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Study of *Salmonella* pathogenicity mechanisms *in vitro* and *in vivo*

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Abstract

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Abstract

Salmonella enterica subsp. *enterica* includes many pathogenic serovars. Models of the study are *S.* Typhimurium and *S.* Abortusovis, a restricted serovar to sheep.

Bacterial pathogen's transmission strategies are usually connected to pathogenesis and limited information is available about the immune response of sheep to *S.* Abortusovis and about flagellin interaction with *S.* Abortusovis. Knowledge about those factors are mainly based on studies of *S.* Typhimurium.

Here we try to understand the role of flagella in *S.* Abortusovis. The idea is that *S.* Abortusovis highly regulates expression of flagella within the host, and we performed studies to clarify how *S.* Abortusovis evade activation of the immune system.

Mutations in *S.* Abortusovis flagella genes were generated and flagella from wild type and mutant's strains of the two different serovars were extracted using diverse media to observe differences in flagellation and host interaction. Samples were analyzed by Western Blot to determine expression of major flagellar protein. Surface expression of flagella was verified by Flow Cytometry and by Scanning Electron Microscopy. To verify the capacity of *S.* Typhimurium and *S.* Abortusovis to stimulate TLR5, a

colorectal carcinoma cell line (T84 cells) was treated with purified flagellin and the transcriptional induction of a CXC chemokine IL-8 was measured.

We showed that *S.* Abortusovis flagella are expressed in specific media, and they induced the transcription of IL-8 in T84 cells.

1. Summary

Pathogenic bacteria have various levels of host specificity. While many bacteria, such as *Salmonella* Typhimurium and *Pseudomonas* aeruginosa can infect a wide range of host, certain bacteria have strict host selectivity. For example, *Neisseria* gonorrhoeae is host restricted to humans, *Escherichia coli* K-88 is specific for pig and *Salmonella* Abortusovis is specific for sheep. This raises the question, which molecular mechanisms underlie host restriction?

In this work *Salmonella enterica* was used as model organism since members of the species *Salmonella enterica* are very closely related on a genetic level, but exhibit differences in host specificity.

Models of this study are a broad host range *Salmonella enterica* subspecies *enterica* serovar Typhimurium, and a highly host restricted serovar *Salmonella enterica* subspecies *enterica* serovar Abortusovis. *S.* Abortusovis infection can seriously damage a sheep-based economy, such as that of Sardinia. *Salmonella* Abortusovis is restricted to ovine and does not naturally infect humans. This serovar causes abortion

in sheep. This is an important health problem in the Mediterranean area, where the sheep industry has a significant economic impact[1].

Previous work comparing adapted and non-adapted *Salmonella* serotypes suggests that persistence of host-adapted serotypes such as *Salmonella* Abortusovis could be based on circumvention of immune-based protective response[2] with the ability to persist at systemic sites[3]. Host adaptation of several serotype such as *S*. Typhi, *S*. Gallinarum, *S*. Dublin and *S*. Pullorum appears to have coevolved with loss of the intestinal lifestyle and the acquisition of the ability to cause systemic infection. Bacterial pathogens' transmission strategies are usually connected to pathogenesis, and host responses are frequently typical for groups of pathogens, rather than being specific to individual pathogens[4] (Figure 1).

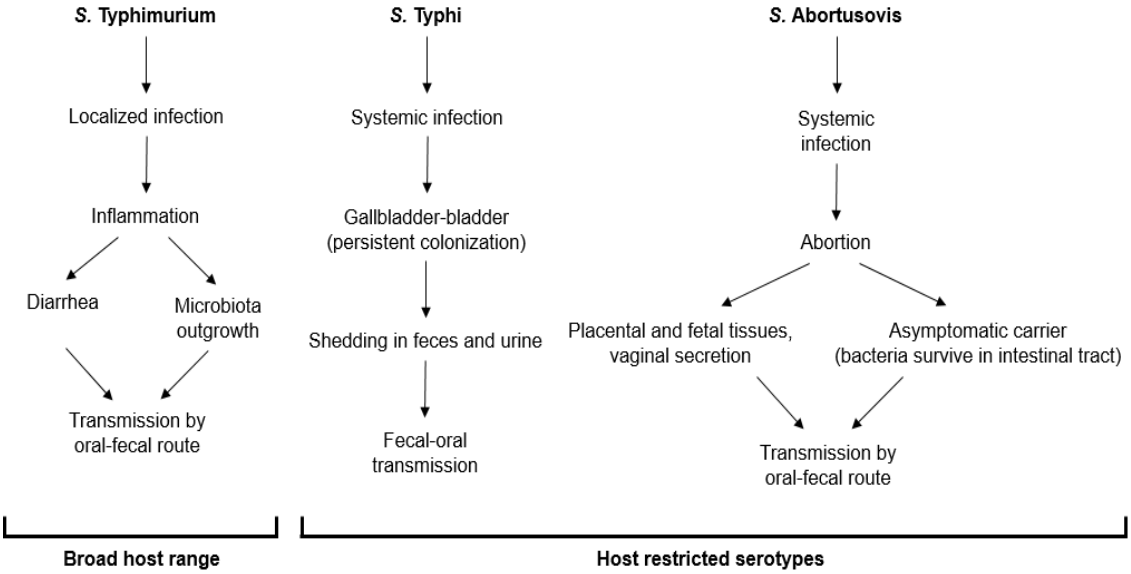

Figure 1. Different transmission strategies in different *Salmonella* serotypes.

See text for details.

In *Salmonella* Typhimurium, bacterial flagellin and bacterial LPS are respectively recognized by TLR5 and TLR4, causing the generation of CXC chemokines in a process that has been implicated in neutrophil influx in the intestinal mucosa during infection[5]. Recently, studies have shown that *S.* Typhi, in contrast to *Salmonella* Typhiumurium, evades the recognition of TLR4 and TLR5. The regulatory protein TviA represses the expression of flagella and the Vi capsular polysaccharide represses the expression of the O-antigen. Through these mechanisms, *S.* Typhi evades receptor recognition and the host responses[6].

However, limited information is available about the immune response in sheep to *S*. Abortusovis. *S.* Abortusovis, like *S*. Typhi, causes a systemic infection which the host is not able to mount a prompt immune response to control infection before *Salmonella* Abortusovis reaches the uterus and causes abortions[7]. Recently, it was shown that LPS of *Salmonella* Abortusovis is not able to induce the expression of the CXC chemokines, like IL-8, suggesting that this serotype is able to evade the TLR4 recognition[8].

Very little is known about flagellin interaction with *Salmonella* Abortusovis. The importance of flagella in host pathogen interaction is mainly based on studies of *S.* Typhimurium (Figure 2).

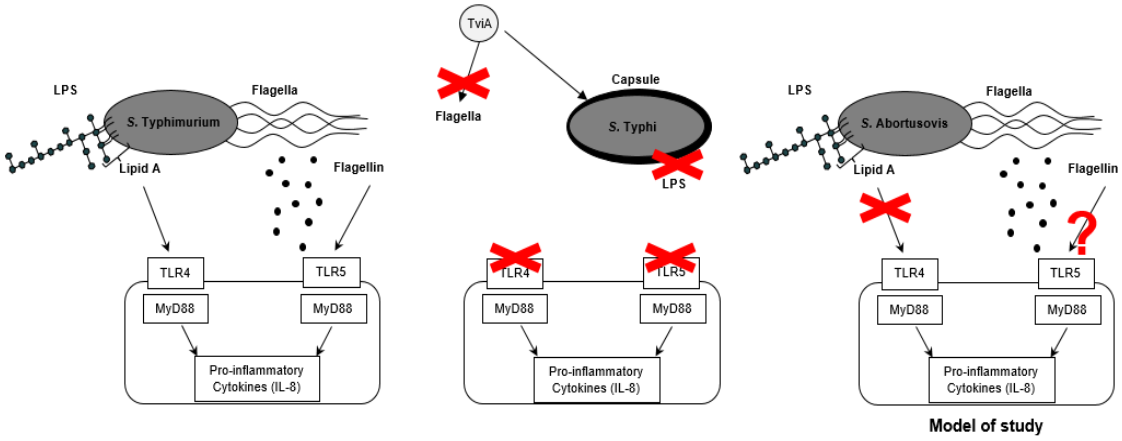

Figure 2. Specific host responses for different *Salmonella* serotypes.

See text for details.

In this work we perform pioneering work to understand the role of flagella in *S.* Abortusovis.

Our central hypothesis is to study how *Salmonella* Abortusovis evade activation of the immune system. To this end, we compared differences between *Salmonella* Typhimurium and *Salmonella* Abortusovis in recognition by TLR5 in *vitro* and in *vivo*. Furthermore, we studied the major bacterial adaptive immune response as the principal target for the humoral antibacterial response during the infection of the host.

The idea is that *Salmonella* Abortusovis highly regulates expression of flagella during infection.

Previous work of studying *Salmonella* Abortusovis flagella *in vitro* had been difficult since flagella are not readily expressed under standard laboratory conditions. In this work we were able to identify a unique *in vitro* growth condition to express *Salmonella* Abortusovis flagella *in vitro* for the first time.

Expression of flagella *in vitro* allowed us to study flagella expression in *Salmonella* Abortusovis. To this end we generated defined mutations in *Salmonella* Abortusovis flagella genes by transducing mutations that had been previously characterized in

Salmonella Typhimurium. We proceeded with the extraction of flagella from wild type and mutant's strains of the two different serovars using diverse media to observe differences in flagellation and host interaction. We analyzed our samples with SDS Page and Western Blot to determine expression of major flagellar protein. Also, we verified surface expression of flagella by Flow Cytometry and by Scanning Electron Microscopy (SEM). These different techniques enabled us to establish a media suitable for inducing the expression of flagella in *Salmonella* Abortusovis, allowing us to study *Salmonella* Abortusovis flagella *in vitro* for the first time.

To verify the capacity of *S*. Typhimurium and *S*. Abortusovis to stimulate TLR5, a colorectal carcinoma cell line (T84 cells) was treated with purified flagellin and the transcriptional induction of a CXC chemokine, IL-8, was measured in response to the bacterial stimulation.

The fact that the expression of flagella only occurs in a particular media suggests that *Salmonella* Abortusovis is able to control expression of flagellin to modulate activation of immune response.

2. Introduction

2.1 *Salmonella*

Salmonella is an enteric pathogen of the *Enterobacteriaceae* family. *Salmonella* is responsible for salmonellosis causing significant morbidity and mortality in humans and animals with a socio-economic impact felt around the world.

The name *Salmonella* derived from Daniel Salmon's name, a veterinary microbiologist who first isolated *Salmonella* Choleraesuis from the intestine of a pig. *Salmonella* is a Gram-negative facultative anaerobic bacterium. Motility is mediated by the presence of peritrichous flagella.

The genus *Salmonella* consists of 2 species, *Salmonella enterica* and *Salmonella bongori. S. enterica* represents a group of 6 subspecies with multiple serovars[9] (Table 1).

Species	Subspecies number	Subspecies name	Number of Serovars
S. enterica			2557
	I	*enterica*	1531
	II	*salamae*	505
	IIIa	*arizonae*	99
	IIIb	*diarizonae*	336
	IV	*houtenae*	73
	V	*indica*	13
S. bongori			22
	Total number of serovars		**2579**

Table 1. *Salmonella* subspecies and serovars.

The different serotypes of *Salmonella* can be divided into two groups, host-specific and non-host specific. The former are those that can infect a single animal species; the latter, however, are able to survive within more species[1] (Table 2).

Serovars	***Host***	***Other Host***
Choleraesuis	pig	human
Dublin	cattle	human, sheep
Typhimurium	birds, mammals	human
Typhi	human	_
Paratyphi A	human	_
Paratyphi C	human	_
Sendai	human	_
Abortusovis	sheep	_
Gallinarum	chicken	_
Typhisuis	pig	_
Abortusequi	horse	_

Table 2. *Salmonella* classification based on host specificity.

2.2 *Samonella enterica*

Salmonella enterica is divided in 6 subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *indica* and *houtenae*.

Salmonella enterica subspecies *enterica* causes disease in vertebrates and is transmitted through ingestion of contaminated food and water[10].

2.3 Host specificity

Salmonella can be divided into two groups, host-specific and broad host range serovars. Curiously, host specific serovars cause systemic disease in their respective host species. For example, *S*. Gallinarum causes fowl typhoid in chicken, *S*. Dublin causes a systemic diseases in cattle, *S*. Choleraesius and *S*. Typhisuis cause septicemic and enteric salmonellosis in pigs and *S*. Abortusovis causes abortion in sheep.

In human, specific serovar can cause severe systemic forms like typhoid in the case of *S*. Typhi and paratyphoid with *S*. Paratyphi A, B and C[11]. In contrast, *Salmonella* broad host range serovar are able to infect different host species, from mammals to birds,

and reptiles, causing infections of varying severity depending on the serotype, the mode of infection, and host characteristics[12].

Salmonella Dublin and *Salmonella* Choleraesuis are non-specific serovars which cause serious systemic diseases in cattle and pigs, but can also cause disease in other mammalian hosts including humans[13, 14].

Serotypes like *S*. Dublin and *S*. Choleraesuis represent those microorganisms that are prevalent in a particular species, but they can also cause disease in other host.

So it is important underscore the difference between host-specifc and host-restricted serovars. The first group includes serovars which cause severe systemic infection in their preferred host, but are usually excreted without any clinical symptoms in accidental hosts.

In the second group are serovars which are restricted to one specific host. Furthermore they exclusively cause systemic infection, which often proves to be fatal for their host[15].

The two serotypes commonly associated with human salmonellosis are *S*. Enteritidis and *S*. Typhimurium; these pathogens are able to cause disease in many mammals, including man[1].

2.3.1 *Salmonella enterica* Typhimurium

S. Typhimurium is an enteric pathogen which cause diarrhea and gut inflammation and during inflammation neutrophils are found in fecal samples[16].

Salmonella enterica subspecies *enterica* serovar Typhimurium (*S.* Typhimurium) is one serovar most widely associated with cases of human infection. Disease symptoms usually develop after between 12 and 72 hours after infection in humans and the transmission is oral-fecal, through the ingestion of contamined food or water. Infected individuals develop gastroenteritis, fever, abdominal cramps, and diarrhea. Infection typically lasts between 4 and 7 days and the majority of people recover without the need for medical intervention[17].

Once ingested, the bacteria travel through the stomach to the mucosa of the intestine where it actively invades. Two type III secretion system, termed type III secretion

system-1 (T3SS-1) and type III secretion system-2 (T3SS-2) are required for causing inflammation. Employing the T3SS-1 *Salmonella* Typhimurium, invades the intestinal epithelium, and it is able to survive in mucosal macrophages using T3SS-2[18].

Initially, following the invasion of intestinal mucosa, the predominant influx cells were the polymorphonuclear leukocytes (PMN)[19, 20].

S. Typhimurium is able to induce the apoptosis of PMN[21], causing the production of pro inflammatory cells and damage in the intestinal mucosa[22].

Recently, it has been better understood an important mechanism of invasive capacity of *Salmonella* Typhiumurium. The activation of gut inflammation is an important activity for *Salmonella* to enhance colonization of the gastrointestinal tract. In fact, *Salmonella* can use oxidation products that become available in this environment for respiration.

During the growth of the microbiota in the gut there is the formation of hydrogen sulfide, a toxic product that is converted by oxidation in thiosulfate by the enterocytes[23, 24].

Thiosulfate is able to interact with reactive oxygen species (ROS), generate in the intestinal lumen during inflammation by neutrophils, oxidizing thiosulfate in tetrathionate.

The genes that allow *Salmonella* to use tetrathionate as an electron acceptor, enable this pathogen to outgrowth the normal microbiota. The outgrowth in the lumen on the intestine permit the transmission of *S.* Typhimurium[25] (Figure 1).

Under anaerobic conditions, tetrathionate respiring *S*. Typhimurium is able to use fermentation end products, which results in a substantial selective advantage for this pathogen.

Salmonella Typhimurium is then able to grow and proliferate in competitive habitats such as the intestine, since it is able to exploit the environment and substances produced by the host[26].

2.3.2 *Salmonella enterica* Typhi

Salmonella enterica subspecies *enterica* serovar Typhi (*S*. Typhi) is the causes of typhoid fever and results in more than 200,000 annual deaths[27].

S. Typhi is a highly adapted human-specific pathogen, usually contracted by ingestion of food or water contaminated by fecal or urinary carriers excreting *S.* Typhi[28].

Unlike other *Salmonella* serovars, which typically cause gastroenteritis characterized by a massive neutrophil influx in the intestinal mucosa, *S.* Typhi causes a systemic disease in which neutrophils are scarce in intestinal mucosa[29].

The bacterium is serologically positive for the polysaccharide capsular antigen Vi which is largely restricted to *S.* Typhi, and Vi-negative strains of this serovar are less infectious and less virulent than Vi-positive strains[27].

Initial colonization and proliferation is not associated with other disease symptoms, suggesting that *S.* Typhi evades induction of immune response during infection.

S. Typhi must survive the gastric acid barrier to reach the small intestine, and a low gastric pH is an important defense mechanism, in fact expression of *tviA*, which is located in the viaB locus of *S.* Typhi, is activated by low tissue osmolarity when the pathogen enters the intestinal mucosa.

The viaB locus has recently been implicated in preventing the generation of host responses in intestinal epithelial cells that lead to neutrophil influx[30].

The mechanism through which the viaB locus reduces IL-8 production in human intestinal epithelial cells is through the modification of the expression of Vi-capsular antigen of TLR ligands on the bacterial cell surface, in fact the regulatory protein TviA activates expression of the virulence-associated (Vi) capsular polysaccharide and represses the expression of flagella. Furthermore activation of complement by the *S.* Typhi O-antigen is obstructed by expression of the Vi capsular polysaccharide, which attenuates neutrophil recruitment[6].

Through these mechanisms, *S.* Typhi prevents initial activation of several pattern recognition receptors that contribute to host responses against non-typhoidal *Salmonella* serovars.

Following entry into the small intestine, the bacteria cross the intestinal epithelial barrier, and then are phagocytosed by macrophages and spread systemically, producing acute disease.

The most common sites of infection are the ileum, liver, spleen, bone marrow and gall bladder. The bacteria reach the gall bladder through the vasculature or the ducts that emanate from the liver[31].

S. Typhi persists in the gall bladder through invasion of gall bladder epithelial cells. Invasive bacteria replicate intracellularly, and shedding could occur as a part of epithelial regeneration. Gall bladder epithelial cells containing *S.* Typhi would be extruded to the lumen, and released bacteria could infect new cells or be shed into the intestine via bile[32].

Human carriers shed *Salmonella* Typhi in their feces, which can then contaminate food and beverage, continuing fecal-oral transmission (Figure 1).

2.3.3 *Salmonella enterica* Abortusovis

Salmonella enterica subspecies *enterica* serovar Abortusovis (*S.* Abosrtusovis) is a host specific serovar. It is restricted to sheep and does not cause any infection in humans. *Salmonella* Abortusovis causes abortion in sheep which is an important health problem in European countries where sheep-farming is the base of the economy. Salmonellosis caused by *Salmonella* Abortusovis is common in France, Spain, Germany, Switzerland and Italy[33]. *Salmonella* Abortusovis is very common in Sardinia, which it breeds more than half of all sheep in Italy.

The damage, both direct and indirect, includes the loss of lambs due to abortions and loss of milk[34].

Salmonella Abortusovis is introduced into a flock by an asymptomatic carrier[35]. When *S.* Abortusovis is introduced for the first time in a flock, abortion becomes epidemic; in later years the infection becomes endemic with sporadic abortions that occur most often in young sheep or new sheep introduced into the flock. In the areas of endemicity, abortion occurs in 30 or 50% of sheep in a flock, generally during the first pregnancy and mainly during the last 2 months of gestation (a total of 5 months), through an unknown mechanism[36]. Furthermore, after the first infection sheep develops a protective immunity against *Salmonella* Abortusovis[37].

The infected animals do not display other symptoms of diseases, in fact the only symptoms is the abortion that usually occurs in the second half of the gestation.

Abortion occur several weeks after infection without any other symptoms before, and sometimes is lethal for the sheep due to placental retention. Furthermore, lambs may also be stillborn or die within a few hours of birth from septicemia. Sometimes, lambs

appear to be healthy but die within 3 weeks; some of them have diarrhea or symptoms of pulmonary infections[33].

During necropsy of infected lambs it is common to observe edema, hemorrhagic sites and necrosis of different organs[34].

After the abortion bacteria can be isolated from placental and fetal tissues as well as from liver, spleen, brain and stomach, suggesting that those are the principal site of multiplication. After 2-4 weeks is possible isolate bacteria from the vaginal secretion of sheep[38].

After a short period of bacteremia, where some organs like liver, spleen and lungs are colonized, bacteria can be expelled by the host or they can survive in the intestinal tract or in the gut, as in the asymptomatic carrier (Figure 1).

It is generally assumed that transmission occurs directly from the aborted fetus, the placenta and uterine excretion. In fact, the fetus and placenta represent the most important sites for the replication of the bacteria.

Nothing is known about a possible indirect transmission through foods and other contaminated materials.

Salmonella Abortusovis is a contagious disease; to prevent its spread there are two different kind of prophylaxis: direct and indirect prophylaxis.

Direct prophylaxis entails the elimination of the contaminated products and the disinfection of all materials and surfaces exposed to this environment.

Indirect prophylaxis is characterized by the administration of attenuated or active vaccine, those are more efficient but they are not available in Europe.

At the moment, no vaccine is available to immunize sheep against *Salmonella* Abortusovis, but there are several promising studies.

2.3.4 *Salmonella enterica* Abortusequi

Salmonella enterica subspecies *enterica* serovar Abortusequi (*S.* Abortusequi) is a host-adapted serotype specific to horses and donkeys that has been isolated in association with abortion and a range of clinical conditions in foals[39].

S. Abortusequi is usually transmitted by direct or indirect contact with pasture, food or water that is contaminated with uterine discharges from carriers. Stallions can also transmit the disease by insemination.

Clinical signs include late abortion (7-8 months) and retention of placenta and metritis in mares. In foals born from infected mother there are clinical signs like acute septicemia in the first week of life and polyarthritis in the second week of life. In stallions symptoms are fever, orchitis with swelling in the prepuce and scrotum and arthritis[40].

The most common symptom is abortion and there are usually no premonitory signs of impending abortion or other clinical signs are observed.

Antibiotic treatments against *S.* Abortusequi are efficient and inactivated vaccines are available.

2.4 Host cell interaction

Host defense against invading microbial pathogens consists of two components: innate immunity and acquired immunity.

Innate immune recognition is based on the detection of constitutive and conserved products of microbes, called pathogen-associated molecular patterns (PAMPs) and

endogenous danger-associated molecular pattern molecules (DAMPs) produced by damaged tissues[41].

Pattern-recognition receptors (PRRs), which include NOD-like receptors (NLRs) and Toll Like receptors (TLRs), comprise the early components of the immune system that function to detect invading pathogens through PAMPs and DAMPs and signal to recruit and activate phagocytic cells such as neutrophils and macrophages[42]. Specifically TLRs recognize microbes on the cell surface and in endosomes, whereas NLRs detect microbial components in the cytosol.

These receptors trigger an immune response and are key to establishing an important network between the innate and adaptive immune systems. Flagella, and LPS are examples of PAMPs, which activate TLR5 and TLR4, respectively, in the host[43].

In contrast the induction of an adaptive immune response begins when a pathogen is ingested by an immature dendritic cell in the infected tissue. The adaptive immune system provides further protection in addition to an immunological memory, which enables a faster response upon repeat exposure to the same pathogen or antigen.

2.5 Toll-Like Receptors

The TLR system provides important protection against microbes and they are present mainly in macrophages, in mast cells, dendritic cells and endothelial cells.

The immediate protection provided by these receptors is based on stimulation of macrophages or mast cells through TLR, through the synthesis and secretion of pro-inflammatory cytokines, and the initiation of the inflammatory process.

TLRs comprise a family of type I transmembrane receptors, which are characterized by an extracellular leucine-rich repeat (LRR) domain and a citoplasmatic domain homologous to the interleukin -1 (IL-1) receptor (TIR) domain[44, 45, 46, 47].

In mammalian species there are at least ten TLRs, and each have a distinct function in innate immune recognition.

The different TLRs can be grouped on the basis of the cellular localization. The receptors that recognize surface components of bacteria are found on the outer membrane and the TLRs that recognize microbial nucleic acids are located on intracellular membranes.

The TLR2 can form heterodimers between TLR2 and either TLR1 or TLR6. For this reason the TLR2 seems to be involved in the recognition of cell wall fragments, such as peptidoglycan from Gram-positive bacteria, bacterial lipoproteins and lipoarabinomannan.

They are found on the cell surface of monocytes, macrophages, dendritic cells, and B cells. It is interesting to note that both TLR1 and TLR6 are expressed constitutively on many cell types, whereas expression of TLR2 is regulated and seems to be restricted to antigen-presenting cells and endothelial cells[48].

TLR4 is found on the cell surface of monocytes, macrophages, myeloid dendritic cells, mast cells and the basolateral side of intestinal epithelium. They are able to recognize lipopolysaccharide (LPS) of Gram-negative bacteria.

Recognition of LPS by TLR4 requires several accessory molecules. LPS is first bound to a serum protein, LBP (LPS-binding protein), which functions by transferring LPS monomers to CD14. Another component of the LPS receptor complex is MD-2 that is required for LPS recognition by TLR4. MD-2 is a small protein expressed on the cell surface in association with the ectodomain of TLR4[49].

TLR5 is located on the cell surface of monocytes, macrophages, dendritic cells, and on the basolateral side of the intestinal epithelial cells. TLR5 recognizes conserved domains of flagellin present on the bacteria.

Recently, it was shown that TLR11 expressed in mice recognizes bacterial flagellin and triggers a protective immune response against *Salmonella* Typhi[50].

Of the 11 types of toll-like receptors TLR3 and TLR4 use the interleukin-1 receptor associated kinase 4 (IRAK-4) as a transmitter for interferon. The IRAK-4 is essential for the early induction of pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1β and interleukin-6.

2.5.1 Toll-Like Receptor 5

The epithelium represents the first line of defense for invading pathogens. TLR5 is the most prominently expressed TLR.

TLR5 specifically recognizes bacterial flagellin, the principal component of bacterial flagella. The activation of this receptor mobilizes the nuclear factor NF-kappaB, which in turn activates a host of inflammatory-related target genes.

Flagellin is the only known activator of TLR5, and until recently flagellin-induced inflammation was believed to be fully dependent on TLR5 expression. TLR5 is responsible for flagellin-induced responses in epithelial cells, endothelial cells, macrophages, dendritic cells (DCs), and T cells[51].

There is strong evidence that the TLR5-activating region of flagellin is located within the conserved domains that are constrained by the need to provide motility.

The structure of flagellin in *Salmonella* Typhimurium consists of four domains, D0, D1, D2 and D3. D0 and D1 are conserved between bacteria species and are located on the outside of the flagellum, D3 and D4 are variable domains that form the inner core of flagellum[52, 53, 54].

Human TLR5 recognize the inner core of flagellum filament which are the conserved domains.

After TLR5-expressing cells are stimulated with flagellin, there is a signaling cascade that involves phosphorylation of interleukin-1 receptor-associated kinase 1, leading to activation of MEK kinases and I-kB kinases, which ultimately activates inflammatory protein production via NF-kB and p38 mitogen-activated protein kinase. After binding

flagellin, TLR5 triggers myeloid differentiation primary response gene 88 (MyD88)-dependent signaling pathway to induce pro-inflammatory cytokine, such as the neutrophil attracting chemokine CXC interleukin-8 (IL-8).

Flagellin activation of TLR5 requires a complicated interaction that could involve additional protein-protein or protein-lipid interactions that are not yet understood.

2.6 Flagella

Flagella are significant for bacterial pathogenesis for two reasons, they allow bacterial cells to move and are essential for microbial survival and additionally they are PAMPs recognized by the immune system.

In *Salmonella*, flagella originate from the bacterial surface and they are dispersed on all the surface of the cell. They vary in number between 5 and 10 per cell.

The flagellar filaments have a consistent diameter of approximately 20 nm but vary in length from 5 to 15 μm[55, 56].

2.6.1 Flagella Structure

The flagella structure consists of three parts: the basal body, the hook and the filament (Figure 3).

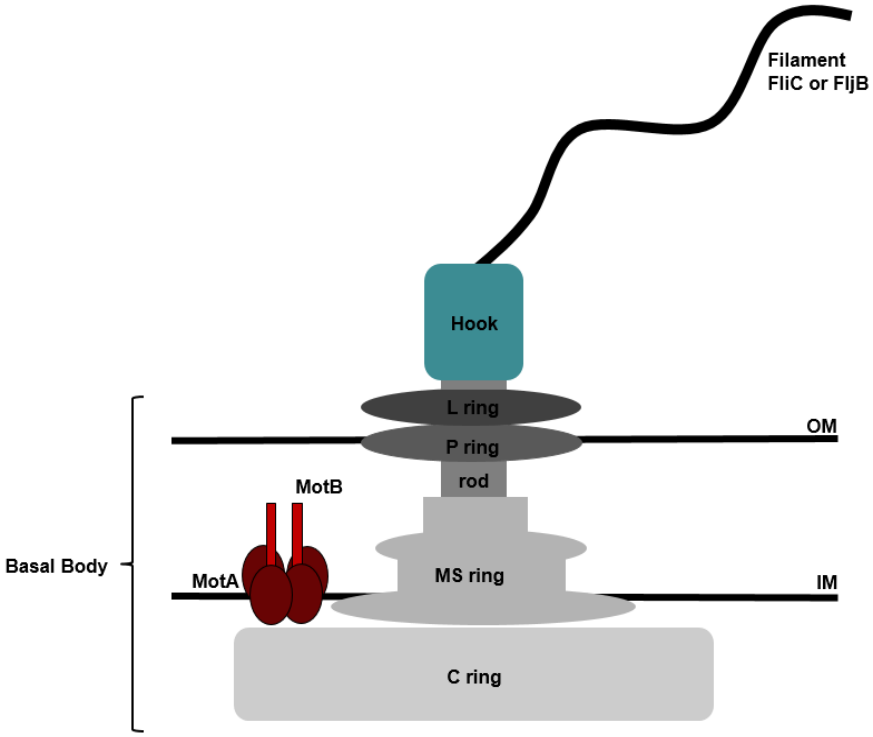

Figure 3. Flagella structure in *Salmonella.*
See text for details.

The basal body connects the flagella to the bacterial cell and consists of a rod and several rings. Some of the rings make up the flagellar motor, which is divided into 2 major parts, the stator (MotA and MotB) that remains stationary, and rotor (C ring, MS ring and rod). The remaining rings L and P located in the outer membrane are stationary[57]. The MS ring is immersed in the cytoplasmic membrane and complexes with proteins MOT (which allow the motion due to a proton-motive force) and protein FLI (which reverse the rotation of the hook).

The hook is a single protein with the function of connecting the filament to the basal body.

It is built in a similar way to the filament, and during the formation the hook-filament junction proteins stay in place and the filament-capping protein (FliD) moves outward as flagellin monomers polymerize.

The filament consist mainly of two proteins, FliC or FljB, and it is a cylindrical structure made up of around 20,000 to 30,000 flagellin (FliC or FljB) subunits[58, 59]. The filament can rotate on the left or on the right direction. The 'normal' form which is more stable is the left side[60].

2.6.2 Flagella Energetics

The energy for flagella is produced using a proton gradient, which produces proton motive force[61, 62]. MotA and MotB proteins form a complex and are part of the stator part of the motor and are involved in proton conduction.

MotA appears to be important for delivery of protons across the membrane and the utilization of those protons in generating torque, MotB appears to be important for the proton channel.

2.6.3 Flagella rotation and motility

The rotary movement is not random, a counter-clockwise movement creates motion and a clockwise arrests motion. Maximum speed varies between 3 and 60 μm/s.

The motor contains a switch which allows the filament to be rotated in counter-clockwise or clockwise directions[56, 63]. When the left handed form is rotated in a counter-clockwise direction the cell is pushed forward.

The motor can alternate through an interaction between a phosphorylated component of the chemosensory system (CheY) and the three proteins subunits that make up the

rotor: FliG that is most directly involved in rotation of the motor, FliN plays a part in rotation and interacts with the stator protein MotA. The MS-ring proteins FliF and FliM have a large role in switching between counter-clockwise and clockwise rotation by binding to CheY phosphate.

2.6.4 Flagella genes

Regulation of flagellar assembly involves a combination of transcriptional, translational and post-translational regulatory mechanisms.

The assembly of the flagellum can be divided into 7 stages: MS-ring formation, assembly of the C-rings and secretion apparatus, rod formation, assembly of the P and L rings, leading hook synthesis, hook completion, secretion substrate specificity switch and filament elongation.

Assembly begins with the insertion of the MS-ring into the inner membrane. The individual extra cytoplasmic flagellar subunits are secreted through the MS-ring after assembly of an associated type III secretion system.

In *Salmonella* the flagellar regulon consists of 17 operons, divided into classes 1, 2 and 3 (Figure 4).

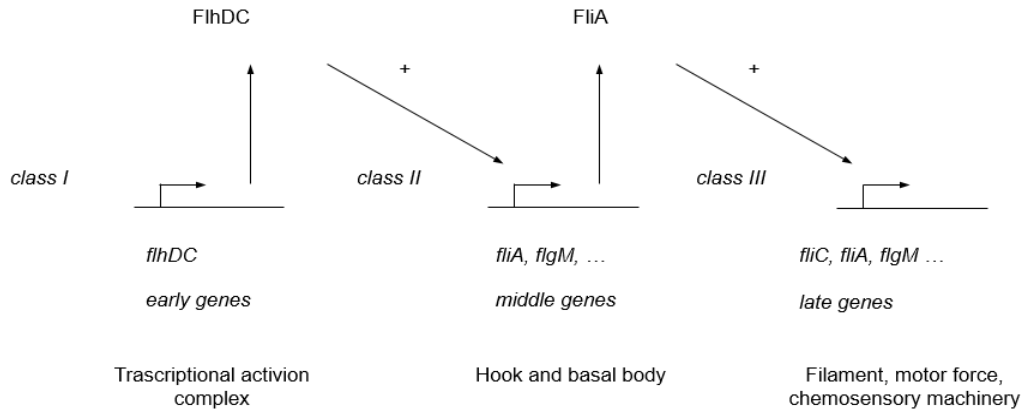

Figure 4. Trascriptional regulation of flagellar assembly in *Salmonella.*

See text for details.

The early genes belong to the class 1 promoters which control the expression of the entire regulon. The middle gene possess class 2 promoters and are important for the production of structural components of the hook and of the basal body. The late genes belong to the class 3 promoters which products include the filament, the motor force generators and chemosensory machinery.

During flagella formation, the first genes to act are the class 1 genes, f*lh*DC.

FlhD and FlhC form $FlhD_4C_2$, an hexameric complex[64, 65]. This complex is a transcriptional activator for σ70-dependent transcription of the class 2 promoters[66].

The class 2 genes include *fliA* (σ28) which is required for transcription of many of the class 3 genes, although some class 3 genes can be expressed independently of *fliA*[67].

In addition, *flgM* and *flgN* genes can be expressed independently of *fliA* by read through of the transcript from *flgA*, a class 2 promoter[68, 69, 70, 71].

*flgM i*s the anti-*fliA* (σ28) factor that negatively regulates fliA by binding it. Upon completion of the basal body-hook structure, the flagellar protein export apparatus switches substrate specificity and transports FlgM out of the cell. Inhibition of class 3

genes is then removed and transcription initiation by FliA dependent class 3 flagellar genes can then proceed[68].

2.6.5 Flagellar phase variation

In *S.* Typhimurium there are two different flagellar filament proteins, FljB and FliC, which are alternately expressed by a mechanism known as phase variation[72]. The molecular mechanism mediating flagellar phase variation occurs by a site-specific DNA inversion event in the chromosome by the stochastic inversion of a promoter, the *hin* switch, which controls both FljB flagellin and an inhibitor (FljA) of FliC flagellin formation. In one orientation the *fljBA* promoter directs transcription of the *fljBA* operon and FljB flagellin is produced. The *fljA* gene is cotranscribed with *fljB* and encodes a transcriptional inhibitor of the *fliC* gene. In the alternate orientation, the *fljBA* operon is not expressed and transcription of the *fliC* gene ensues. So when the *fljB-fljA* operon is expressed, only FljB flagellar filaments are produced; when the operon is not transcribed, the gene for FliC flagellin (*fliC*) is released from inhibition and FliC flagellar filaments are formed[73] (Figure 5).

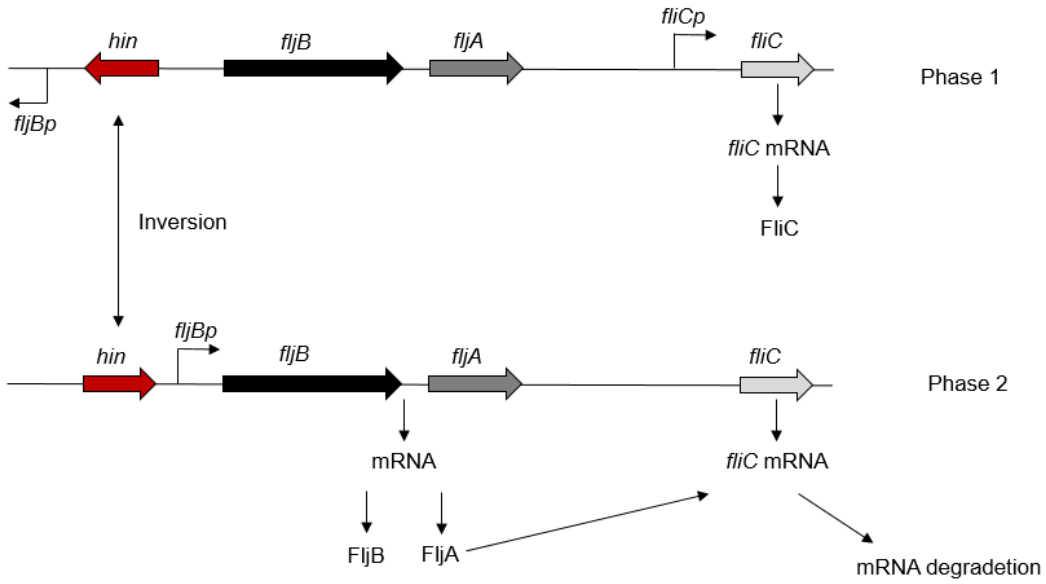

Figure 5. Flagellar phase variation in *Salmonella* Typhimurium.

See text for details.

The presence of a second phase type of flagella may help *Salmonella* to evade the immune system.

3. Hypothesis and aims

Limited information is available about the immune response of sheep to *Salmonella* Abortusovis.

The objective of this application is to study the differences of innate immune recognition and host responses to this pathogen compared with *S.* Typhimurium.

Our central hypothesis is that direct contact of *Salmonella* Abortusovis with host cells does not trigger an initial innate immune response allowing the organism to reach systemic sites.

Normally, epithelial cells initially herald the presence of invasive pathogens. After penetration of the intestinal epithelium, bacteria encounter mononuclear cells, whose stimulation through TLRs results in the release of cytokines (e.g. IL-8 and TNF-α).

We hypothesized that *Salmonella* Abortusovis is able to escape the recognition by TLR5 with an 'on/off' mechanism, in which initially flagellin is 'off', but later during the pregnancy is 'on' causing infection and abortion.

In this work we want test our hypothesis that recognition of PAMPs, which normally contributes to the initiation of inflammatory response in the intestinal mucosa, is different during the infection of *Salmonella* Abortusovis in sheep.

To this end, we decided to study the initial interaction of *Salmonella* with the epithelium using a colonic epithelial cancer cell line T84 (American Type CultureCollection) to study if the serotypes Typhimurium and Abortusovis during infection of intestinal epithelium are able to invade equally, and to understand if flagella in *S.* Abortusovis are able to activate the TLR5.

The result of this analysis clarified the fundamental mechanisms leading to systemic infection, which improved our understanding of systemic disease in general.

Our results provided first an important mechanistic insight into how *S*. Abortusovis is able to modulate host responses that distinguish abortions from infections with *S*. Typhimurium and we elucidated how serovar Abortusovis is able to not trigger the first innate immune response, which is important in the early recognition of this pathogen.

This outcome was significant because it established key events and provided insights into basic mechanisms of the innate immune response and the systemic disease,

thereby refining a paradigm for innate immune host-response to invasive enteric pathogens.

The picture emerging in this study is that multiple factors are involved in initiating a pro-inflammatory response in the intestinal mucosa during *Salmonella* Typhimurium infection but not during *Salmonella* Abortusovis infection.

4. Materials and methods

4.1 Bacterial strains and growth conditions

The bacterial strains used in the present study are *Salmonella* Typhimurium, *Salmonella* Abortusovis and *Salmonella* Abortusequi (Table 3).

Subspecies	Strain	Parent	Genotype	Source
S. Typhimurium	SR11	SR11	wild type	Strain Collection
S. Typhimurium	TH1077	LT2	*fliC*5050::Mu*d*J	[74]
S. Typhimurium	TH714	LT2	*fljB*5001:: Mu*d*J	[75]
S. Typhimurium	TH4623	LT2	*flgM*5222::Mu*d*Cm	[76]
S. Typhimurium	TH4054	LT2	*flhC*5456:: Mu*d*J	[74]
S. Abortusovis	SS44	SS44	wild type	[77]
S. Abortusovis	LS1	SS44	*fliC*5050:: Mu*d*J	This study
S. Abortusovis	LS2	SS44	*fljB*5001:: Mu*d*J	This study
S. Abortusovis	LS3	SS44	*flgM*5222::Mu*d*Cm	This study
S. Abortusovis	LS4	SS44	*flhC*5456:: Mu*d*J	This study
S. Abortusequi			wild type	Strain Collection

Table3. *Salmonella* Typhimurium and *Salmonella* Abortusovis strains used in this study.

Strains (Table 3) were routinely grown at 37°C in LB broth. Undiluted serum of sheep, young sheep and pregnant sheep were used to grow bacterial strains to test if, in specific media, *S.* Abortusovis is able to express flagella *in vitro.* Strains were grown over night in different media, and depending on the experiment directly used or diluted 1:50 in LB or in serum for 3 h.

Blood from sheep, young sheep and pregnant sheep was collected from a flock in the Sardinian region, by a veterinarian that collaborated previously in this study.

Before blood collection, individual sheep were analyzed and no *Salmonella* infection was detected as determined by rectal swab tests and plating on *Salmonella-Shigella* agar plates (SS agar). Those plates contains lactose. If lactose fermentation occurs, the medium will turn red due to the acidic pH. *Salmonella*, as non-lactose fermenters, appears as transparent or translucent colorless colonies on SS agar. Samples were also streaked on non-selective but differential medium such as MacConkey Agar.

Serological confirmation tests using polyvalent antisera for flagellar (H) and somatic (O) antigens were performed to exclude the presence of *Salmonella*.

Blood was collected in heparin tubes, and it was treated in lab by centrifugation at 5,000 rpm for 30 min. Serum was collected and stored at -20°C.

4.2 M9 minimal medium

M9 minimal medium was used in this study to analyze the expression of flagella in *Salmonella* Typhiumurium and in *Salmonella* Abortusovis.

This media was prepared using a final volume of 100 ml, 20 ml of M9 salts 5X (Sigma-Aldrich), 2 ml of Glucose 20 % (Sigma-Aldrich), 200 µl of MgSO4 1M (Fisher Scientific), 10 µl of CaCl2 1 M (Fisher Scientific) and 78 ml of H_2O.

M9 minimal medium was filter sterilized and used to grow different serovars.

4.3 Phage transduction

Mutations of flagella genes were transduced in *Salmonella* Abortusovis to study changes in motility using samples of *Salmonella* Typhimurium strains that had been previously characterized.

A P22 lysate of *S.* Typhimurium *fljB*5001::Mu*d*J, *S.* Typhimurium *fliC*5050::Mu*d*J, *S.* Typhimurium *flhC*5456::Mu*d*J, *S.* Typhimurium *flgM*5222::Mu*d*Cm was used to transduce in serotype Abortusovis flagella proteins mutations. The mutations were confirmed by PCR using specific primers (Table 4).

Gene	Sequence (5' –3')	Source
fliC	AAGTCAGGTTGTTTACGGTGTTGC TGTCGCTGTTGACCCAGAATAAC	This study
flhC	CAGCATCTCGGGAAAGTTTACG GCTTTATCTTGAGCAGTGTCCGC	This study
fljB	GACAGATTGTTTACGGTATTGCCC GTATTACGCCGCAGATTACGATG	This study
flgM	TGAGCGAGTCTGCTATTTTTCCC TGAGCATTGACCGTACCTCACC	This study

Table 4. Primers used in this study.

Phage lysates were typically prepared by growing a 5 ml culture of the donor strain in LB broth with the appropriate antibiotics and incubate at 37°C over night (O/N) with aeration.

A volume of 4 ml of P22 broth were added to 1 ml of bacterial culture and incubate at 37°C for 16 h with aeration.

A volume of 1.4 ml of culture was centrifuged for 2 min at 12,000 g and the supernatant was transferred to a cryovial, and 0.2 ml chloroform was added and vortexed for at least 1 min.

For the transduction, the recipient strain was growth in LB broth with the appropriate antibiotics and incubate at 37°C O/N with aeration.

Three 10-fold serial dilutions were prepared of the phage lysate in 0.1 ml PBS, and 0.1 ml of the recipient strain was added to each of the diluted phage lysate and incubate at RT for 1 h.

Samples were centrifuged at 20,000 g for 1 min and the pellet was resuspended with 0.1 ml of LB and plated on LB plates containing the appropriate antibiotics and incubate O/N at 37°C.

Individual single colonies were streaked for single colonies on EBU plates, containg 25% (w/v) K_2HPO_4, 1% Evan's Blue and 1% Sodium Fluoresceine, and incubated for 16 h at 37°C.

One colony from each streak was cross-streaked with 0.01 ml of P22 H5 on EBU plates, incubated at 37°C for 8 h and the positive colonies were streak for single colonies on LB plates containing the appropriate antibiotics.

4.4 Motility analysis in Motility plates

For motility assays, plates containing 10 g of tryptone/liter, 5 g of NaCl/liter, and 3 g/L agar were inoculated with 10 µl of the indicated O/N cultures and incubated at 37°C 16 h.

4.5 Purification of bacterial flagella

The protocol followed for the flagella extraction is based on trichloroacetic acid precipitation (TCA)[78].

S. Typhimurium and *S*. Abortusovis were grown for 16 h at 37°C with aeration, diluted 1:50 in 6 ml of LB broth, and grown for 3 h at 37°C with aeration.

Flagella were sheared by treating the bacterial culture with a homogenizer (IKA) on ice for 1 min. The suspension was centrifuged at 3,220 g for 10 min at 4°C, and the pellet was washed with trichloroacetic acid to a final concentration of 10 %, followed by centrifugation at 12,000 g for 30 min at 4°C. The pellet was washed two times with ice-cold acetone and resuspended in 0.05 ml of water.

4.6 Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis (SDS-PAGE)

The samples were boiled for 5 min with loading buffer (1x) containg 62.5 mM Tris-HCl pH 6.8, 2.5 % SDS, 0.002 % Bromophenol Blue, 5 % β-mercaptoethanol, 10 % glycerol.

A volume of 0.01 ml of sample was separated by sodium dodecyl sulfate 12 % polyacrylamide gel electrophoresis. Gels were composed of 10.72 ml of H_2O, 8 ml of Tris-HCl 1.5 M (pH 8.8), 12.8 ml of acrylamide, and 320 µl of 10 % SDS. 0.05 % TEMED and 10 % APS were added for the polymerization.

The 4 % stacking gel was composed of 6.1 ml of H_2O, 2.5 ml of Tris-HCl 0.5 M (pH 6.8), 1.3 ml of acrylamide, 10 % SDS and 10 % TEMED were added for the polymerization.

Gel were visualized with Coomassie Blue G-250 (Sigma), 0.2g of Coomassie Blue G-250 were dissolved in 100 ml of H_2O warmed to approximately 50°C. 100 ml of 2N H_2SO_4 were added. The solution was filtered and 22.2 ml 10N KOH and 28.7g TCA were added.

To stain the gel were immersed in the solution for 15 minutes, and stored in 7 % HOAC.

4.7 Western Blotting

Protein extracted and separated by 12 % (v/v) SDS-PAGE gels were blotted onto nitrocellulose membrane using a semi-dry method.

Blots were blocked in 3 % (w/v) skimmed milk powder (Sigma), 0.1 % (v/v) Tween20 (Sigma) in Dulbecco PBS at 4°C for 1h.

Primary antibodies, anti-FliC monoclonal rabbit (Difco), were diluted in 5 % (w/v) Bovine Serum Albumin (Sigma), 0.1 % (v/v) Tween20 in PBS and incubated with blots for 2 h at 4°C, with gentle rocking.

Blots then were washed three times in 3 % milk solution at 5 min intervals with gentle rocking at RT.

Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (BD) secondary antibodies was diluted in 5 % (w/v) Bovine Serum Albumin, 0.1 % (v/v) Tween in PBS and were incubated with blots for 1h at RT with gentle rocking.

The membrane was washed with 3 % milk solution at 5 min intervals three times and three times with PBS at 5 min intervals.

Blots were developed using a chemi-luminescence reagent (EuroClone), and the signal from the blots was captured using a specific software, Image Lab Software (BioRad).

4.8 Flow Cytometry

Samples were grown over night at 37°C with aeration in the indicated growth media.

The samples were centrifuged at 3,000 rpm for 10 min and resuspended in 0.1 ml of PBS. 4 % paraformaldehyde was added and the samples incubated at RT for 20 min.

The samples were washed two times with PBS containing 0.02 % of gelatin, resuspended in 0.5 ml 2 % Normal Goat Serum, and incubated for 30 min at RT.

1:250 dilution of the primary antibody was added directly and incubated at RT for 1 hour. After the incubation the samples were centrifuged at 3,000 rpm for 10 min, and washed three times with PBS containing 0.02 % gelatin.

The samples were resuspended ml 0.5 ml 2 % Normal Goat Serum and 1:250 FITC secondary antibody and 0.025 ml of Propidium Iodide Solution (1 µg/ml). They were incubated for 1 hour in the dark at room temperature, and after the incubation washed 3 times with PBS containing 0.02 % gelatin.

After centrifugation at 3,000 rpm for 10 min, the final pellet was resuspended in 10 ml of PBS.

BD Accuri C6 Flow Cytometer (BDBioScience) was used for this experiment and the gate of interest was designed using *Salmonella* Abortusovis growth in LB as a blank.

4.9 Scanning Electron Microscopy (SEM)

Processing and imaging of samples for scanning electron microscopy (SEM) was carried out at the Electron Microscopy facility run by Salvatore Marceddu in the University of Sassari.

The samples were prepared by growing *S.* Typhimurium and *S.* Abortusovis for 16 h at 37°C with aeration in different media, diluted 1:50 in 6 ml of media, and growth for 3 h at 37°C with aeration.

Flagella were sheared by treating the bacterial culture with a homogenizer (IKA) on ice for 1 min. The suspension was centrifuged at 3,220 g for 10 min at 4°C, and the pellet was washed with trichloroacetic acid to a final concentration of 10 %, followed by centrifugation at 12,000 g for 30 min at 4°C. The pellet was washed two times with ice-cold acetone and air-dried. The samples were send to the microscopy facility for image acquisition.

4.10 T-84 cell line infection

Human colorectal carcinoma cell line (T-84) (Invivogen) was cultured in DMEM/F12 media supplemented with Hepes buffer solution, Glutamax and 10 % FBS (Giboco).

Cells were split and a 24 wells plate was prepared for the experiment. Cells were used at a density of $1*10^6$ cells/well. After 24 h the media was changed in media without serum.

After a total of 48 h flagella were added for 60 min (flagella samples 5 µl / PBS control 10 µl).

The flagella used for the T84 stimulation was extracted after growing the bacteria culture O/N in LB or serum. A volume of 0.1 ml of overnight culture was inoculated in 5 ml of LB or serum, and grown at 37°C for 3 h. The samples were treated with a homogenizer (IKA) on ice for 1 min and centrifuged at 3,220 g for 10 min at 4°C, and 5 µl of the filtered supernatant was added in every well, and incubated 60 min.

4.11 RNA extraction

Total RNA was extracted with TRI Reagent (Molecular Research Center), according to the recommendation of the manufacturer.

Briefly, 1 ml of TRI REAGENT was added to each wells and the cell lysate was passed several times through a pipette.

The homogenate was stored for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes, and the homogenate was supplemented with 0.2 ml chloroform and shaken vigorously for 15 seconds. The resulting mixture was incubated for 15 min and centrifuged at 12,000 g for 15 min at 4°C.

Aqueous phase was transferred to a fresh tube and 0.5 ml of isopropanol was added per 1 ml of TRI REAGENT used for the initial homogenization. The samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 g for 8 min at 4°C.

The RNA pellet was washed with 75% ethanol and centrifuged at 7,500 g for 5 min at 4°C.

RNA pellet was air-dried for 15 min and dissolved in sterile water made RNase-free by diethyl pyrocarbonate (DEPC) treatment.

For RT-PCR analysis, DNase treatment was necessary for optimal results.

4.12 DNase treatment

A volume of 20 µl of 10X DNase I Buffer and 1 µl rDNase I (Ambion) were added to the RNA, and incubated at 37°C for 30 min. A volume of 1 µl of resuspended DNase Inactivation Reagent was added and incubated 2 min at RT.

The samples were centrifuged at 10,000 g for 1 min, and the RNA was transferred to a fresh tube.

The RNA obtained was used for the RT-PCR.

4.13 RT-PCR

Briefly, 2 µg of RNA was added to 1 µl of random primers (InvivoGen) and 1 µl to 3 µl of H_2O, for a final volume of 5 µl, and incubated at 70° C for 5 min followed by 10 min on ice.

A volume of 5 µl were added, for a final volume of 20 µl, to a mixture contained 4 µl of H_2O, 3 µl of $MgCl_2$, 4 µl of buffer 5X, 2 µl RNasi inhibitor, 1 µl of nucleotides and 1 µl of Taq (InvivoGen).

The samples were incubated for the following RT-PCR cycle: 5 min at 25° C, 60 min at 42° C and 15 min at 70°C.

The samples were used for the Real-Time PCR.

4.14 Real-Time PCR

Real-time PCR was performed using SYBR green (Promega) and the 7900HT Fast real-time PCR system.

To determine transcription of the *IL8* gene, the following primers for *GAPDH* gene and for *IL8* were used: GAPDH, 5'-3' FW: CTGCTTTGATGTCAGTGCTGCTAC and RW: CTGCCGTGTGAAGCCCACAATAAA; IL-8, 5'-3' FW: GCCAACACAGAAATTATTGTAAAGCTT and RW: CCTCTGCACCCAGTTTTCCTT.

The analysis was performed in triplicate using the following cycle: 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min.

The fold change in mRNA levels was determined using the comparative threshold cycle (CT) method (Bio-Rad).

For statistical analysis (fold increases in IL-8 expression), data were transformed logarithmically to calculate geometric means. A parametric test (paired Student's *t*-test) was used to calculate differences in the increases in IL-8 expression. A two-tailed *P* value of < 0.05 was considered to be significant.

5. Results

5.1 The right media is necessary to express *Salmonella* Abortusovis flagella *in vitro*

Our goal of studying the role of flagella in host-pathogen interaction was initially hampered by the fact that flagella are not expressed by *Salmonella* Abortusovis under standard laboratory conditions. Thus, we sought to experimentally identify a growth condition suitable for the expression of flagella *in vitro*. Specifically, we varied carbon sources, pH, growth phase and temperature. Motility was used as a functional read-out for flagella expression (Table 5).

	Media	16 h 37°C aerobic	12 h 37°C aerobic	6 h 37°C aerobic	16 h 37°C micro - aerobic	12 h 37°C micro - aerobic	6 h 37°C micro - aerobic
***Salmonella* Typhimurium**	**LB broth**	+	+	+	+	+	+
	LB pH 3	-	-	-	-	-	-
	LB pH 5	+	+	+	+	+	+
	LB pH 9	+	+	+	+	+	+
	Minimal Medium	+	+	+	+	+	+
	Minimal Medium pH 3	-	-	-	-	-	-
	Minimal Medium pH 5	+	+	+	+	+	+
	Minimal Medium pH 9	+	+	+	+	+	+
	Sheep serum	+	+	+	+	+	+
***Salmonella* Abortusovis**	**LB broth**	-	-	-	-	-	-
	LB ph 3	-	-	-	-	-	-
	LB ph 5	-	-	-	-	-	-
	LB ph 9	-	-	-	-	-	-
	Minimal Medium	-	-	-	-	-	-
	Minimal Medium pH 3	-	-	-	-	-	-
	Minimal Medium pH 5	-	-	-	-	-	-
	Minimal Medium pH 9	-	-	-	-	-	-
	Sheep serum	+	+	+	+	+	+

Table 5. Flagella expression in different media.

As shown in Table 5, *Salmonella* Typhimurium expresses flagella in almost all media condition tested. In contrast, no expression of flagella was observed in *S.* Abortusovis serotype. The same results were observed using the same media at 30°C.

We reasoned that the absence of a specific inducer may prevent flagella expression and motility *in vitro*. Therefore we tested motility of *Salmonella* Abortusovis in a media that better resembles *in vivo* growth condition, for example, the serum from sheep.

Interestingly, we observed that when sheep serum was used for growing bacteria *Salmonella* Abortusovis was able to express flagella.

On the basis of those results different kind of sheep serum were tested. Additionally, we tested the serum of other farm animals (Table 6).

	Media	16h 37°C aerobic
***Salmonella* Abortusovis**	LB broth	-
	Sheep serum	+
	Young sheep serum	+
	Pregnant sheep serum	+
	Lamb serum	-
	Pig serum	-
	Cow serum	-
	Horse serum	+
	Human serum	-

Table 6. Expression of *Salmonella* Abortusovis flagella *in vitro* using different serum.

As shown in Table 6, *Salmonella* Abortusovis expressed flagella in serum obtained from sheep, but no expression was observed using other media as lamb serum, pig serum, cow serum and human serum. Expression of flagella in this serotype was observed using horse serum.

The status of the sheep and their pregnancy does not seems to play a role for the expression of flagella and for the motility.

Those results were collected by studying the expression of flagella in motility agar plates, and when flagella was expressed, a motility halo was observed. In addition to the motility LB agar plates, in this study motility plates containing an equal mixture of LB agar and each serum were used.

Salmonella Abortusovis is not able to move using LB media, in contrast to *Salmonella* Typhimurium that showed motility in LB (Figure 6A). However, *S.* Abortusovis is able to move in agar plates if serum of sheep is use to pre-grow this serotype. An interesting result was also observed using horse serum, in this media *Salmonella* Abortusovis showed a positive result, which means that flagella are expressed in this media too (Figure 6B).

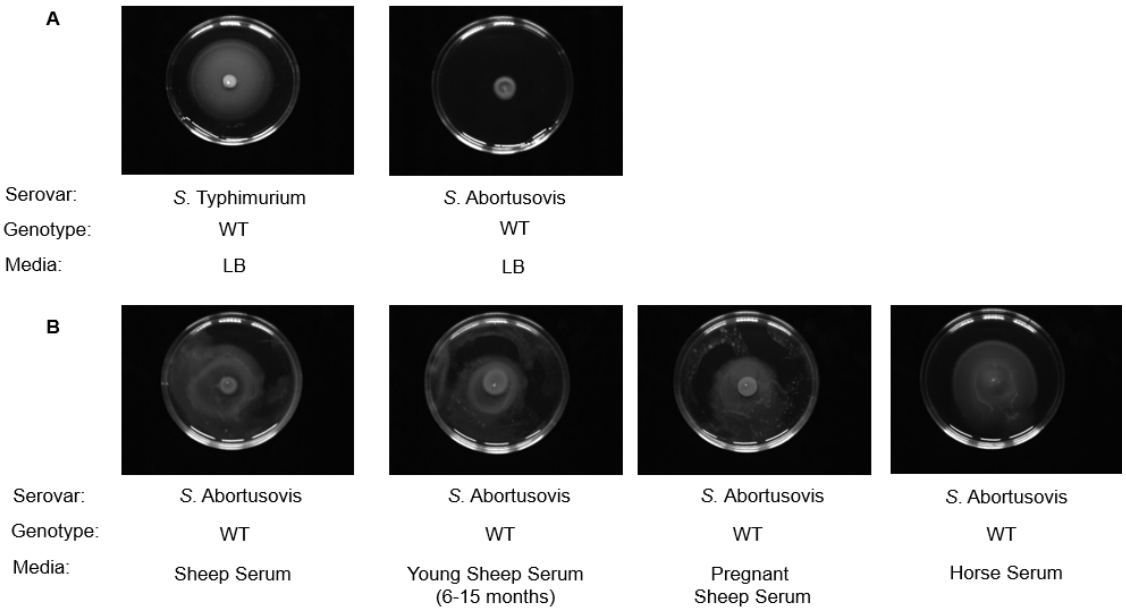

Figure 6. Representative images of *Salmonella* Typhimurium (SR11) wild type (WT) and *Salmonella* Abortusovis (SS44) WT in motility plates. A) SR11 WT and SS44 WT were grown 16 h in LB media at 37°C with aeration. A volume of 10 µl was inoculated on LB motility agar plates for 16 h at 37°C. This experiment was repeated three times. B) SS44 WT was grown 16 h in undiluted sheep serum, young sheep serum, pregnant sheep serum and horse serum, at 37°C with aeration. A volume of 10 µl was inoculated on motility agar plates containing an equal mixture of LB agar and each serum. This experiment was repeated three times.

Growth media that closely mimics the host is suitable to induce expression of flagellin in a host-restricted serovar.

On the basis of this result *Salmonella* Abortusequi was growth using horse serum and LB (Figure 7).

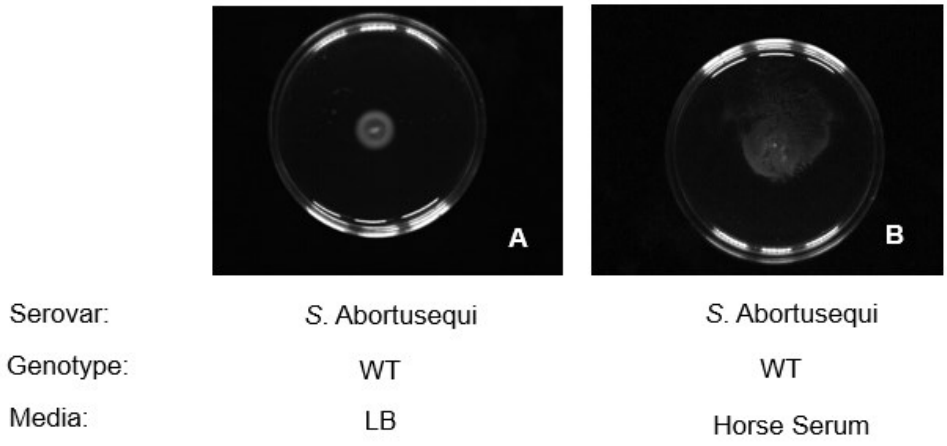

Figure 7. Representative images of *Salmonella* Abortusequi wild type (WT) in motility plates. A) *Salmonella* Abortusequi WT was grown 16 h in LB media at 37°C with aeration. A volume of 10 µl was inoculated on LB motility agar plates for 16 h at 37°C. This experiment was repeated three times. B) *Salmonella* Abortusequi WT was grown 16 h in undiluted horse serum at 37°C with aeration. A volume of 10 µl was inoculated on motility agar plates containing an equal mixture of LB agar and horse serum. This experiment was repeated three times.

For this serotype it was observed that *S.* Abortusequi is not able to swim in motility agar plate if grow in LB but motility was observed when the bacteria was grown in horse serum.

As a control *Salmonella* Typhiumurium and *Salmonella* Abortusovis mutants were tested on motility plates.

Salmonella Typhimurium non-flagellated mutants were not able to move (Figure 8A), and the same was observed for *Salmonella* Abortusovis non-flagellated mutants using sheep serum as growth media (Figure 8B).

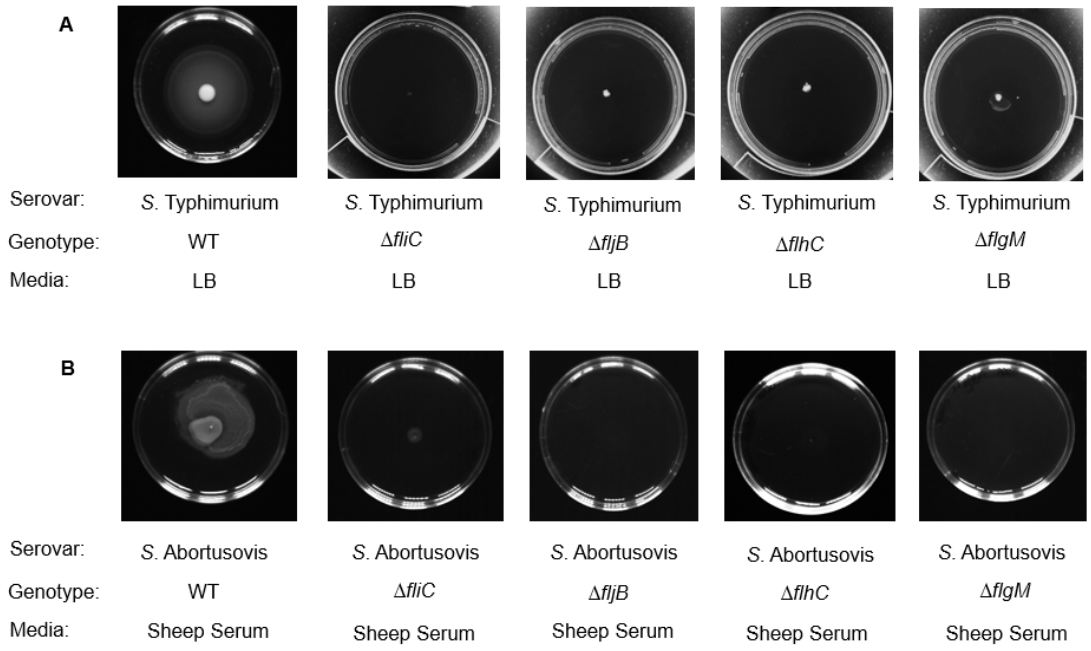

Figure 8. Representative images of *Salmonella* Typhimurium (SR11) and *Salmonella* Abortusovis (SS44) in motility plates. A) SR11 wild type (WT), *S.* Typhimurium Δ*fliC* (TH1077), *S.* Typhimurium Δ*fljB* (TH714), *S.* Typhimurium Δ*flgM* (TH4623) and *S.* Typhimurium Δ*flhC* (TH4054) were grown 16 h in LB media at 37°C with aeration. A volume of 10 µl was inoculated on LB motility agar plates for 16 h at 37°C. This experiment was repeated three times. B) SS44 WT, SS44 Δ*fliC* (LS1), SS44 Δ*fljB* (LS2), SS44 Δ*flgM* (LS3) and SS44 Δ*flhC (*LS4) were grown 16 h in sheep serum at 37°C with aeration. A volume of 10 µl was inoculated on motility agar plates containing an equal mixture of LB agar and sheep serum. This experiment was repeated three times.

So we were able to express *Salmonella* Abortusovis flagella *in vitro* using a media that was as close as possible to the *in vivo* condition.

The next results will answer the question if now we can study this expression *in vitro*.

5.2 Flagellin expression *in vitro*

Although it is likely that the observed motility phenotype were due to flagella based motility, other modes of motility have been reported in *Salmonella*, like *Salmonella* Typhi has type IVB pili. To confirm that exposure to serum indeed resulted in the upregulation of flagella proteins, we analyzed expression of the major subunit of phase I flagella, the flagellin FliC by western blotting.

To understand if it is possible analyze flagella expression in *Salmonella* Abortusovis, we decided to extract flagella from serovar Typhimurium and Abortusovis, both wild type and mutant.

Following the trichloroacetic acid precipitation we were able to extract flagella, and analyze them by Western Blotting using anti-FliC monoclonal rabbit antibody.

Our analysis confirmed our previous results obtained with motility agar plates. Expression of flagella was detected in *Salmonella* Typhimurium growth in LB, and any detection was observed for *Salmonella* Abortusovis growth in LB, but when *S.* Abortusovis serotype was grown in different sheep serum we were able to observe the expression of flagella (Figure 9A).

As a control, the same analysis was performed for the non-motile mutants in *S.* Typhimurium and *S.* Abortusovis, and any flagellin detection was observed (Figure 9B).

On the basis of the results obtained with motility agar, we also analyzed the expression of flagella in *Salmonella* Abortusovis growth in horse serum and of *Salmonella* Abortusequi growth in LB and horse serum (Figure 9C).

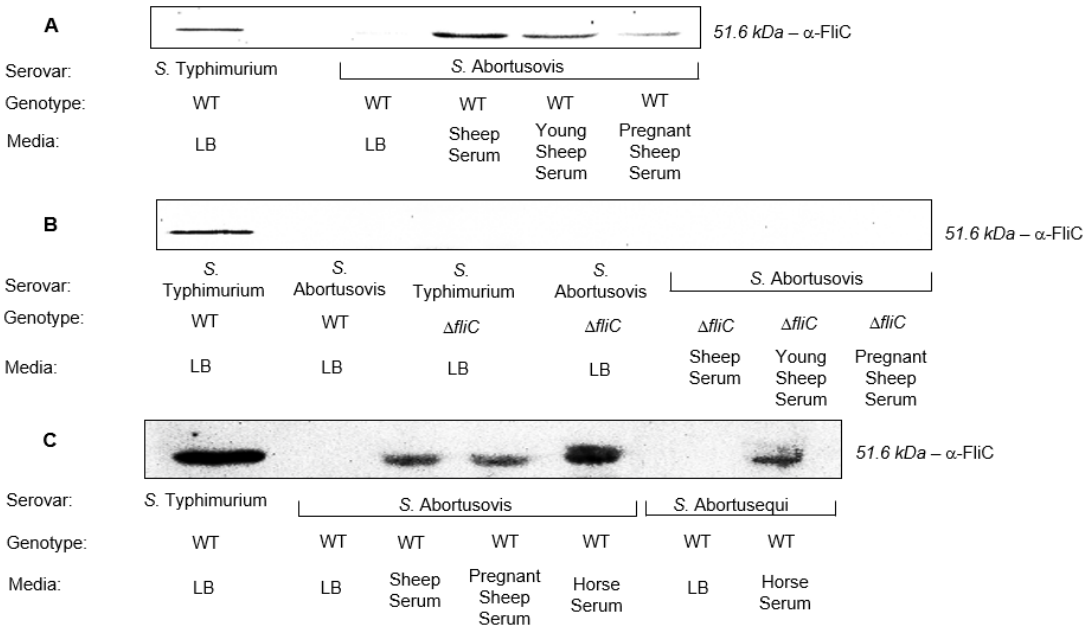

Figure 9. Expression of the major subunit of phase I flagella, FliC, by Western Blotting. A) *Salmonella* Tyhpimurium (SR11) wild type (WT) and *Salmonella* Abortusovis (SS44) WT were grown in LB over night (O/N) at 37°C with aeration. SS44 WT was also grown in undiluted sheep serum, young sheep serum and pregnant sheep serum O/N at 37°C with aeration. Flagella were precipitated with trichloroacetic acid (TCA) and analyzed by Western Blotting with anti-FliC monoclonal rabbit (α-FliC). The molecular mass is indicated on the right. This experiment was repeated three times. B) SR11 WT, SS44 WT, *S.* Typhimurium Δ*fliC* (TH1077) and SS44 Δ*fliC* (LS1), were grown in LB over O/N at 37°C with aeration. LS1 (Δ*fliC)* was also grown in undiluted sheep serum, young sheep serum and pregnant serum O/N at 37°C with aeration. Flagella were precipitated with TCA and analyzed by Western Blotting with α-FliC. The molecular mass is indicated on the right. This experiment was repeated three times. C) SR11 WT, SS44 WT and *Salmonella* Abortusequi WT were grown in LB O/N at 37°C with aeration. SS44 WT was also grown in undiluted sheep serum, young sheep serum, pregnant sheep serum and horse serum O/N at 37°C with aeration. *Salmonella* Abortusequi WT was also grown in undiluted horse serum O/N at 37°C with aeration. Flagella were precipitated with TCA and analyzed by Western Blotting with α-FliC. The molecular mass is indicated on the right. This experiment was repeated three times.

We showed that flagella expression was detected in *S.* Abortusovis and *S.* Abortusequi growth in horse serum, but nothing was observed when *Salmonella* Abortusequi was grown in LB media.

That suggested that this phenomenon probably occurs in host restricted serovar and might be applicable to other host restricted serovars as well.

5.3 Quantification of flagella expression in *Salmonella*

Expression of flagella on the surface in *Salmonella* Typhiumurium and in *Salmonella* Abortusovis was quantified using flow cytometry.

We were able to confirm our previous results, expression of flagella was observed for *Salmonella* Typhimurium growth in LB. As expected, no flagella was observed in *Salmonella* Abortusovis growth in LB, but significant differences were observed when *Salmonella* Abortusovis was grown in sheep serum (Figure 10A). Specifically, data in Figure 10A, show flagella expression in this serovar after growth in pregnant sheep serum, but the same was observed after growing in sheep serum and young sheep serum (data not show).

With this technique we also confirmed the results previously obtained for flagella mutants. In *Salmonella* Typhimurium mutants no expression of flagella was detected (data not show), and no expression was observed also for *Salmonella* Abortusovis mutants (Figure 10B).

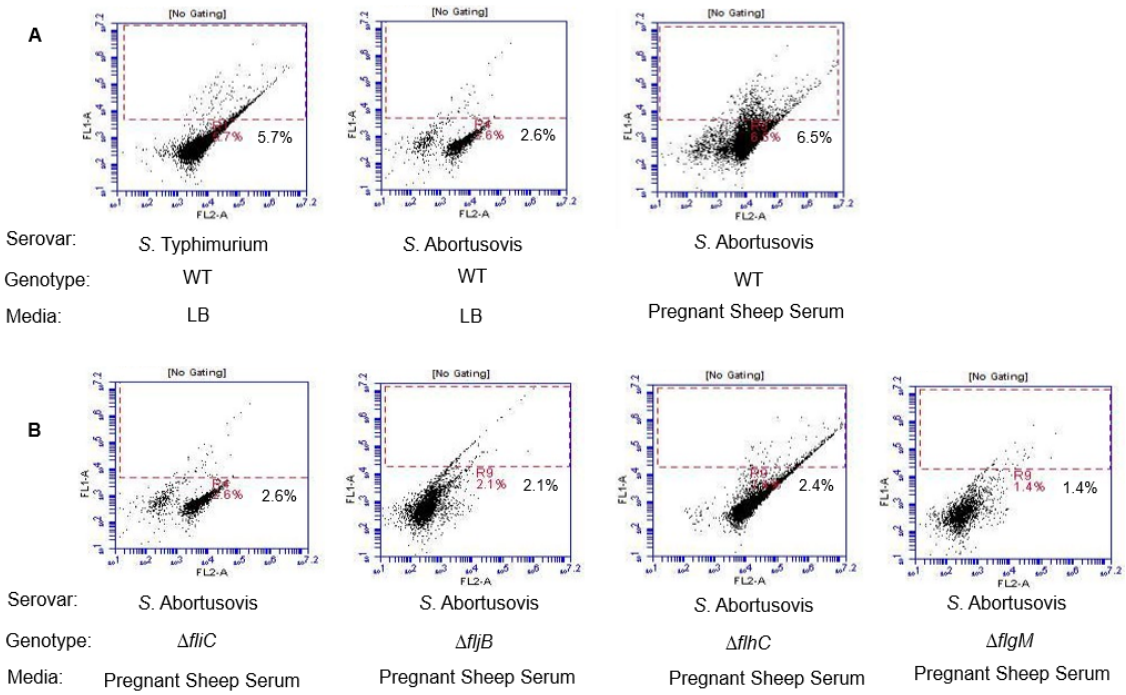

Figure 10. Quantification of flagella expression on the surface in *Salmonella* serovars. A) *Salmonella* Tyhpimurium (SR11) wild type (WT) and *Salmonella* Abortusovis (SS44) WT were grown in LB over night (O/N) at 37°C with aeration. SS44 WT was also grown O/N in pregnant sheep serum at 37°C with aeration. Propidium Iodide (PI) protocol was followed to quantify the expression of flagella, and SS44 WT in LB was used as blank for the gate construction. B) SS44 Δ*fliC* (LS1), SS44 Δ*fljB* (LS2), SS44 Δ*flgM* (LS3) and SS44 Δ*flhC (*LS4) were grown O/N in pregnant sheep serum at 37°C with aeration. PI protocol was followed to quantify the expression of flagella, and SS44 WT in LB was used as blank for the gate construction.

5.4 *Salmonella* Abortusovis flagella: visualization of structures

Previous results were confirmed by Scanning Electron Microscopy. Specifically we were able to see for the first time flagella expressed by *Salmonella* Abortusovis *in vitro*. *Salmonella* Typhiumurium growth in LB was used as a positive control, and flagella expression was observed by SEM. As expected, no flagella were observed in *Salmonella* Abortusovis growth in LB, but flagella were observed after growth in sheep serum, young sheep serum and pregnant sheep serum, in particular in this figure we showed expression after growth in pregnant sheep serum (Figure 11A).

As a control flagella mutants in *Salmonella* Typhimurium and *Salmonella* Abortusovis were confirmed too. Any flagella mutant for *Salmonella* Typhimurium showed flagella expression (data not show), and the same was observed for *Salmonella* Abortusovis (Figure 11B).

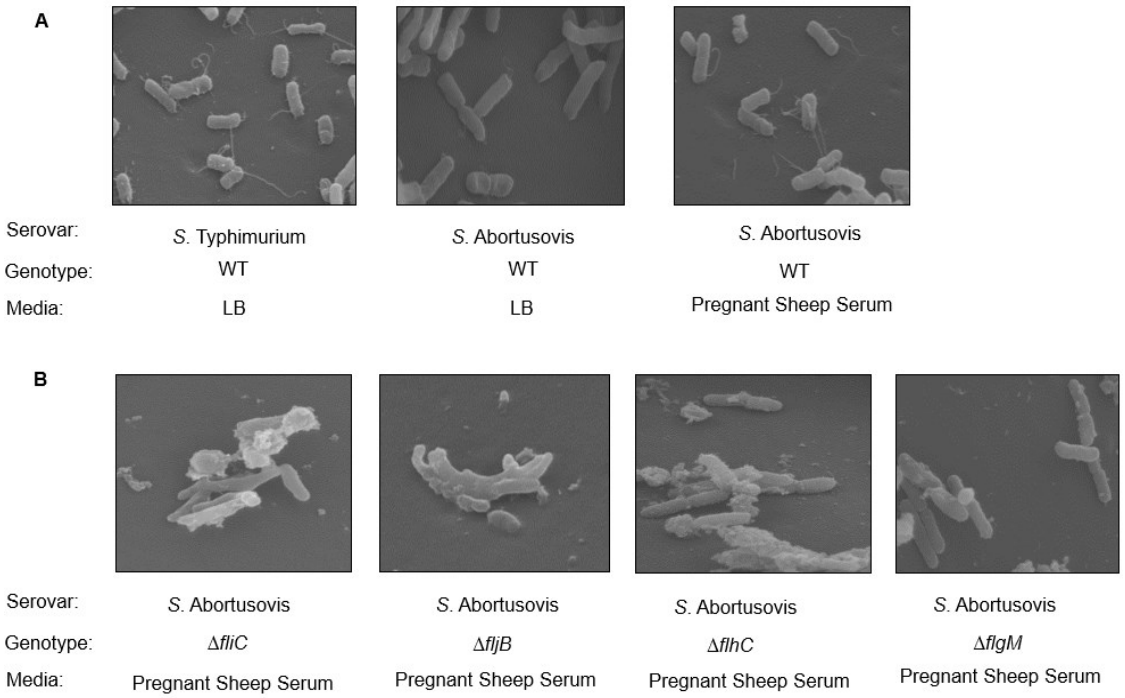

Figure 11. *Salmonella* Tyhpimurium (SR11) wild type (WT) and *Salmonella* Abortusovis (SS44) WT were grown in LB over night (O/N) at 37°C with aeration. SS44 WT was also grown O/N in pregnant sheep serum at 37°C with aeration. Flagella were precipitated with trichloroacetic acid (TCA) for image acquisition. B) SS44 Δ*fliC* (LS1), SS44 Δ*fljB* (LS2), SS44 Δ*flgM* (LS3) and SS44 Δ*flhC* (LS4) were grown O/N in pregnant sheep serum at 37°C with aeration. Flagella were precipitated with TCA for image acquisition.

5.5 Induction of TLR5 *in vitro* experiment

IL-8 transcription was measured to test whether the flagellin of *Salmonella* Abortusovis is recognize by TLR5. Studies have shown that molecular structures that are highly conserved in PAMPs can be mutated during evolution to avoid the pathogen from innate immune recognition, such as *Campylobacter jejuni*[79].

Alignment of *Campylobacter jejuni* and *Salmonella* Typhimurium FliC sequences show that amino acids 89-96, that are required for TLR5 activation, are mutated in *Campylobacter jejuni*, which is consistent with the observation that the flagellin of *Campylobacter jejuni* is a poor ligand for TLR5 and inducer of IL-8 production (Figure 12).

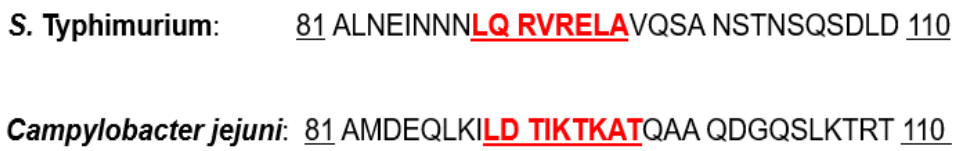

Figure 12. Amino acids 89-96 are required for TLR5 activation. Sequence alignment of flagellin proteins from *Salmonella* Typhimurium and *Campylobacter jejuni* that activate TLR5.

Our results shown that *Salmonella* Abortusovis flagellin is recognized by TLR5. Specifically data show that flagellin extracted from *Salmonella* Typhimurium growth in LB induced transcription of IL-8. As expected, no transcription was measured for *Salmonella* Abortusovis growth in LB, but a significant increase of transcription was measured for *S.* Abortusovis after growth in sheep serum, young sheep serum and pregnant sheep serum. In particular transcription of IL-8 was more induced when *Salmonella* Abortusovis was growth in pregnant sheep serum. (Figure 13)

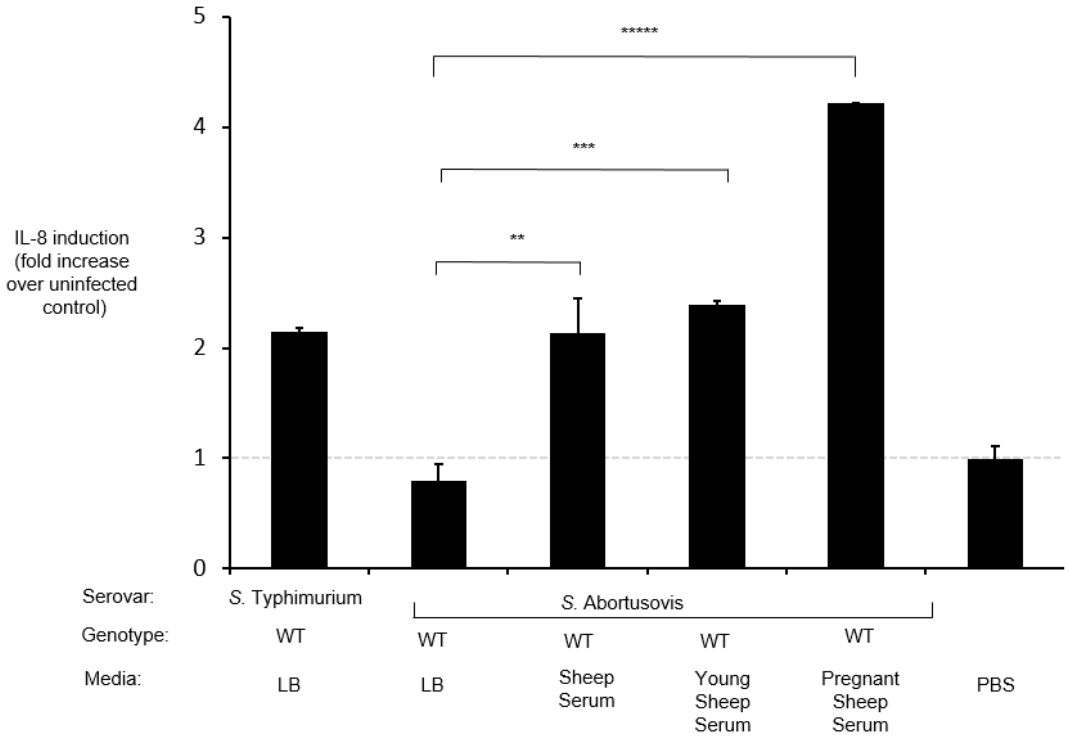

Figure 13. IL-8 transcription in T84 cells. *Salmonella* Typhimurium (SR11) wild type (WT) and *Salmonella* Abortusovis (SS44) WT were grown 16 h in LB media at 37°C with aeration. SS44 WT was also grown 16 h in undiluted sheep serum, young sheep serum, pregnant sheep serum at 37°C with aeration. Flagella were precipitated with trichloroacetic acid and filter sterilized. T84 cells were stimulated for 60 min with 5 µl of the filtered supernatant, and PBS was used as a control. RNA was extracted and relative gene expression was determined by real-time qRT-PCR. The data are expressed as fold increases of gene expression in infected cells over uninfected cells. The data are shown as geometric means (bars) from three independent experiments +/- the standard error. **, 0.01, ***, 0.001, *****, 0.00001. The dashed line indicates no change in *IL8* transcription (fold change of 1).

Those data suggested that maybe there are some components in the serum which regulate the expression of flagella in host-restricted *Salmonella* serovar.

6. Discussion

Salmonella flagella have an important role in bacterial physiology and virulence, and generally *Salmonella enterica* serovars are considered motile. However differences between serovars in motility and in host interaction has been reported. The role of flagella in *Salmonella* has been studied in serovars like Typhimurium, Paratyphi A, Typhi and other, but not in *Salmonella* Abortusovis.

Based on those facts we decided to characterize the motility phenotype and the role of flagella in *Salmonella* Abortusovis.

In *Salmonella* Typhimurium, flagella are appendages that are always expressed on the surface. Recognition of flagellin, the major subunit of the flagellum, results in the activation of inflammatory responses and secretion of pro-inflammatory cytokines and chemokines during infection. Intestinal inflammation enhances fitness and transmission of *Salmonella* Typhimurium by the fecal-oral route.

A different scenario is observed in *Salmonella* Typhi. This serovar has virulence mechanisms that enable it to evade many of the host pathways that are used to detect non-typhoidal serovars. TviA, a regulatory protein specific to *Salmonella* Typhi,

represses expression of flagellin as the pathogen enter the host, thus limiting inflammatory responses at the site of entry. Evasion of host immune response allows for systemic dissemination of *Salmonella* Typhi[80].

It has been recently shown for *Salmonella* ParatyphiA, that in this serovar there is lower expression of flagella regulon, compared to *Salmonella* Typhimurium, and functional flagella are required for invasion, suggesting that these regulatory differences affect host-pathogen interactions and contribute to the distinct clinical manifestations resulting from typhoidal versus nontyphoidal infections in humans[81].

Furthermore, in those serovars the conditions in which expression of flagella can be activated or repressed are known.

Salmonella Typhimurium variant Copenhagen is the main cause of a bacterial disease resembling human typhoid fever in pigeons. Interestingly in this serotype expression of flagella is repressed as temperature exceeding 41°C[82].

In *Salmonella* Typhi, expression of flagella is regulated by osmolarity. In high osmolarity conditions, as in the tissues, the omosensitive EnvZ/OmpR system regulates expression of the flagellin repressor TviA, resulting in decreased

transcription of the flagellar operon. In contrast, flagella are expressed at lower osmolarity, such in the gut lumen[83].

Very little is known about regulation of flagella in *Salmonella* Abortusovis. Here we report that *Salmonella* Abortusovis is able to express flagella *in vitro* using media that reproduces *in vivo* conditions, indicating the presence of an unidentified compound in sheep serum that induces the expression of flagella in this serovar. Specifically, we observed that exposure to serum resulted in the upregulation of flagella proteins through various methods, including western blotting, flow cytometry and scanning electron microscopy.

Additional studies are needed to understand which compounds in serum induce flagella expression.

Salmonella Abortusovis appears to highly regulate expression of flagella in the host and this serovar seems to be able to control expression of flagellin to modulate activation of immune response.

Our results showed that *Salmonella* Abortusovis flagellin is expressed in sheep serum and young sheep serum. Expressed flagellin is able to induce transcription of IL-8, but

the most prominent increase in the transcription of flagellin was observed when *Salmonella* Abortusovis was cultured in pregnant sheep serum.

We speculate that maybe flagellin expressed by *Salmonella* Abortusovis during pregnancy induces transcription of IL-8 creating an environment not compatible with the life of the fetus, causing abortion.

We can propose that the following scenario unfolds *in vivo*. *Salmonella* Abortusovis is introduced into a flock by an asymptomatic carrier and other sheep may become infected by the fecal-oral route.

Once ingested, *Salmonella* Abortusovis does not express flagella and it is not recognized by the host. Upon entry of the mucosal tissue *S.* Abortusovis bacteria arrive in the intestine where two different situations can happen. Presumably, *Salmonella* Abortusovis can transiently colonize the intestinal tract, and the pathogen can be expelled through feces by the asymptomatic carrier. In the second situation, *Salmonella* Abortusovis invades the intestinal mucosa, evades activation of the local immune system and spreads to systemic sites such as liver, spleen and placenta.

In this scenario, we hypothesize that *Salmonella* Abortusovis is able to express flagella, causing abortion in sheep. *Salmonella* Abortusovis in this way is secreted by the sheep trough vaginal secretion and fetal tissues and bacteria can be ingested by naïve hosts (Figure 14).

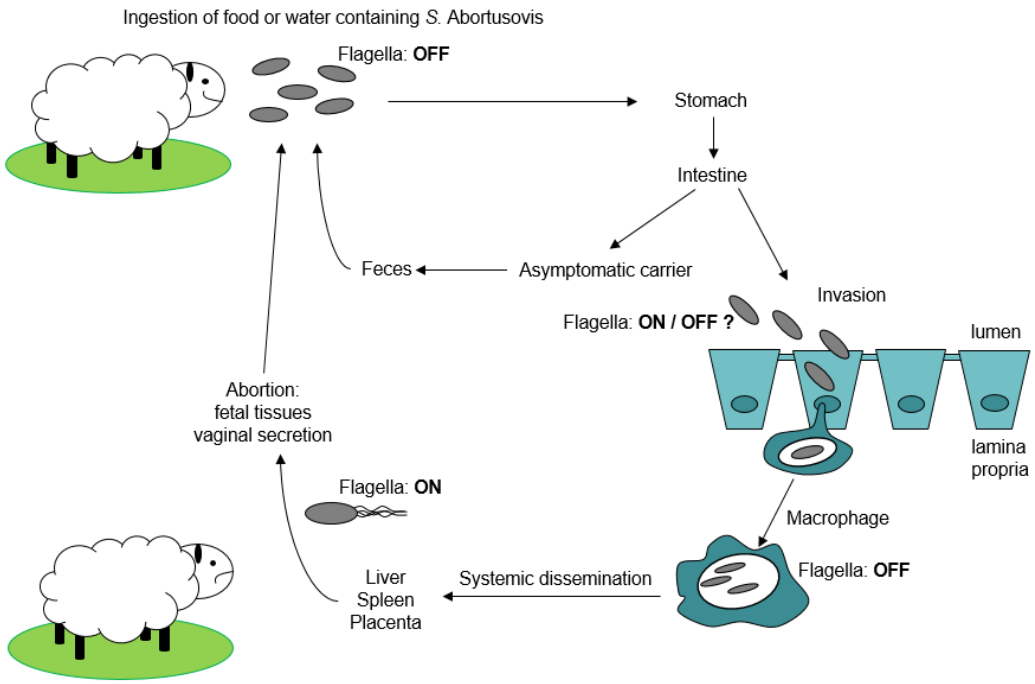

Figure 14. Proposed life cycle of *Salmonella* Abortusovis in sheep.
See text for details.

Addition studies are needed to understand if our predicted scenario is really occurring *in vivo*.

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