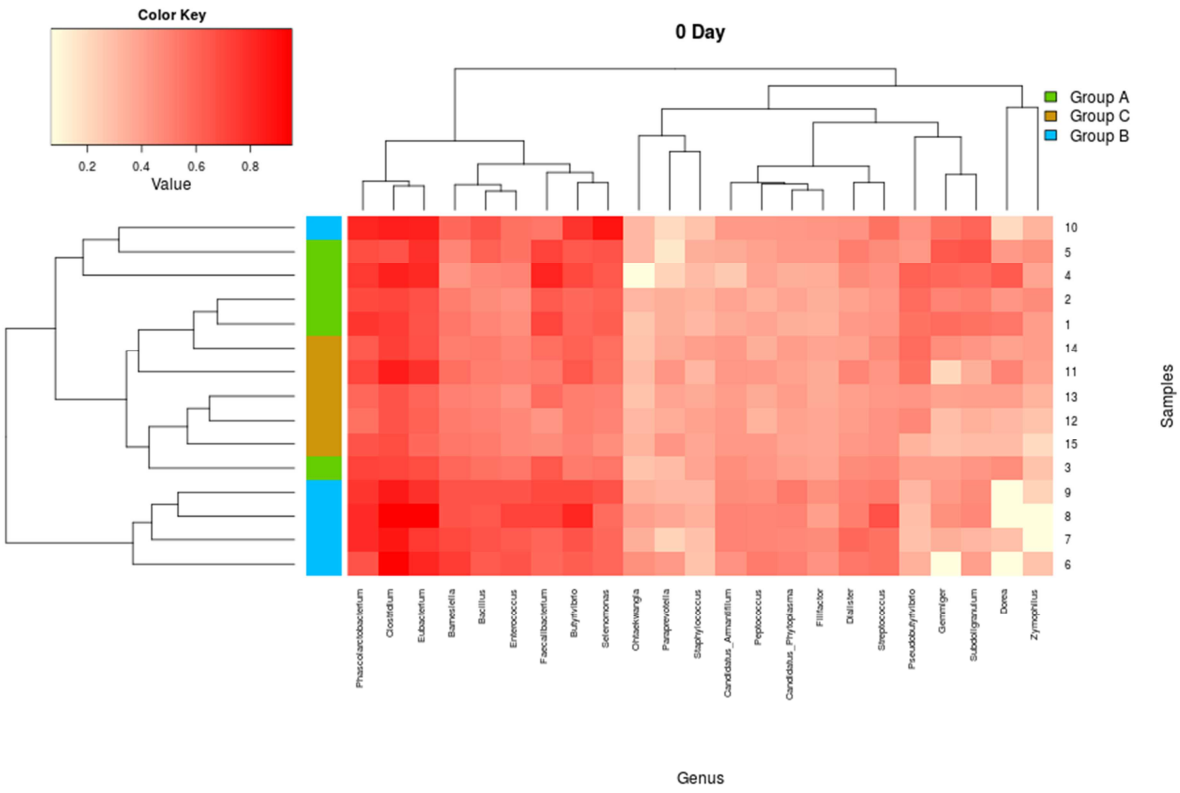
Supplementary Figure 3 (A-B). TNF-α, IL1-α, IL1-β and INF-γ expression in the ileocecal lymph nodes at 1 and 12 dpi, was measured by real time RT-PCR. Grey bars and black bars represent, $STM^{\Delta znuABC}$-(group B) and STM^{wt} -infected piglets (group C), respectively. The asterisk indicates statistical significance $*P \leq 0.05$, Mann-Whitney test.



Supplementary Figure 4 (A-E). Quantitative analysis of specific commensal bacterial groups in the feces of piglets infected with $STM^{\Delta znuABC}$ or STM^{wt} at different timepoints. White bars represent uninfected controls. Grey bars and grey-black bars represent $STM^{\Delta znuABC}$- (group B) and STM^{wt}-infected piglets (group C), respectively. P-values were calculated using one-way ANOVA with Bonferroni's post-test. Significant differences between groups are indicated by $*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.001$. Eub, all bacteria; Lacto, *Lactobacillus/Lactococcus* group; Clost, *Eubacterium rectale/Clostridium coccoides*; Bact, *Bacteroides* sp.; Bifido, *Bifidobacterium*; Prev, *Prevotellaceae*; Ent, *Enterobacteriaceae* other than *Salmonella*; STM, *S. enterica* serovar Typhimurium.



Supplementary Figure 5 (A-B). Quantitative analysis of specific commensal bacterial groups in the colonic content of piglets infected with $STM^{\Delta znuABC}$ or STM^{wt}, at 1 and 12 dpi. White bars represent uninfected controls. Grey bars and grey-black bars represent $STM^{\Delta znuABC}$- (group B) and STM^{wt}-infected piglets (group C), respectively. P-values were calculated using one-way ANOVA with Bonferroni's post-test. Significant differences between groups are indicated by $*P \leq 0.05$), $**P \leq 0.01$) and $***P \leq 0.001$. Eub, all bacteria; Lacto, *Lactobacillus/Lactococcus* group; Clost, *Eubacterium rectale/Clostridium coccoides*; Bact, *Bacteroides* sp.; Bifido, *Bifidobacterium*; Prev, *Prevotellaceae*; Ent, *Enterobacteriaceae* other than *Salmonella*; STM, *S. enterica* serovar Typhimurium.



Supplementary Figure 6. Heatmap indicating genus-level changes in the microbiota composition of piglets naive (group A), and piglets infected with STM$^{\Delta znuABC}$ (group B) or with STM^{wt} (group C) at 2 and 12 dpi. The relative abundance of the most represented genera is indicated by a gradient of color from white (low abundance) to red (high abundance). The hierarchical clustering analysis of the samples, based on the similarity of the microbiota composition, are displayed on the left. Animals 1-5: group A (Naïve), green; animals 6 -10: group B (STM$^{\Delta znuABC}$), blue; piglets 11-15: group C (STM^{wt}), orange.

Chapter 8

Salmonella Typhimurium infection primes a nutriprive mechanism in piglets

From originally published article:

Miarelli M, Drumo R, Signorelli F, Marchitelli C, Pavone S, Pesciaroli M, Ruggieri J, Chirullo B, Ammendola S, Battistoni A, Alborali GL, Manuali E, Pasquali P. Salmonella Typhimurium infection primes a nutriprive mechanism in piglets. Vet Microbiol. 2016 Apr 15;186:117-25. Epub 2016 Mar 4.

http://dx.doi.org/10.1016/j.vetmic.2016.02.006

Abstract

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is an important cause of acute food- borne zoonoses worldwide, typically carried by pigs. It is well known that *Salmonella* has evolved a wide array of strategies enabling it to invade the host, but little information is available on the specific host responses to *Salmonella* infections. In the present study, we used an *in vivo* approach (involving piglets infected with a virulent or an attenuated *S.* Typhimurium strain) coupled to histological and proteomic analysis of the cecum mucosa, to highlight the host pathways activated during *S.* Typhimurium infection. We confirm the complex host-pathogen interaction. Our data showed that the metabolic and the cytoskeleton organization functions were the most significantly altered. In particular, the modifications of energy metabolic pathway could suggest a "nutriprive" mechanism, in which the host reduce its metabolic and energetic status to limit *Salmonella* infection. This study could represent a preliminary approach, providing information useful to better understand the host-*Salmonella* interaction.

1. Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a Gram-negative bacterium able to colonize the lower intestinal tract of a wide range of animals, including humans (Fedorka-Cray et al., 1995). It is an important cause of acute food-borne zoonoses worldwide and responsible of numerous cases of human gastroenteritis and bacteremia annually. Pigs are typically asymptomatic carriers of *S.* Typhimurium and this commensal-like state establishes a significant reservoir of infection (Bearson and Bearson, 2011). Approximately 15% (range 7%-20%) of all cases of enteric salmonellosis in industrialized countries originate from pork products (Burel et al., 2013), therefore, a better control of *Salmonella* infection in pigs is important to reduce health risks for humans. Proteome approaches are a useful

tool to investigate the host–pathogen interaction, allowing the detection of pathophysiological alterations that occur during infection (Encheva et al., 2007; Arce et al., 2014). Most of previous studies have investigated the translational changes occurring in *S.* Typhimurium during infection (Sonck et al., 2009), while little information is available on the host response. However, recently, some studies have focused on the dynamic response of porcine mesenteric lymph nodes, ileum and intestinal mucosa to *S*. Typhimurium infection (Martins et al,. 2012; Collado-Romero et al., 2012). The aim of the present study, was to highlight the alterations induced by two different strains of *Salmonella*, a fully virulent and an attenuated strain, in the porcine cecum proteome. The attenuated *S.* Typhimurium strain, lacking the high affinity zinc transporter ZnuABC, (Ammendola et al., 2007) was chosen because it represents a promising mucosal vaccine against salmonellosis in pigs (Gradassi et al., 2013; Pesciaroli et al. 2013; Ruggieri et al., 2014). The identified differentially expressed proteins were investigated by bioinformatic tools in order to identify the molecular pathways and the biological functions which are altered during infection. Our results provide general information that may be useful to better understand the host- *S.* Typhimurium interaction.

2. Materials and methods

2.1. Salmonella spp. cultures

Virulent *S.* Typhimurium ATCC 14028 (STM^{wt}) and its isogenic attenuated *znuABC* mutant ($STM^{\Delta znuABC}$) (Ammendola et al., 2007) were grown overnight at 37°C in Brain Heart Infusion broth (Oxoid Ltd, Basingstoke, UK), harvested by centrifugation and washed twice in ice-cold phosphate buffer solution (PBS) (Sigma-Aldrich, St. Louis, MO).

2.2. Animals and samples collection

Thirty-one post-weaned piglets of 28 days of age were enrolled in the study. All were the offspring of *Salmonella*-free sows (negative for *Salmonella* by serological and microbiological tests); similarly, the study animals had proved to be *Salmonella*-free. Piglets were divided into three groups, each of which were allocated in separate rooms in biosafety level facility of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, and pellet fed was administered ad libitum. A total of 9 animals composed the naïve group (control) which was inoculated by oral route with sterile sodium bicarbonate buffer. Two groups of 11 piglets were orally infected with 2×10^9 CFU of $STM^{\Delta znuABC}$ ($STM^{\Delta znuABC}$) or 2×10^9 CFU of STM^{wt} (STM^{wt}), respectively.

Four naïve control piglets and 5 animals of each of the two groups of infected piglets were euthanized using a captive bolt pistol and exsanguination at 1 day post infection (dpi); 5 piglets of naïve and 6 piglets of groups $STM^{\Delta znuABC}$ and STM^{wt} were euthanized at 12 dpi. Samples of ileum, cecum and colon were fixed in 10% neutral buffered formalin for subsequent histological analyses. In addition, sections of cecum were frozen by immersion in liquid nitrogen and stored at -80° for subsequent proteomic analyses. All the experiments were authorized by national authority and were conducted according to European Directive (2010/63/EU). The approval number is: 54/2012.

2.3. Histology

Formalin-fixed tissues were embedded in paraffin wax and stained with haematoxylin and eosin according to standard procedures. Features taken in consideration: the presence of eosinophilic, neutrophilic and lympho-plasmacytic infiltrate in the lamina propria and in the submucosa. It was also evaluated the severity of the intestinal epithelial damage.

2.4. Protein extraction, two dimensional gel electrophoresis and image analysis

Pools containing equal amounts (50 mg) of each of the cecum samples were prepared for the three experimental groups, immediately after thawing, generating 6 sample pools. Protein extraction was performed as previously described (Signorelli et al., 2010), with minor modifications. Briefly, samples were homogenized in lysis buffer (7M urea, 2M thiourea, 2% CHAPS, 1% DTT, and inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), sonicated on ice, shaken for 1 h at 15°C and centrifuged for 5 min at 20000 x g at 15°C. Total protein concentration was quantified by 2D Quant Kit (GE Heathcare, Niskayuna, NY). Pellet was precipitated using cold acetone; then it was dried and resuspended in lysis buffer.

Samples (100 µg/strip) were loaded on rehydrated IPG strips (11 cm, pH 3-10, NL, Bio-Rad) for isoelectric focusing using a Protean IEF cell (Bio-Rad Laboratories, Inc., Hercules, CA) for a total of approximately 27 kVh. After focusing, the IPG strips were gently soaked for a total of 15 min in equilibration solution (6 M urea, 50 mM Tris-HCI buffer pH 8.8, 30% v/v glycerol, 2% w/v SDS and 0.002% bromophenol blue) containing 2%, w/v DTT, followed by 2 step incubation in equilibration solution containing 2.5% w/v iodoacetamide for a total of 15 min. Second-dimension gel electrophoresis was carried out in a 4-15% polyacrylamide Ready Gel precast gels using the CRITERION™ Cell (Bio-Rad). Equilibrated strips were placed onto gels and run at a constant voltage of 200V for about an hour. Gels were stained overnight in cCBB (colloidal Coomassie Brilliant Blue (Neuhoff et al., 1988). Each pool was analyzed in triplicate.

The proteins were visualized by gel scanning with the Molecular Imager Pharos FX scanner (Bio-Rad Laboratories). To identify valid spots, PD Quest advanced program software (Bio-Rad Laboratories) was used. Spots were automatically detected on the basis of the spot parameters chosen such as the faintest, smallest, and largest spot on

the gel scan, and only those well-resolved were taken into account. Spot photodensity was normalized for the total quantity of all valid spots. Spots photodensities of the $STM^{\Delta znuABC}$ and STM^{wt} groups were compared with the control group, separately.

2.5. Statistical analysis of spot data

The spots with a photodensity fold change larger than 2 and a *p*-value below 0.05 (t-test), between at least a paired spot (control and sample, at 1 dpi or at 12 dpi), were considered differentially expressed and identified by mass spectrometry (MS). Significant differences in spot photodensity were confirmed by one-way analysis of variance of data (ANOVA). ANOVA was performed with GLM procedure, using the SAS 9.1 statistical software (SAS, Statistical Analysis with SAS/STAT® Software V9.1. SAS Institute Inc. 2009), considering each animal group as factor. Data are expressed as estimate mean and compared by Duncan's test with $p < 0.05$ as significant level.

2.6. Protein identification by mass spectrometry

Protein spots were excised manually from the gels, and destained with 50 mM ammonium bicarbonate pH 8.0 in 50% acetonitrile. Gel pieces were re-suspended in 50 mM ammonium bicarbonate pH 8.0 containing 100 ng of trypsin, incubated for 2 h at 4° C and then overnight at 37°C. The supernatant containing the resulting peptide mixtures was removed and the gel pieces were re-extracted with acetonitrile. The 2 fractions were then collected, freeze-dried, and analyzed by LC-MS/MS using the LC/MSD Trap XCT Ultra (Agilent Technologies, Palo Alto, CA) equipped with an 1100 HPLC system and a chip cube (Agilent Technologies). Mass spectral data were used to search a non-redundant protein database (National Center for Biotecnology Information – NCBI mammals database) using an in-house version of the Mascot software (Matrix Science, Boston, MA, USA). Each spectrum from

mass spectrometry was searched against the mammalian protein database. Proteins of the bacterial origin were not identified.

2.7. System biology analysis

To gain information about the biological significance of the identified proteins, we used the bioinformatic tools. Integrated functions and interactions of the proteins were explored using Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/pathway.html) (Kanehisa et al., 2004), Search Tool for the Retrieval of Interacting Genes/Proteins (STRING v 10) (http://string-db.org/) (Szklarczyk et al., 2015) and Database for Annotation, Visualization and Integrated Discovery (DAVID v6.7) (http://david.abcc.ncifcrf.gov/) (Huang et al., 2009). KEGG is a database resource for understanding high-level functions of the biological system. STRING is a meta-resource that aggregates known and predicted interaction data between proteins derived from four sources: genomic context, high-throughput experiments, co-expression and previous knowledge. We set up the STRING organism both as *H. sapiens* and *S. scrofa* (using both the official gene symbols) and we set up the confidence score (the approximate probability that a predicted link exists between two enzymes) at the medium value (40%). DAVID bioinformatics resources consist of an integrated biological knowledgebase and analytic tools aimed at systematically extracting biological meaning from large gene/protein lists. The program compares a list of genes with functional annotation databases, including GO (gene ontology) terms, KEGG pathways, and SPPIR (Swiss-Prot and Protein Information Resource) keywords. By comparing the prevalence of proteins belonging to these categories in their respective database and in the input gene list, DAVID generates a *p*-value highlighting significantly overrepresented annotation terms (Dennis et al., 2003). The full *H. sapiens* and *S. scrofa* genomes were set as the reference list, however for a full analysis *H. sapiens* was used because all genes of

our input list were recognized (14 of 14), while the *S. scrofa* (7 of 14) and the classification stringency was set to medium (default value). We used the functional annotation tools to determine overrepresented annotation groupings among our input genes.

3. Results

3.1. Salmonella Typhimurium induces histological changes with a magnitude paralleling its virulence

We compared the cecum, colon and ileum histopathological findings from control, STM$^{\Delta znuABC}$ and STM^{wt} infected piglets, at 1 and 12 dpi. At 1 dpi, sections from control piglets showed only a mild eosinophilic and lympho-plasmacytic infiltrate (Fig. 1 a-c); conversely, STM$^{\Delta znuABC}$ and STM^{wt} infected piglets showed neutrophilic infiltrate in the lamina propria and submucosa (Fig. 1 d-i). The ileum and cecum were the most affected intestinal sections, showing crypt abscess formation and crypts distortion. An apparent more severe grade of neutrophilic inflammation was detected in animals treated with virulent strain (Fig. 1 g-i). Moreover, flattened surface epithelium with diffuse enterocyte loss in piglets infected with STM$^{\Delta znuABC}$ was observed (Fig. 1 d-f); on the other hand, STM^{wt} infected piglets showed an increased epithelial damage ranging from foci of erosion to ulceration with accumulation of abundant necrotic debris in the gut lumen (Fig. 1 g-i). At 12 dpi, all the groups of piglets showed mild eosinophilic and lympho-plasmacytic infiltrate (Fig. 2), but only piglets infected with STM^{wt} and with STM$^{\Delta znuABC}$ also presented neutrophilic infiltrate (Fig. 2 d-i). The neutrophil inflammation was mild and in a multifocal pattern in piglets infected with STM$^{\Delta znuABC}$ (Fig. 2 d-f), while mild and diffuse in piglets infected with STM^{wt} (Fig. 2 g-i). Moreover, enterocyte loss was observed in piglets infected with STM$^{\Delta znuABC}$ (Fig. 2 d-f) while more severe epithelial

damage characterized by epithelial cell degeneration and extensive erosion of mucosa were highlighted in piglets infected with STM^{wt} (Fig. 2 g-i).

3.2. Salmonella Typhimurium induces cecum proteome changes

Using the proteomic approach, we identified the protein profiles expressed in the *cecum* of piglets infected with the attenuated or the virulent strain of *S.* Typhimurium. Differences in spot photodensity (fold change larger than 2 and $P<0.05$ (*t-test*)) between gels from infected samples and control, either at 1 dpi or at 12 dpi were identified in fourteen spots (Fig. 3) which were processed by LC-MS/MS analysis. The corresponding proteins are listed in the Table 1. At 1 dpi, we detected that 6 and 3 proteins were changed when piglets were infected with $STM^{\Delta znuABC}$ and STM^{wt} strains, respectively (Fig. 4A); while at 12 dpi, 10 and 5 proteins changed in piglets infected with $STM^{\Delta znuABC}$ and STM^{wt} strains, respectively (Fig. 4B). On the whole, we observed an overall predominance of proteins that decreased their abundance in both infected groups compared to the control at 1dpi. On the contrary, at 12 dpi, the proteins showed either increase or decrease when compared to the control. In addition, very few proteins were induced by both *S.* Typhimurium strains, as evidenced by the overlapping areas in Figure 5. According KEGG classification, the 14 proteins detected were grouped in the following categories: 5 in metabolism, 4 in cellular processes, 2 in environmental information processing, 2 in genetic information processing; albumin is not precisely classified in KEGG database, being involved in many cellular pathways. STRING v10 network in Figure 8.6 showed that the 14 identified proteins formed two functional tightly connected clusters, including 5 proteins (GAPDH, ATP5B, TPI1, UQCRC1, CKB, where GAPDH had a central role), and 9 proteins (ACTB, ALB, GSN, MYL9, ANXA2, ANXA5, HSPB1, HARS, COL6A3, where ACTB had a central role), respectively. DAVID v6.7 software package for functional analysis revealed that the

dysregulated proteins were involved in acetylation processes (71%), in duplication processes (29%) and 86% of them were phosphoproteins. Moreover, the more enriched Functional Gene Ontology categories were: soluble fraction as cellular component (29%), generation of precursor metabolites and energy (29%) as biological processes, calcium ion binding (36%) as molecular function (Table 2). The main functions of the identified proteins are reported in Table 3.

4. Discussion

In this study we aimed to obtain insights into the *in vivo* response of the porcine intestinal mucosa after infection with an attenuated and a virulent *S.* Typhimurium strains. For this purpose, at 1 and 12 dpi, we investigated the histological features of three different sections of intestinal tract (cecum, colon and ileum) and we analyzed the cecal proteins which were dysregulated during *Salmonella* infection. Our histological results support the concept that *S.* Typhimurium induces mucosal lesions with severity paralleling its virulence. In addition, we found eosinophils infiltration in the lamina propria of the intestine in piglets, irrespective to the experimental groups. We think, however, that this phenomenon has a negligible significance because it is a frequent finding in young healthy pigs (Tsukahara et al., 2010).

Proteomic analysis showed that energy metabolism and cytoskeleton related proteins are significantly perturbed after *Salmonella* infection. In fact, we observed the down regulation of enzymes such as ATP5B, UQCRC1 and CKB involved in oxidative phosphorylation, and of GAPDH, involved in gluconeogenesis/glycolysis, which could suggest a reduction of the metabolic status of the host. In fact, these alterations could be the attempt of the host to create environmental conditions that are detrimental for the pathogen. Our findings are in agreement with data already reported in literature. A study focused on the response of the murine colon mucosa to

Salmonella infection has revealed the shut-off of oxidative phosphorylation and the down regulation of genes involved in many metabolic pathways (Liu et al., 2012); moreover, Appelberg (Appelberg, 2006) has observed that macrophages use a "nutriprive" mechanism to deprive intracellular pathogens of nutrients inhibiting their growth. At the same time, it is known that pathogens can induce and modulate metabolic responses in the host aiming to provide an increased nutrient supply useful for their own invasion (Eisenreich et al., 2013). In line with these evidences, our observation that piglets infected with STM^{wt} showed a minor down-regulation of proteins involved in metabolic pathways in comparison to $STM^{\Delta znuABC}$-infected piglets, could indicate that in the first, the nutriprive mechanism is less efficient, because STM^{wt} is more able to oppose itself to it. System biology analysis also revealed the enrichment of three functional categories: acetylation, duplication and phosphoprotein which take part in modulation of many enzymes of the pathways of carbon, nitrogen and energy metabolism. In such context, we also could expect a lowering of protein synthesis, which might explain the down regulation of HARS, an enzyme involved in the proteins synthesis. It is well documented that *Salmonella* uses effectors to manipulate the cytoskeletal machinery of the host in order to induce its own internalization (Guiney and Lesnick, 2005; Hallstrom and McCormick, 2011; Ramos-Morales, 2012). They cause a remarkable series of events culminating in a marked rearrangement of the epithelial actin filaments (Criss et al., 2001) and in the formation of actin-rich membrane ruffles extensions enveloping *Salmonella*, for bacterial internalization (Patel and Galán 2005). In our study, we observed a substantial down regulation of several structural and regulatory proteins involved in actin cytoskeleton rearrangements. For example, at 1dpi, ACTB showed a reduction in piglets infected with STM^{wt} and $STM^{\Delta znuABC}$; this finding is in agreement with the results obtained by Prado Martins et al. (Prado Martins et al., 2012), which showed a

decrease of abundance of ACTB in porcine mesenteric lymph nodes after *Salmonella* Typhimurium infection. A possible explanation could be attributable to the binding of actin by a specific effector of *Salmonella* (SipA) determining the reduction in the concentration of its monomeric conformation (Galan and Zhou, 2000). Similarly, the down regulation of GSN could be attributable to the ability of SipA to interfere with cellular mechanisms of actin turnover (McGhie et al., 2004), and the reduction of MYL9, a protein involved in the maintaining of the cellular integrity and in cellular adhesion, could contribute to increase the intestinal permeability (Park et al., 2011). In the same way of the proteins of metabolic pathways, cytoskeleton related proteins showed a major down regulation in $STM^{\Delta znuABC}$-infected piglets compared to STM^{wt}-infected piglets (Fig. 4A). This observation could indicate that STM^{wt} is faster than $STM^{\Delta znuABC}$ in inducing and carrying out its own internalization process probably because it has a completely efficient set of effectors, so that while the $STM^{\Delta znuABC}$-infected cells are still working in ruffles formation, STM^{wt}-infected cells are recovering their original shape. *Salmonella* is also able to decrease the transcellular resistance by altering the adhesive glycoproteins responsible for the attach of the cells to the collagen of the extracellular matrix (Ramos Morales et al., 2012). A previous study has demonstrated that *Salmonella* exposure causes modifications of extracellular matrix in the bird's gut consisting in the degradation of pre-existing matrix proteins with the consequent collagen release (Berndt et al., 2009). Our data seems to be in agreement with these evidences suggesting that the increased COL6A3 could represented the free quota of proteins resulting by the matrix degradation caused by *Salmonella*.

In addition to cytoskeleton related proteins, we also observed changes in ANXA2 abundance. ANXA2 functions as a platform for dynamic actin-driven membrane processes such as the membrane ruffling and the internalization of

bacteria (Gerke and Moss, 2002; Hayes et al., 2006). It is mostly found in a complex with p11, and the ANXA2-p11 complex has a higher affinity for membranes than ANXA2 alone (Jolly et al., 2014). At 1 dpi, the prominent reduction of ANXA2 observed, could suggest that the monomeric ANXA2 is reduced because the largest quota of the protein is bind to p11 to contribute to the ruffling of the membranes. In conclusion, we found that cytoskeleton modification is another of the most significantly perturbed process during *Salmonella* infection, as also corroborated by bioinformatic findings: the enrichment of calcium binding cluster showed by DAVID, because it is known that calcium is important during the process of invasion and internalization of *Salmonella* (Furukava, R. et al., 2003), and the central role played by ACTB displayed by STRING (Fig. 6).

Finally, we have also found alterations of HSPB1, which is known to be induced by environmental stress and involved in anti-apoptotic signaling (Charette et al., 2000; Collado Romero et al., 2012). At 1 dpi, the decrease of HSPB1 could represent the defense attempt of the host to reduce the bacterial infection causing apoptosis. On the contrary, at 12 dpi, the high increase of HSPB1 as well as that of most of the proteins mentioned above, in piglets infected with $STM^{\Delta znuABC}$, could indicate that the infection is substantially resolved and that the normal physiological processes are being restoring.

5. Conclusion

This study allowed to investigate the response of porcine cecum to two different strains of *Salmonella* Typhimurium, aiming to characterize the molecular pathways involved during infection. Our results indicate a significant modification of metabolic pathway and of cytoskeleton. We revealed a “nutriprive” mechanism by which the host tries to control *Salmonella* infection depriving pathogen of energy and nutrients; moreover, the alterations of cytoskeleton related proteins reflect the active

modulation of the cellular factors due to the pathogen invasion. In the whole, the observations reported in this study provide insights that may be useful to better understand the interaction of *Salmonella* with vertebrate hosts.

Acknowledgments

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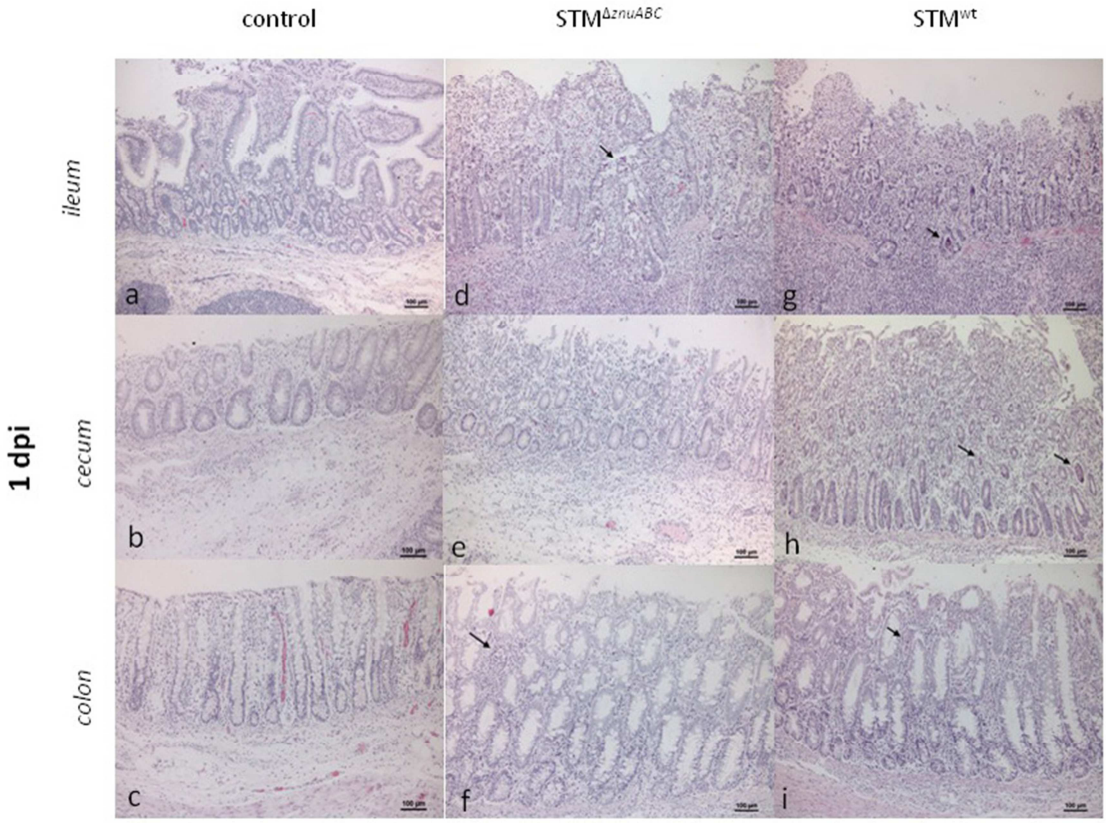

Fig. 1. Photomicrographies showing histological findings of the ileum, cecum and colon from control piglets and STM$^{\Delta ZnuABC}$, STM^{wt} strain infected piglets at 1 dpi. Control piglets: mild lymphocytic and eosinophilic infiltrate in the lamina propria of ileum, cecum and colon (a, b, c). STM $^{\Delta ZnuABC}$ infected piglets: diffuse neutrophilic infiltrate in the ileum and cecum lamina propria and submucosa (d, e) and diffuse and moderate lymphoplasmacytic infiltrate in the lamina propria of colon with scattered foci of neutrophilic inflammation (f (arrow)). Epithelial cell degeneration, necrosis of lymphoid follicles with focal ulceration of mucosa in the ileum (d, arrow) and enterocyte loss in the cecum and (e, f) can also be seen. STM^{wt} infected piglets: diffuse neutrophilic infiltrate in the lamina propria and submucosa with dilated crypts filled with neutrophils (crypt abscess) in the ileum and cecum (g (arrow), i (arrow)), and moderate lymphocytic inflammation in colon (i). Diffuse enterocyte loss in all intestinal tracts (g, h, i) and focal erosion in the ileum (g (arrows)) can also be seen. (bars 100 µm).

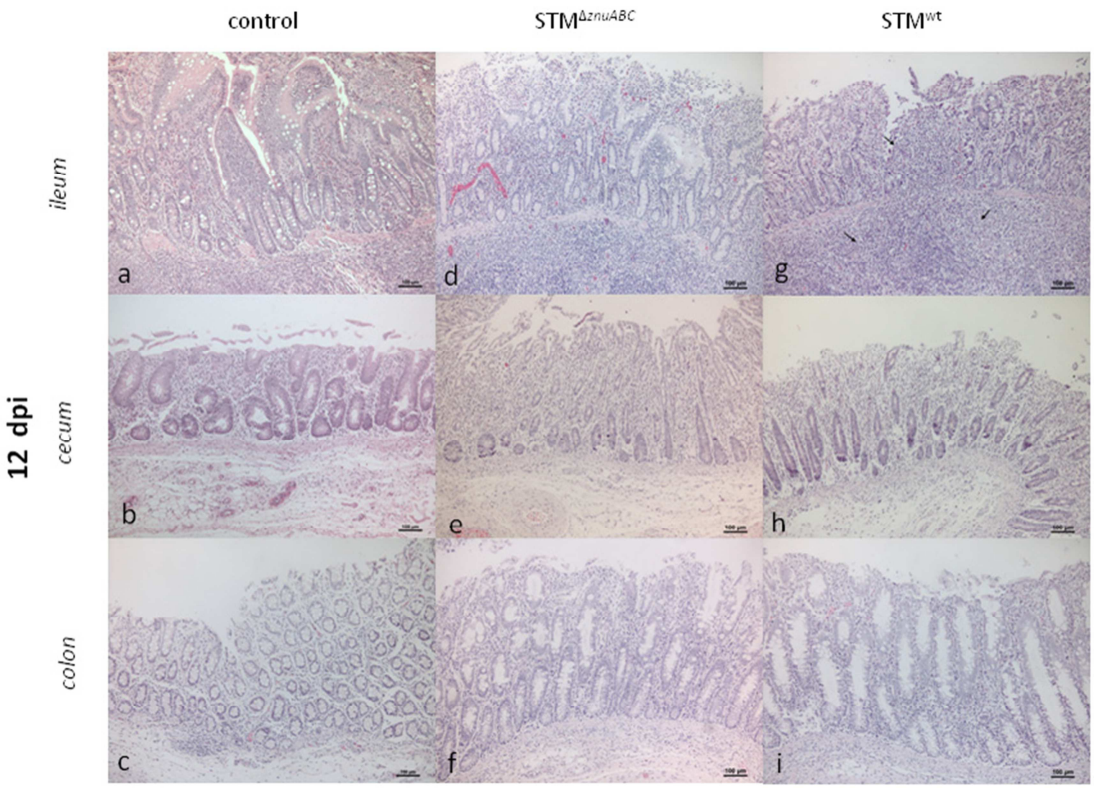

Fig. 2. Photomicrographies showing histological findings of the ileum, cecum and colon from control piglets and STM $^{\Delta znuABC}$, STM^{wt} strain infected piglets at 12 dpi. Control piglets: diffuse mild lymphoplasmacytic and eosinophilic inflammatory infiltrate in the ileum, cecum and colon. STM$^{\Delta znuABC}$ infected piglets: diffuse eosinophilic and lymphocytic infiltration and mild neutrophilic multifocal inflammation in the ileum (d); diffuse mild neutrophilic infiltration in the cecum (e); diffuse moderate lymphoplasmacytic infiltration with scattered foci of neutrophilic inflammation and isolated dilated crypts filled with neutrophils (crypt abscess) in colon (f). Epithelial cell degeneration, enterocyte loss with scant accumulation of necrotic debris in the lumen can be seen in the ileum, cecum and colon. STM^{wt} infected piglets: diffuse marked lymphoplasmacytic inflammation and neutrophilic infiltration in a multifocal pattern in the ileum (arrows, g); diffuse mild neutrophilic and lymphocytic infiltration in the cecum (h), moderate lymphocytic inflammation in the colon (i). Diffuse epithelial cell degeneration with ulcerative area in the ileum (g), and flattened surface epithelium with extensive areas of erosion can also be seen in the cecum and colon (h, i). (bars 100 µm).

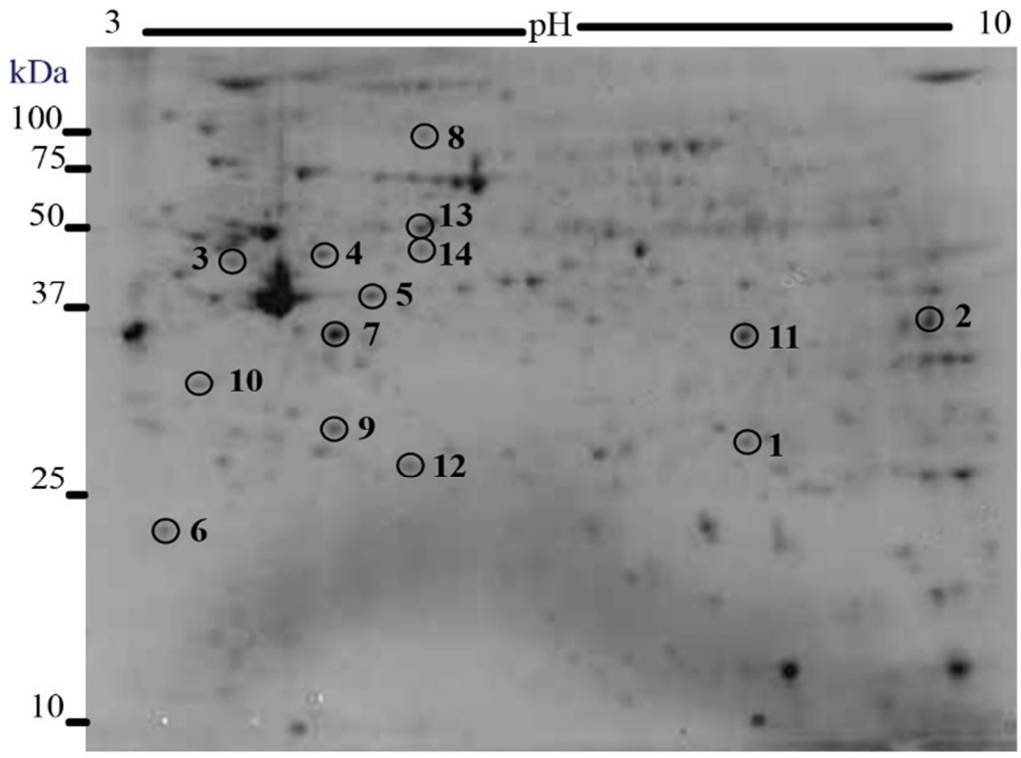

Fig. 3. Two-dimensional gel image of one individual sample of pooled piglet cecum tissue. The numbers on top of the gels indicate the isoelectric point and the numbers on the left give the position of the proteins in the molecular mass standard. Circles indicate the protein spots selected for LC/MS_MS identification. The numbers correspond to protein listed in Table 1.

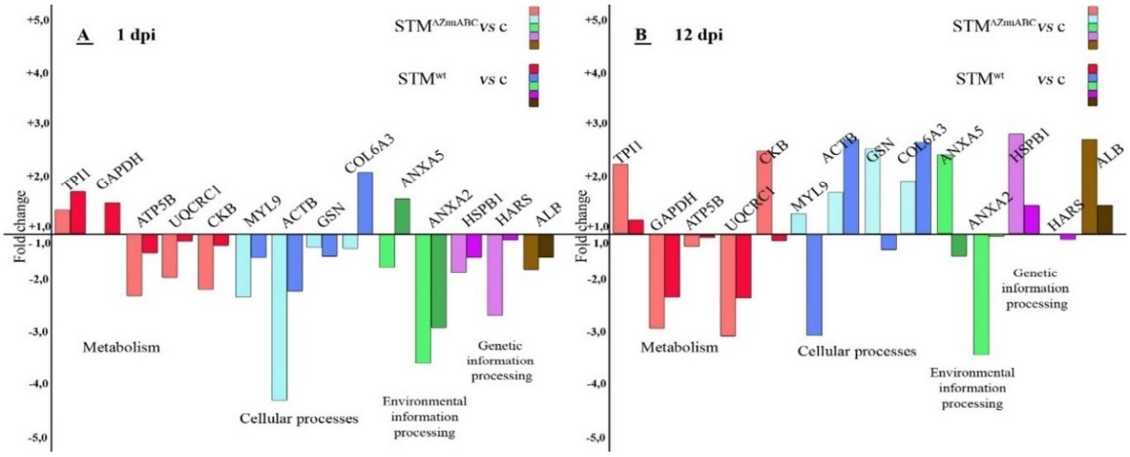

Fig. 4. Fold change of the 14 identified proteins at 1 dpi (A) and 12 dpi (B). from attenuated ($STM^{\Delta znuABC}$) and virulent (STM^{wt}) strains infected piglets, compared to uninfected piglets (c). Y axis represents the *ratio* of spot photodensity (= fold change) of infected *versus* control piglets. The *ratio* was classified as positive if it the protein concentration increased after inoculation, negative if it decreased after infection.

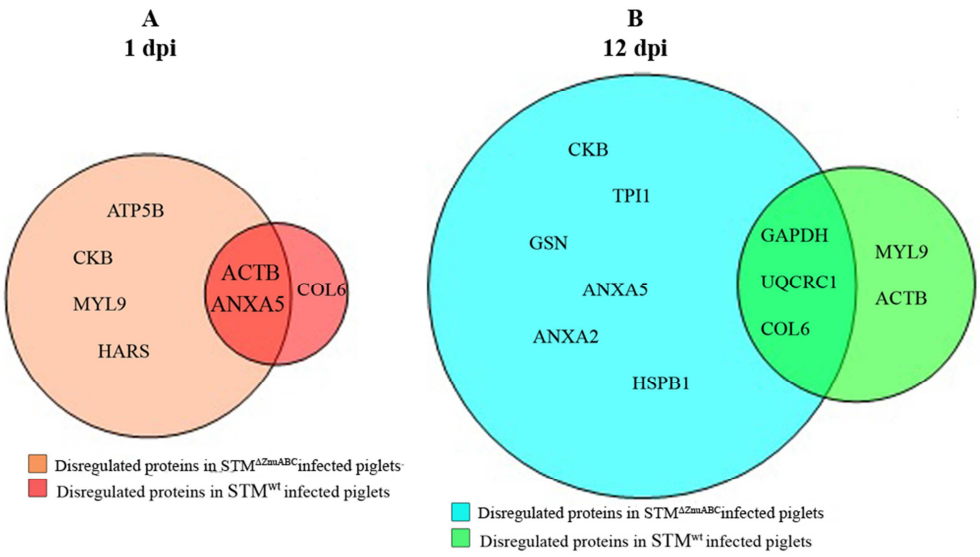

Fig. 5. VENN diagram depicts the differentially expressed proteins in cecum from attenuated ($STM^{\Delta znuABC}$) and virulent (STM^{wt}) strains infected piglets at 1 dpi (A) and 12 dpi (B). The overlapping area of the two circles contains the commonly identified proteins either in attenuated or virulent strain infected piglets at the two time-points considered.

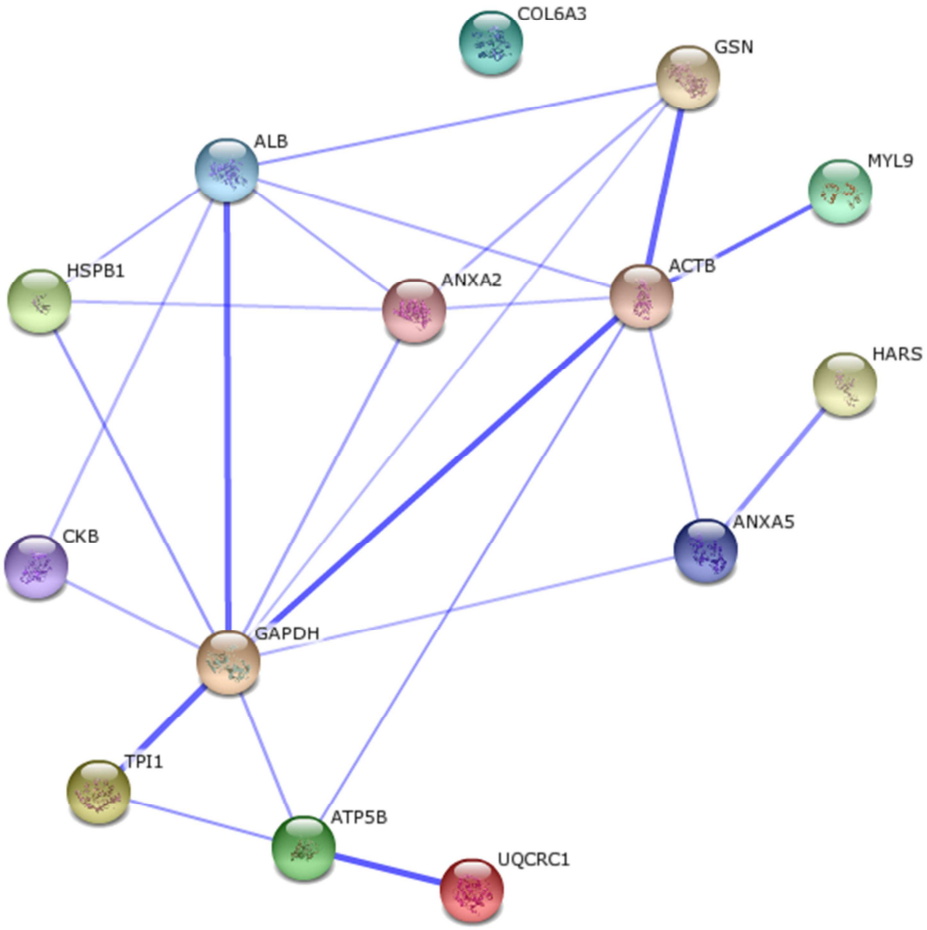

Fig. 6. Visual representation of the STRING v10 network. The figure shows interactions between the 14 differentially expressed proteins, obtained by setting *H. sapiens* as genome (by setting *S. scrofa*, only GSN was excluded). Each identified protein corresponded to a node. Solid or dotted line between nodes represents respectively a direct and indirect protein association. Stronger associations are represented by thicker lines. The network showed two tightly connected clusters, forming two functional modules. The first module included 5 proteins: GAPDH, ATP5B, TPI1, UQCRC1 and CKB, where GAPDH had a central role; the second module included 9 proteins: ACTB, ALB, GSN, MYL9, ANXA2, ANXA5, HSPB1, HARS and COL6A3, where ACTB had a central role.

Table 1

List of dysregulated proteins in the cecum of piglets infected with Salmonella attenuated strain ($STM^{\Delta ZnuABC}$) and virulent strain(STM^{wt}), at one day (1 dpi) and twelve days (12 dpi) post infection.

Spot no[a].	gi Number[b]	Mass Da[c]	pI[c]	Sequence coverage (%)	Mascot score	Name	Gene	1 dpi				12 dpi			
								$STM^{\Delta ZnuABC}$	*vs* c	STM^{wt} *vs* c		$STM^{\Delta ZnuABC}$	*vs* c	STM^{wt} *vs* c	
								Ratio[d]	*p*-value	Ratio[d]	*p*-value	Ratio[d]	*p*-value	Ratio[d]	*p*-value
Metabolism															
1	gil262263205	26879	6.54	52	489	Triosephosphate isomerase 1	TPI1	1.44	0.310	1.79	0.084	2.33	<.0001	1.25	0.336
2	gil329744642	36041	8.51	36	456	Glyceraldeyde-3-phosphate dehydrogenase	GAPDH	-1.00	0.997	1.58	0.086	-2.79	<.0001	-2.19	0.0004
3	gil89574051	45198	4.99	34	513	Mitochondrial ATP synthase H^+ transporting F1 complex beta subunit	ATP5B	-2.18	0.049	-1.34	0.328	-1.21	0.599	-1.02	0.958
4	gil335299041	53349	5.76	10	104	Cytochrome b-c1 complex subunit 1, mitochondrial	UQCRC 1	-1.83	0.102	-1.12	0.683	-2.93	0.023	-2.21	0.050
5	gil343790893	42916	5.47	7	579	Creatine kinase, brain	CKB	-2.05	0.274	-1.20	0.718	2.60	0.006	-1.10	0.855
Cellular processes															
6	gil264748	19740	4.80	7	474	Myosin regulatory light chain, LC20	MYL9	-2.20	0.279	-1.43	0.546	1.37	0.405	-2.92	0.155
7	gil60389477	42174	5.30	36	482	B-actin	ACTB	-4.18	<.0001	-2.08	0.002	1.78	00.48	2.81	0.0003
8	gil121118	85065	5.93	16	455	Gelsolin	GSN	-1.23	0.644	-1.40	0.481	2.63	0.036	-1.27	0.767
9	gil335303566	344591	6.47	3	463	PREDICTED: collagen alpha-3(VI) chain isoform 1	COL6A3	-1.25	0.629	2.18	0.012	2.00	0.266	2.75	0.064
Environmental information processing															
10	gil335293906	36169	4.94	56	715	PREDICTED: annexin 5-like	ANXA5	-1.61	0.429	1.66	0.178	2.51	0.0003	-1.40	0.359
11	gil54020966	38795	6.49	66	1155	Annexin A2	ANXA2	-3.46	<.0001	-2.77	<.0001	-3.30	0.009	-1.02	0.919
Genetic information processing															
12	gil50916342	14268	5.94	27	168	Heat shock protein 27kDa, partial	HSPB1	-1.72	0.2619	-1.43	0.4167	2.91	0.017	1.53	0.452
13	gil311250313	57837	5.79	6	157	PREDICTED: histidyl-tRNA synthetase, cytoplasmic-like	HARS	-2.54	0.054	-1.09	0.770	1.00	1.000	-1.28	0.575
14	gil164318	71348	5.92	23	676	Albumin partial	ALB	-1.65	0.229	-1.42	0.360	2.81	0.034	1.53	0.500

[a] Refers to the proteins labeled in Fig. 3
[b] NCBI accession number
[c] Theoretical value
[d] Ratio (=fold change) is classified as positive if the protein concentration increased after infection, negative if it decreased after infection

Table 2

Functional classification of dysregulated proteins in the *cecum* of piglets infected with $STM^{\Delta ZnuABC}$ and STM^{wt}.

Functional annotation	Functional cathegory	n°of input protein	% of input protein	Input protein	*p*-value	Benjamin
SP_PIR[a]	acetylation	10	71	ATP5B, TPI1, ACTB, ANXA2, ANXA5, GAPDH, HSPB1, HARS, MYL9, UQCRC1	7.1E-6	8.3E-4
	duplication	4	29	ALB, ANXA2, ANXA5,GSN	4.3E-4	2.5E-2
	phosphoprotein	12	86	ATP5B, TPI1, ACTB, ANXA2, ANXA5, GAPDH, HSPB1, HARS, MYL9, UQCRC1, GSN, CKB	7.4E-4	2.8E-2
GOTERM_CC[b]	soluble fraction	4	29	TPI1, ANXA2, ACTB, HSPB1	2.7E-3	2.8E-1
GOTERM_BP[c]	generation of precursor metabolites and energy	4	29	ATP5B, TPI1, GAPDH, UQCRC1	3.0E-3	5.9E-1
GOTERM_MF[d]	calcium ion binding	5	36	ATP5B, ANXA2, ANXA5, GSN, MYL9	1,1E-2	6.7E-1

[a]Swiss_Prot_Protein_Information_Resource;

[b]Gene_Ontology_Term_Cellular_Component;

[c]Gene_Ontology_Term_Biological_Process;

[d]Gene_Ontology_Term_Molecular_Function

Table 3

List of main functions of the dysregulated proteins in the cecum of piglets infected with *Salmonella* attenuated strain ($STM^{\Delta znuABC}$) and virulent strain (STM^{wt}).

Spot number [a]	Name	Gene	Putative functions[b]
1	Triosephosphate isomerase 1	TPI1	* glycolytic enzyme that catalyzes the interconversion dihydroxyacetone phosphate and glyceraldehyde-3-phosphate.
2	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	*glycolytic enzyme that catalyzes the convertion of D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate. Participates in nuclear events including transcription, RNAtransport, DNA replication and apoptosis. Modulates the organization and assembly of the cytoskeleton.
3	Mitochondrial ATP synthase H+ transporting F1 complex beta subunit	ATP5B	*enzyme of the respiratory chain. Produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes
4	Cytochrome b-c1 complex subunit 1, mitochondrial	UQCRC1	*enzyme of the respiratory chain. Component of the ubiquinol-cytochrome c reductase complex (complex III or cytochrome b-c1 complex), which is part of the mitochondrial respiratory chain. This protein may mediate formation of the complex between cytochromes c and c 1.
5	Creatine kinase, brain	CKB	**reversibly catalyzes the transfer of phosphate between ATP and various phosphogens (e.g. creatine phosphate). Creatine kinase isoenzymes play a central role in energy transduction in tissues with large, fluctuating energy demands.
6	Myosin regulatory light chain, LC20	MYL9	**plays an important role in regulation of both smooth muscle and nonmuscle cell contractile activity via its phosphorylation.
7	β-actin	ACTB	*beta actin is an isoform of globular actin (G-actin), the globular monomeric form. Polymerization of globular actin (G-actin) leads to a structural (F-actin) in the form of a two-stranded helix.
8	Gelsolin	GSN	*calcium-regulated, actin-modulating protein that binds to the plus (or barbed) ends of actin monomers or filaments, preventing monomer exchange. It can promote the assembly of monomers into filaments (nucleation) as well as sever filaments already formed.
9	PREDICTED: collagen alpha-3(VI) chain isoform 1	COL6A3	*is a constituent of collagen fibrils constituting of extracellular matrix, an intricate lattice that forms in the space between cells and provides a structural support.
10	PREDICTED: annexin A5-like	ANXA5	* belongs to the annexin family of calcium-dependent phospholipid binding proteins some of which have been implicated in membrane-related events. Furthermore annexins participate in regulating membrane-cytoskeleton dynamics.
11	Annexin A2	ANXA2	* belongs to the annexin family of calcium-dependent phospholipid binding proteins some of which have been implicated in membrane-related events. Furthermore annexins participate in regulating membrane-cytoskeleton dynamics.
12	Heat shock protein 27 kDa, partial	HSPB1	*is a molecular chaperones that suppress protein aggregation and protect against cell stress,
13	PREDICTED: histidyl-tRNA synthetase, cytoplasmic-like	HARS	*enzyme that charges tRNAs with the histidyne amino acids, plays an accessory role in the regulation of protein biosynthesis.
14	Albumin partial	ALB	*functions primarily as a carrier protein for steroids, fatty acids, and thyroid hormones and plays a role in stabilizing extracellular fluid volume.

[a] Refers to the proteins labeled in Fig. 3

[b] As given in *NCBI, ** UniprotKB/Swiss-Prot

Chapter 9

DISCUSSION

9.1 General discussion

S. Typhimurium is a pathogen of great clinical relevance, representing one of the principal causes of food borne illness. The disease is characterized by a severe inflammation of the intestinal epithelium associated with a significant neutrophils influx into intestinal lumen, resulting in a usually self-limiting enterocolitis. Despite a marked host immune response and the altered environmental conditions encountered by the pathogen in the inflamed gut, *S.* Typhimurium can colonize intestine to high number (Behnsen et al., 2015).

9.1.1 *S.* Typhimurium promotes an active infection exploiting LPS-induced inflammation

Inflammation plays a key role in the host response aiming to reduce the colonization and eventually eliminate pathogens. A growing body of evidences about *S.* Typhimurium draw a fascinating picture of a pathogen that is able to survive in the inflamed gut and to exploit inflammatory response gaining a competitive advantage over the intestinal microbiota (Borewicz et al., 2015).

Salmonella inflammation-adapted lifestyle was driven by acquisition of new genetic traits by horizontal gene transfer at distinct evolutionary times. Approximately 100-140 million years ago, the genus *Salmonella* acquired SPI-1 encoding for the T3SS-1, essential for the invasion of intestinal epithelium, after its separation from the related *Escherichia coli* lineage. Later on, 70-100 million of years ago *S. enterica* genus separeted from *S. bongori* and acquired SPI-2, whose

genes are required for replication and survival (Vernikos et al., 2007; Santos et al., 2009). The successful assimilation of virulence traits through SPI-1 and SPI-2 enabled *Salmonella* serovars to trigger intestinal inflammation and to thrive in the inflamed intestine (Santos et al., 2009).

It is known that there are many differences between vertebrate hosts regard the response to *S.* Typhimurium (Santos et al., 2001). Most of the studies about the central role played by inflammation on the fitness of *S.* Typhimurium have used mice for experimental infections, including streptomycin-treated mouse model characterized by an altered microbiota (Stecher et al., 2007; Liu et al., 2012). However, both the inability of mouse to reproduce gastroenteritis and the lack of an intact microbiota, make instrumental the utilization of a more suitable animal models for advancing in our comprehension on *Salmonella* pathogenesis. For this reason, we used pig, the natural host of *S.* Typhimurium, as model for salmonellosis in our whole work.

In Chapter 6 of this thesis, we sought to explore if inflammation favors an active infection of *Salmonella* (STM14028) using an *in vivo* pig model and *in vitro* porcine cell lines. In both cases, inflammation was induced by LPS, which is known to be an activator of immunity and a potent stimulator of pro-inflammatory cytokines production (Meng & Lowell, 1997).

- *In vivo* experiment has shown the induction of an early host response post STM14028 infection in LPS-treated piglets; the increased cytokines level and body temperature registered already at 4 hours after infection, are consistent with the acute inflammatory stimulus induced by LPS. This data is confirmed by the observation that LPS-no treated piglets reach relevant inflammation level only at 48 h post infection (Fig. 3 and Fig. 4, Chapter 6).

LPS treatment enhances also the progression of *S.* Typhimurium, making the pathogen more capable to colonize at higher numbers either the gastrointestinal tract and systemic organs (Fig. 5, Chapter 6). This result agrees with previous studies carried out on mice, showing that the administration of LPS can exacerbate the growth of *S. enterica* (Hormaeche, 1990). LPS-induced inflammation, as well as that induced by chemical treatments or by infectious agents themselves, breaks the balance intestinal determining a profound perturbation of microbiota, which in turn creates the favorable conditions for *S.* Typhimurium colonization (Lupp et al., 2012).

- Similarly, our *in vitro* experiments have revealed an increased STM14028 colonization, at 3h and 24h after infection, in LPS-pretreated porcine monocytes/macrophages and IPEC-J2 cells. In contrast, when LPS stimulation was inhibited using its natural antagonist, the RS-LPS, cells showed a reduced colonization with values similar to the ones of LPS untreated cells (Fig. 2A-D, Chapter 6). These data suggest that LPS is able to facilitate the intracellular penetration of *S.* Typhimurium in both enterocytes (IPEC-J2) and monocytes/macrophages cells.

  Previous studies conducted by using intestinal epithelial cells (Caco-2) monolayers have shown that LPS has no direct effects on the enterocytes structure (Wells et al., 1993; Yeung et al., 2013); thus, the LPS-induced bacterial uptake has to involve mechanisms other than an increased ruffling and micropinocytosis. On the contrary, we can speculate that LPS might promote TLR4-mediated internalization. Neal et al. (2006) have demonstrated that human endothelial kidney (HEK)-293 cells, which lack of TLR4, are not able to promote the bacterial uptake; however, they acquired a phagocytic phenotype following transfection with functional TLR4, indicating the important role of TLR4-associated internalization (Neal et al., 2006).

In monocytes/macrophage, bacteria are internalized through phagocytosis. The high colonization level by *S.* Typhimurium observed in monocytes/macrophages cells could seem an unexpected result, given their phagocytic function. Actually, *S.* Typhimurium, as well as many facultative intracellular pathogens, has developed mechanisms to escape macrophages killing and to favor intra-macrophage multiplication (Belon et al., 2015). For example, *Salmonella* exploits TLRs activation on macrophages to establish a successful infection. In fact, the maturation and the acidification of the *Salmonella* containing vacuole (SCV) via TLR, is required to induce SPI-2 genes, which are involved in survival and replication of the pathogen (Arpaia et al., 2012).

9.1.2 *S.* Typhimurium modifies microbiota in a virulence-dependent manner

It is well known that *Salmonella* infection consists in an interactive tripartite relationship between pathogen, host response and the resident microbiota. The intestinal microbiota is involved in the maintaining of intestinal homeostasis through a balanced symbiosis with the host, and contributes to many biological functions including the resistance to pathogens invasion (Stecher et al. 2007). *S*. Typhimurium utilizes specific virulence factors for inducing the inflammatory host response, which, in turn, modifies microbiota composition breaking colonization resistance and favoring the pathogen colonization (Stecher et al. 2007).

In Chapter 7, we investigated if and how virulence of *S*. Typhimurium influences the shift of porcine intestinal microbiota composition upon experimental infection. For this purpose, we compared the effects of two different strains of *Salmonella*, a wild type and an attenuated strain deleted of the whole *znuABC* operon. Our data suggest a positive correlation among *Salmonella* virulence, the degree of intestinal inflammation and the colonization. We saw that *S*. Typhimurium

wild type induces a robust inflammatory response and results in a major colonization in gut; on the contrary, the attenuated strain causes a milder inflammation and is associated with a reduced organs colonization (Fig. 2, Fig. 4 Fig. 5 and 9, Chapter 7).

The different virulence of the two strains is also reflected in a different alteration of the microbiota composition. The analysis of microbiota has showed the reduction of SCFA-producing bacteria (*Ruminococcaceae* and *Clostridium* genera) and lactate-fermenting bacteria (*Megasphaera*), and the increases of lactic acid-producing bacteria (*Lactobacillus* genus) in *S*. Typhimurium wild type-infected piglets. These alterations of microbiota could explain the high colonization level of the fully virulent strain of *Salmonella*. In fact, our data are in agreement with previous studies which have revealed that the depletion of *Ruminococcaceae* and the increase of *Lactobacillus* make the host more susceptibile to enteric infections (Antharam et al., 2013; Ling et al., 2014). It seems, in fact, that the alteration of these bacterial genera may cause the dysfunction of epithelial intestinal barrier and the increase of osmotic load in the intestinal lumen, determining diarrhea (Antharam et al., 2013). It is well known, in fact, the protective effect of SCFAs in counterbalancing intestinal dysbiosis (Gantois et al., 2006; Sokol et al., 2008). We have also observed a reduction of lactate-fermenting bacteria such as *Megasphaera*. It can be speculated that this alteration may act in parallel with the increase of *Lactobacillus* genus, contributing to the accumulation of lactic acid/lactate and thus, to the increase of osmotic pressure in the gut.

In contrast, the increase of SCFA-producing and lactate-fermenting bacteria observed in *S*. Typhimurium attenuated-infected piglets, may contribute to enhance intestinal defense barriers allowing to the microbiota to successfully compete with the pathogen. Taken together, the increased SCFAs production and the impossibility

of *S*. Typhimurium attenuated to acquire zinc in the environment, could explain the reduced ability of this strain to colonize host.

Overall, our data show that *S*. Typhimurium infection modifies specific members of microbiota, the SCFAs- and lactate-producing/fermenting bacteria, which are key determinants of colonization resistance against invading pathogen. These changes seem to be related with the virulence of *S*. Typhimurium. We can conclude that there is a direct relationship among virulence, inflammation and microbiota composition; the degree of virulence of *Salmonella* strains influences the intestinal inflammation, determining the ability of *Salmonella* to compete with the microbiota in order to establish infection.

9.1.3 **Effects of *S*. Typhimurium infection on the porcine cecal mucosa**

The studies about the host-pathogen functional interface support our understanding of *Salmonella* complex pathogenesis. The using of high throughput technologies has allowed to detect global dynamics that occur in the host during infection (Rodland et al., 2008).

In Chapter 8, we have sought to capture the pathophysiological alterations induced by wild type and attenuated *S*. Typhimurium strains in the porcine cecum, through the utilization of a proteomic approach.

Our findings have shown a modulation of the normal host functions upon *Salmonella* infection; metabolism, cellular processes, environmental and genetic information processing are the most significantly perturbed biological functions (Table 1, Chapter 8). In particular, the identified proteins belonging to metabolism are involved in oxidative phosphorylation and gluconeogenesis/glycolysis which are crucial pathways for the production of nutrients and energy. In addition, it is also observed the perturbation of an enzyme involved in protein synthesis. Overall, their

downregulation suggests a reduction of metabolic and energetic status of the host that may be seen as a defense strategy against the nutrient theft by *Salmonella*. In response to *S*. Typhimurium, host could adopt a "nutriprive mechanism" in which deprives environment of nutrients, creating intestinal conditions that are detrimental for *Salmonella* growth. This hypothesis is in agreement with the study of Appelberg (2006) that demonstrates that in addition to the microbial killing via ROS/RNS, macrophages exert their antimicrobial activity also preventing access to essentials nutrients and growth factors for the pathogen survival (Appleberg, 2006).

At the same time, it is well known that during infection, *S*. Typhimurium itself, interferes with and modulate the metabolic processes of the host trying to steal nutrients for its bioenergetic and biosynthetic requirements (Eisenreich et al., 2008). This evidence could be useful to explain our data regarding the minor alteration of metabolism proteins after infection with *Salmonella* wild type in comparison with the attenuated strain. Probably, thanks to its pathogenicity, *Salmonella* wild type would be more capable to oppose to the nutriprive mechanism of the host and to modulate metabolic responses.

Our findings have also shown that cytoskeleton related proteins are another biological category significantly downregulated by *S*. Typhimurium infection (Fig. 4A, Chapter 8). This biological category includes structural and regulatory proteins involved in the maintaining of the cellular integrity and in cell-cell adhesion. In line with data present in literature (Collado-Romero et al., 2012; Martins et al., 2012; Arce et al., 2014), we can speculate that their modulation is probably associated with functions, such as cytoskeleton rearrangements and destruction of membrane integrity, requested for a successful *S*. Typhimurium invasion.

Moreover, later during infection, the function of the most of proteins seem to be restored especially in *Salmonella* attenuated-infected piglets (Fig. 4B, Chapter 8), suggesting that the infection is being resolving.

Finally, our general pattern reveals the global effects of *Salmonella* infection on the porcine cecum contributing to shed light on the molecular mechanisms used by the pathogen to cause disease.

9.2 Conclusions

In this study we investigated the interactions among *S*. Typhimurium, the vertebrate host and the resident microbiota.

Using piglet as model for salmonellosis, we demonstrated that:

- *Salmonella* induces a host inflammatory response which is correlated with its degree of virulence;
- intestinal inflammation is actively exploited by the pathogen to promote its own intestinal colonization;
- the degree of inflammation could contribute to the progression and to the outcome of salmonellosis through the modulation of microbiota;
- proteomic analysis of the intestinal mucosa response to *S*. Typhimurium has shown a significant modulation of the normal host functions, metabolic pathway and cytoskeleton-involving processes in particular;
- in response to *S*. Typhimurium, host seems to use a "nutriprive" mechanism to reduce the availability of nutrients and energy in the intestine inflamed and to inhibit *Salmonella* infection.

Overall, we can conclude that the interaction among *S*. Typhimurium, the host and intestinal microbiota represents one of the most dynamic and intricate biological

system existing in nature. The outcome of each infection is the result of this interactive tripartite relationship.

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RESEARCH EXPERIENCES

School of Veterinary Medicine and Science - University of Nottingham, Sutton Bonington Campus, UK September-December 2013. Investigation of the swine immune response to the oral administration of a live attenuated strain of *Salmonella enterica* serovar Infantis by monitoring the clinical conditions of the animals (body temperature, fecal shedding) and by the use of histological and immunological assays. It was also evaluated the ability of the same strain to induce protection against infection with a virulent strain of *Salmonella enterica* serotype Choleraesuis and / or Typhimurium.

AWARDS

Winner of a fellowship for a Short-Term Scientific Missions provided by MedVetNet Association. 1st February- 1st April. Institute for Microbiology, Department of Infectious Diseases, University of Veterinary Medicine Hannover (TiHo). Hannover (Germany). Acquisition of the technique of isolation of bone marrow cells and their differentiation into dendritic cells. Study of the interaction of dendritic cells with different strains of *Salmonella enterica* serovar *Typhimurium*.

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ERRATA CORRIGE

The present document integrated the PhD thesis of Drumo Rosanna, entitled: "Study of host-pathogen-microbiota interactions on a *Salmonella enterica* serovar *Thyphimurium* piglet model". 2016. Scuola di Dottorato di Ricerca in: Scienze Veterinarie. Università degli Studi di Padova.

Chapter 1

- **Page 9, line 2:** ***At the end of the line, add*** "(Singh, 2013)."
- **Page 13, line 4:** ***Instead to*** "(Hugas and Beloeil, 2014)" ***insert*** "(Hoelzer et al., 2011)".
- **Page 13, line 11:** ***After*** "they include pork meat, poultry, eggs, raw milk and dairy products" ***add*** "(Hoelzer et al., 2011)".
- **Page 13, line 11:** "In recent years, the role of food of vegetable origin, as potential vehicles of gastrointestinal infection, has been highlighted" ***should be replaced with*** "Recently, the role of food of vegetable origin (most commonly salads and tomatoes), as potential vehicles of *Salmonella* for humans, has been highlighted (Freitas Neto et al., 2010)."

Chapter 2

- **Page 15, line 7:** ***After*** "Cathelicidins are also cationic proteins that act as potent lipopolysaccaride (LPS)- neutralizing factors" ***add*** "(Patel & McCormick, 2014)"
- **Page 15, line 8:** ***After*** "Lactoferrin is a multifunctional protein that functions sequestering iron and destabilizing microbial membranes" ***add*** "(Legrand et al., 2008)".
- **Page 17, line 8:** ***Instead to*** "(Broz et al., 2012)" ***insert*** "(Godinez et al., 2008)".

Chapter 9

- **Page 126, line 8:** ***Instead to*** "(Lupp et al., 2012)" ***read*** "(Lupp et al., 2007)".
- **Page 127, line 10:** ***Instead to*** "(Arpaia et al., 2012)" ***read*** "(Arpaia et al., 2011)".
- **Page 130, line 11:** ***Instead to*** "(Eisenreich et al., 2008)" ***read*** "(Eisenreich et al., 2013)".
- **The following references should be added to the "References" section (section 9.3, Pag.133):**

Freitas Neto OC, Penha Filho RAC, Barrow P, Berchieri Junior A. Sources of human non-typhoid Salmonellosis: a review. *Brazilian Journal of Poultry Science*. 2010. 12(1): 1-11.

Godinez I, Haneda T, Raffatellu M, MD, Paixão TA, Rolán HG, Santos RL, Dandekar S, Tsolis RM, Bäumler AJ. T cells help to amplify inflammatory responses induced by *Salmonella enterica* serotype Typhimurium in the intestine. *Infection and Immunity*. 2008. 76(5): 2008–2017. doi: 10.1128/IAI.01691-07.

Legrand D, Pierce A, Elass E, Carpentier M, Mariller C, Mazurier J. Lactoferrin structure and functions. *Advances in Experimental Medicine and Biology.* 2008. 606:163-94.

- **The following reference should be removed from the "References" section (section 9.3), Pag.137, line 18:** "Hugas M, Beloeil P. Controlling Salmonella along the food chain in the European Union - progress over the last ten years. *Euro Surveillance*. 2014. 19(19). pii 20804".