Early protection against *Salmonella* infection in chickens by modification of the initial host-pathogen interactions

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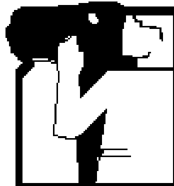

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How does a chick,
Who's not been about,
Discover the trick
Of how to get out?

Aileen Fisher

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LIST OF ABRREVATIONS

AMP	adenosine monophosphate
BGA	brilliant green agar
BHI	brain heart infusion broth
BPW	buffered peptone water
Cfu	colony forming units
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
CDC	Centre for Disease Control and Prevention
CE	competitive exclusion
DHB	2,3 dihydrobenzoate
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
IFN-γ	interferon-gamma
FAFLP	fluorescence amplified fragment length polymorphism
FOS	fructo-oligosaccharides
GTP	guanosinetriphosphate
Ins(1,4,5,6)P_4	inositolphosphate
IL	interleukin
kGy	kiloGray
LB	Luria-Bertoni
LPS	lipopolysaccharides
MAb	monoclonal antibody
MALT	mucosa -associated lymphoid tissues
MLEE	multilocous enzyme electrophoresis
MOS	manno-oligosaccharides
NADPH	nicotinamide adenine dinucleotide phosphate
NalR	nalidixin acid resistant
PABA	para-amino benzoic acid
PBS	phosphate buffered saline

PEEC	pathogen elicited epithelial chemoattractant
PMN	polymorphonuclear leukcoytes
RNA	ribonucleic acid
SCFA	short-chain fatty acids
SCV	Salmonella containing vacuole
SE-ILK	Salmonella Enteritidis immune lymphokines
SPI	Salmonella pathogenicity island
SPF	specific pathogen free
TMB	3,5,3',5' tetramethylbenzidine
TNF-α	tumor necrosis factor alpha
Th-cell	T-helper cell
TTSS	type three secretion system
VAR	Veterinary and Agrochemical Research Centre
VFA	volatile fatty acids
XLD	xylose lysine decarboxylase

GENERAL INTRODUCTION

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Salmonella : a brief introduction with emphasis on host range

Salmonellae were first described at the end of the nineteenth century and are named after D.E. Salmon, who isolated *Salmonella* choleraesuis, from pigs (Salmon and Smith, 1886; Le Minor, 1994). Strains belonging to the genus *Salmonella* comply with the definition of the family Enterobacteraceae : these are straight rods, aero-anaerobes, generally motile with peritrichous flagella, ferment glucose, often with the formation of gas and reduce nitrate into nitrite (Grimont *et al.*, 2000). Some serotypes have peculiarities : the avian-specific serotype Gallinarum, for example, has no flagellae and is therefore non-motile.

Salmonella nomenclature is complex and different systems are used in the literature. The nomenclature of the genus *Salmonella* started with the identification by White (1926) and Kaufmann (1941) on the basis of the serological identification of O (somatic) and H (flagellar) antigens. This 'one serotype – one species' concept was improved when it was demonstrated by DNA-DNA hybridization that there were only two species within the genus *Salmonella*, and that one of the species could further be divided in subspecies (Crosa *et al.*, 1973 ; Stoleru *et al.*, 1976 ; Le Minor *et al.*, 1982, 1986). These species are named *enterica* and *bongori*. *Salmonella enterica* is divided into six subspecies, which are referred to by a Roman numeral and a name (Brenner *et al.*, 2000). All subspecies can be further divided in numerous serotypes, based on their antigenic formula (Popoff and Le Minor, 1997; Popoff *et al.*, 2000, 2001, 2003). The serotypes of species *enterica* subspecies *enterica* are given names, such as for example *Salmonella enterica* subspecies *enterica* serovar Enteritidis, or *S.* Enteritidis in short. Of a total of more than 2450 serotypes currently known, *Salmonella enterica* subspecies *enterica* has more than 1450 (Brenner *et al.*, 2000). More recently, multilocous enzyme electrophoresis (MLEE, Boyd *et al.*, 1996), fluorescence amplified fragment length polymorphism (FAFLP, Scott *et al.*, 2001, 2002) and the use of DNA-microarrays (Chan *et al.*, 2003) were introduced for more precise identification of the genetic structure of *Salmonella*. Fields *et al.* (2002) already succeeded in specific identification of most common serotypes, by sequencing genes encoding O and H antigens and production of antigen-specific sequences to be used as probes in PCR reactions.

Salmonella strains can be classified according to the association with host animal species. Members of *Salmonella enterica* subspecies *enterica* are mainly associated with warm-blooded vertebrates, while strains of species *bongori* and the other subspecies of species *enterica* are usually isolated from cold-blooded animals (Brenner *et al.*, 2000, Uzzau *et al.*, 2000). *Salmonella* serotypes which are exclusively associated with one particular host species are referred to as being host-restricted. Examples are *S.* Typhi, host-restricted to humans and chimpanzees, *S.* Gallinarum, restricted to fowl, *S.* Abortusovis, restricted to sheep and *S.* Typhisuis, host-restricted to swine (Uzzau *et al.*, 2000). All these host-restricted serotypes produce systemic infection with different clinical signs. Generally there is no evidence of enteritis. Serotypes, which are prevalent in one particular host species but which can also cause disease in other host species are called host-adapted (Uzzau *et al.*, 2000). The major host-adapted serotypes are Dublin and Choleraesuis, adapted to cattle and swine, respectively. Although rare, natural transmission of these serotypes may occur to humans, in which they are rather virulent (Threlfall *et al.*, 1992). Therefore Kingsley and Baumler (2000) state that host-adaptation can be viewed as the ability of a pathogen to circulate and cause disease in a particular host population, a property that is unrelated to its virulence for other host species. Serotypes that usually induce a self-limiting gastroenteritis in a broad range of unrelated host species, while being capable of inducing systemic disease in a wide range of host animals, are called un-restricted or broad host-range serotypes (Uzzau *et al.*, 2000). Examples are *S.* Typhimurium and *S.* Enteritidis, the two most frequently isolated serotypes from poultry.

Poultry as a source of *Salmonella* infection in man

In humans, disease caused by the host-restricted serotype *S.* Typhi is called typhoidal salmonellosis, whereas disease caused by any other serotype is called non-typhoidal salmonellosis. Worldwide, two major changes in the epidemiology of non-typhoidal salmonellosis have occurred during the second half of the 20th century. First, *Salmonella* Typhimurium strains resistant to multiple antibiotics, such as *S.* Typhimurium DT104, have emerged and second, *Salmonella* Enteritidis has emerged as a major chicken and egg associated pathogen (Rabsch *et al.*, 2001). This literature review will be restricted to the emergence of *Salmonella* Enteritidis and the role of poultry as a source of *Salmonella* infection in humans.

Humans

In the United States, approximately 40000 cases of human salmonellosis are reported annually, and an estimate of the real number of non-typhoidal salmonellosis cases by the Centre for Disease Control and Prevention (CDC) is about 1.4 million (Mead *et al.*, 1999). Indeed, the degree of reporting salmonellosis seems to be an important factor in under-estimating the number of cases, since a small country as The Netherlands report a mean of 50000 cases annually (Van Pelt and Valkenburgh, 2001). With more than 500 deaths per year, *Salmonella* is the most lethal food borne pathogen in the US. The majority of the isolates are Enteritidis (24.7%) and Typhimurium (23.5%) (Glynn *et al.*, 1998). In the US, *S.* Enteritidis steadily increased in frequency from being the sixth most common serotype in 1960 to the most frequently isolated serotype from humans in 1990 (Aserkoff *et al.*, 1970 ; Mishu *et al.*, 1994). In European countries, the same trends are being observed comparable with North-America. In Germany, *S.* Enteritidis (61.3%) and *S.* Typhimurium (23.4%) accounted for more than 80% of human isolates in 1995 (data from National Reference Centre for *Salmonellae* and other enteric pathogens, Robert Koch Institute, Berlin, Germany). Also in England and Wales *S.* Enteritidis isolates are the most frequent, with a percentage of 70,7% of human isolates in 1997 (Schroeter *et al.*, 1998).

In Belgium, a strong increase in *Salmonella* isolates in humans has been observed since 1987, especially caused by an increase in *S.* Enteritidis isolates. A peak was reached in 1999 with 15774 cases, while 10873 isolates were observed in 2001 (National Reference Centre for *Salmonella* and Shigella, annual report). Of these isolates, 64.2% were of the serotype Enteritidis and 21.5% were *S.* Typhimurium in 2001. For the serotype Enteritidis, phage type four was most frequently isolated (55% in 2000, 44.5% in 2001), followed by phage type 21 (29.5% in 2000, 35.5% in 2001). Of all isolates, 96% were of faecal origin, followed by isolates from blood (1.9%), urine (0.9%), pus, sputum, and a wide variety of sources. Enteritidis was the most frequently isolated serotype in all kinds of human samples.

Chickens

The increase in number of *S*. Enteritidis isolates in humans is directly related with an increase in number of isolates from chicken meat and eggs. The increase in *S*. Enteritidis isolates from chicken origin started between 1965 and 1980 in Western countries (Lee, 1974 ; Rabsch *et al.*, 2000). Interestingly, the increase in *S.* Enteritidis isolates coincided with a decrease in *S.* Gallinarum in poultry. It has been proposed that eradication of *S.* Gallinarum resulted in loss of flock immunity against the O9-antigen, enabling *S.* Enteritidis to spread (Bäumler *et al.*, 2000 ; Rabsch *et al.*, 2000). During the last 20 years, *S.* Enteritidis has replaced other serotypes as commonest serotype in poultry worldwide (Poppe, 2000). A study from McIlroy and McCracken (1990) showed an increase in *S.* Enteritidis isolates from chickens ranging from 3.3% in 1985 to almost 50% of all *Salmonella* isolates in 1988 in the United Kingdom. The number of isolates in The Netherlands increased from about 5,5% in 1986 to 15% in 1992 and about 20% in 2000, being the most predominant serotype (Van Duijkeren *et al.*, 2002).
Poultry is still the main reservoir for *Salmonella* in Belgium. In 2001, 890 *Salmonella* strains from poultry origin were isolated, which is 52% of all isolates from animals (Veterinary and Agrochemical Research Centre (VAR), annual report). Of all poultry isolates, serotype Enteritidis was the most predominant (29%), as in former years, followed by Virchow (16%) and Typhimurium (5%). In layers, more than half of the isolates in 2001 were Enteritidis (59%). In hatcheries, breeding farms and during production Virchow and Enteritidis were both frequently isolated. In broilers, a wide spectrum of serotypes was isolated : Enteritidis (23%), Agona (15%), Infantis (11%), Virchow (9.9%), Anatum (9.4%) and Hadar (8.9%) were the most predominant. In an epidemiological study of Heyndrickx *et al.* (2002), 10 of 18 investigated Flemish broiler flocks were *Salmonella* positive. Of these *Salmonella* positive flocks, most were positive for multiple serotypes. In Belgium, among poultry isolates, *S.* Enteritidis strongly increased from 1992 until 1997, after which it stabilized. In 1996, in finished chicken products 15.1% of prepared poultry products was contaminated with *Salmonella*, while for chicken parts and laying hen carcasses contamination was both 4.2% (Uyttendaele *et al.*, 1998).

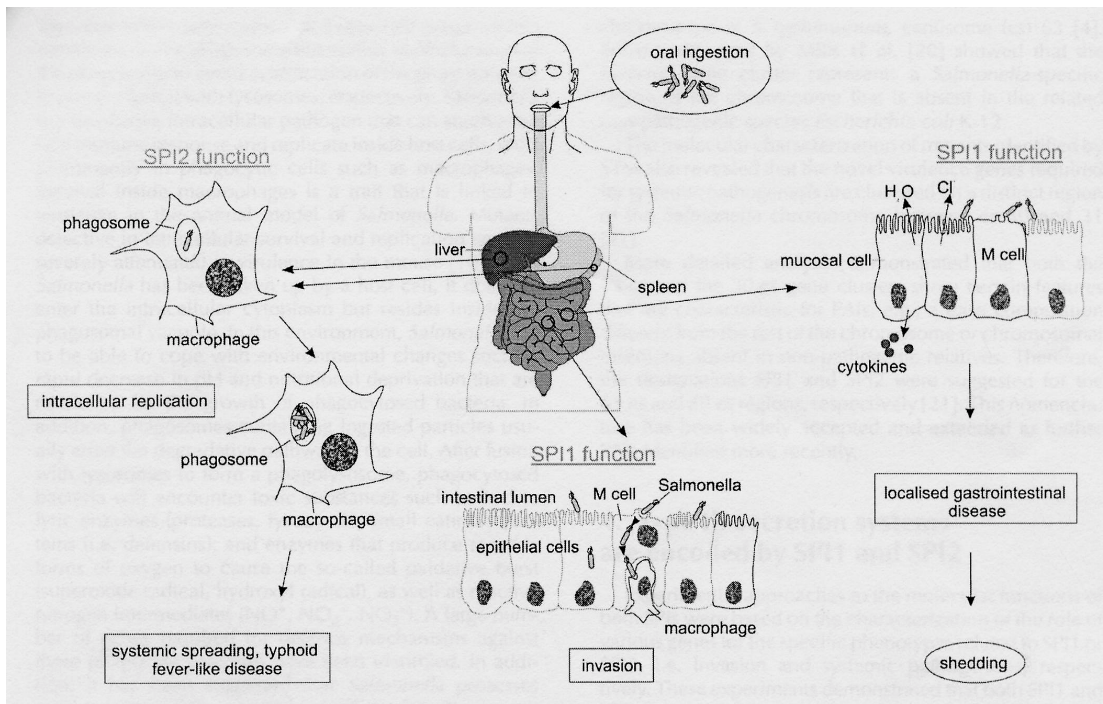

Figure 1. Schematic representation of host-pathogen interactions during pathogenesis of *Salmonella* infections. *Salmonella* Pathogenicity Island I (SPI-1) function is required for the initial stages of salmonellosis, i.e. the entry of *Salmonella* in non-phagocytic cells by triggering invasion and the penetration of the gastrointestinal epithelium. Furthermore, SPI-1 function is required for the onset of diarrhoeal symptoms during localized gastrointestinal infections. The function of SPI-2 is required for later stages of the infection, i.e. systemic spread and the colonization of host organs. The role of SPI-2 for survival and replication in host phagocytes appears to be essential for this phase of pathogenesis.

Host-pathogen interactions in *Salmonella* infections

Chickens usually are infected by oral uptake of bacteria from the environment. Contaminated litter is the most important source of infection. Besides this horizontal transmission, vertical transmission through eggs is also considered as a source of infection in chickens (Poppe, 2000). Most of the data mentioned below with respect to host-pathogen interactions are derived from *in vitro* studies in mammalian cell lines and *in vivo* studies in mice. Chicken specific data are scarce, and it is possible that the bacteria-host interactions are host-dependent. It is known that *S.* Enteritidis can persist in chicken organs and intestine and predominantly colonizes the caeca of the animal (Desmidt et al., 1997, 1998). Less is known on interactions with chicken epithelial cells and macrophages, but the majority of the mechanisms described below for the in vitro and mouse models may also apply to the chicken.

The pathogenesis of *Salmonella* can be divided in two distinct phases, i.e. the intestinal and the systemic phase of the infection, both regulated by genes of a different *Salmonella* pathogenicity island (SPI-1 and SPI-2) (Figure 1).

The intestinal phase of the infection (summarized in Figure 2)

Salmonella bacteria are able to survive gastric acidity and can therefore pass the stomach to reach the intestinal tract of the animal (Kwon and Ricke, 1998). In the chicken, the caeca are the predominant sites of *Salmonella* colonization (Desmidt *et al.*, 1997, 1998). The bacteria can adhere to epithelial cells by specific adhesin-receptor interactions in which bacterial fimbriae and mannosylated residues of the mucous and resorptive epithelial cells are involved (Dibb-Fuller *et al.*, 1999; Vimal *et al.*, 2000). An important step in the pathogenesis of *Salmonella* is the process of invasion into intestinal epithelial cells. Upon contact with intestinal epithelial cells, *Salmonella* bacteria inject a set of bacterial proteins into host cells via the bacterial pathogenicity island I (SPI-1) type three secretion system (TTSS) (Zhou and Galán, 2001). Type three secretion systems are specialized organelles whose core function is the delivery of bacterial proteins into eukaryotic cells (Hueck, 1998; Galán and Collmer, 1999; Cornelis and Van Gijsegem, 2000). A subset of structural components of TTSSs, evolutionary related to the flagelar export apparatus, has been shown to form a supramolecular structure termed the needle complex (Kubori *et al.*,

1998;2000). This structure spans both the inner and outer membranes of the bacterial envelope and resembles the flagelar hook basal body complex. The needle complex is composed of two pairs of membrane-localized rings joined by a hollow cylindrical structure that serves as the base for an externally localized needle-like protruding structure. The pathogenicity island I of *Salmonella* is a large genetic element on centisome 63 of the chromosome encoding multiple proteins necessary to assemble a complex TTSS apparatus as well as some effector proteins, injected by the needle complex into intestinal epithelial cells (Lostroh and Lee, 2001). The effector proteins of SPI-1 interact with host proteins of the intestinal epithelial cells and cause cellular changes associated with pathogenesis. *Salmonella* entry into intestinal epithelial cells is strictly dependent on the function of the host cell actin cytoskeleton. Upon establishing contact with epithelial cells, *Salmonella* induces actin cytoskeletal rearrangements at the site of bacterial-host cell contact that directs bacterial internalization (Finlay *et al.*, 1991; Francis *et al.*, 1993; Ginocchio *et al.*, 1994). In epithelial cells, the monomeric globular form of actin (G-actin) and the ordered multimeric form termed filamentous actin (F-actin) are maintained at a dynamic state by a number of actin-binding proteins (Theriot and Mitchison, 1993; Sun *et al.*, 1999; Bamburg, 1999; Bartles, 2000). The concentration of free G-actin is kept below the concentration required for actin polymerization (critical concentration). In fact, a constant process of depolymerization and polymerization keeps the net length of the actin polymers constant and this dynamic process ensures rapid responsiveness to environmental changes (Zhou and Galán, 2001). All the regulatory steps for actin organization in the eukaryotic cell provide opportunities for *Salmonella* effector proteins to intercept and redirect the actin cytoskeleton to mediate bacterial entry. The effector proteins SopE, SopE2 and SopB, that are injected in the epithelial cells by the TTSS of SPI-1, activate the Rho family GTPases CDC42 and Rac, that trigger a series of signal transduction events that lead to actin cytoskeleton rearrangements (Chen *et al.*, 1996; Hobbie *et al.*, 1997; Hardt *et al.*, 1998; Bakshi *et al.*, 2000; Stender *et al.*, 2000; Zhou *et al.*, 2001). These cytoskeleton rearrangements lead to the so-called membrane ruffles and the internalization of the bacteria by the epithelial cells. The cells quickly recover from the actin rearrangements and regain their normal architecture, mediated by the *Salmonella* protein SptP, which opposes the CDC42 and Rac activating function of the bacterial Sop proteins (Takeuchi, 1967; Fu and Galán, 1998, 1999). Another family of *Salmonella* SPI-1 effector proteins involved in

bacterial entry in epithelial cells is the Sip family. SipA modulates the internalization process by decreasing the critical concentration for actin polymerization, inhibiting depolymerization of actin filaments and increasing the actin bundling activity of T-plastin (Zhou *et al.*, 1999, 1999b, Hayward and Koronakis, 1999). In this way, SipA increases the stability of actin bundles that drive and support the growth of membrane ruffles and filopodia that engulf and internalize the bacteria by modulating the actin bundling activity of plastin.

Once inside the epithelial cell, the bacteria are thought to suppress apoptotic pathways in the cell. It is suggested that the SPI-5 effector protein SigD, regulated by the SPI-1 protein HilA, may play a role in delaying the onset of apoptosis (Steele-Mortimer *et al.*, 2000). This could create a niche in which the bacteria can multiply extensively.

Salmonella bacteria induce an inflammatory response in the intestinal epithelium that leads to the infiltration and transmigration of polymorphonuclear leukocytes (PMN), mediated by interleukin 8 (IL-8) and pathogen-elicited epithelial chemoattractant (PEEC) production (McCormick *et al.*, 1998). Both bacterial internalization and the infiltration of PMN are the consequence of the activation of CDC42 and Rac and therefore require the same bacterial effectors, i.e. SopE, SopE2 and SopB (Chen *et al.*, 1996; Hobbie *et al.*, 1997; Hardt *et al.*, 1998; Zhou *et al.*, 2001). These bacterial proteins thus play an essential role in the production of pro-inflammatory and other cytokines (Hobbie *et al.*, 1997). Although the same bacterial effector proteins of the SPI-1 TTSS stimulate both bacterial internalization and cytokine production, this does not mean that invasion per se stimulates pro-inflammatory cytokine production. Indeed, although CDC42 and Rac have to be activated for both responses, the actual downstream effectors to reach both responses are different (Chen *et al.*, 1999). As an example, cytokine production is mediated by stimulation of MAP kinase pathways (Erk, p38 and Jnk) that follow the activation of CDC42 and Rac. These eukaryotic proteins, however, are not necessary for actin rearrangements, so invasion is theoretically not needed for cytokine production (Galán, 2001). Moreover, also SipA has been reported to induce PMN transmigration when it was applied exogenously to cell culture systems (Lee *et al.*, 2001). Finally, the SPI-1 TTSS is not the only mechanism by which *Salmonella* can stimulate cytokine production by epithelial cells. Indeed, like many other bacteria, *Salmonella* encodes pathogen-associated molecular patterns (PAMPs), such as flagella and lipopolysaccharides (LPS), that are capable of stimulating the innate immune system (Medzhitov and Janeway, 2000a,

2000b). This requires the presence of functional Toll-like receptors for the bacterial PAMPs in the infected cell. It is likely that the apical side of the epithelial cells may not display functional Toll-like receptors to prevent potentially harmful responses to the gut flora. Therefore, when *Salmonella* bacteria are in the lumen, possibly only the SPI-1 TTSS may be the mechanism to stimulate cytokine production. In addition to the induction of pro-inflammatory cytokines and PMN transmigration, *Salmonella* induces other cellular changes that can apparently modulate chloride secretion, directly contributing to diarrhea (Eckmann *et al.*, 1997). Modulation of chloride secretion seems to be a direct consequence of products of the inositol phosphate metabolism. In particular, *Salmonella* infection of intestinal epithelial cells leads to the accumulation of Ins(1,4,5,6)P_4, that exerts its function by antagonizing the closure of chloride channels in the gut. Accumulation of Ins(1,4,5,6)P_4 requires the activity of the bacterial inositol phosphatase SopB (Norris *et al.*, 1998; Wallis and Galyov, 2000; Zhou *et al.*, 2001). The ability of *Salmonella* to cause a self-limiting diarrhea is of great importance for the bacteria itself, since cross-infection by the fecal-oral route is the main infection route (Wallis and Galyov, 2000).

Transcriptional regulation of SPI-1 genes is a primary mechanism for controlling the production of SPI-1 TTSS factors in response to environmental and physiological parameters (Bajaj *et al.*, 1996; Lucas and Lee, 2000). The expression of genes encoding the SPI-1 TTSS apparatus and most of its effectors requires HilA, a transcription factor encoded on SPI-1, that is influenced by environmental factors such as pH, osmolarity, oxygen tension and growth phase (Bajaj *et al.*, 1995, 1996; Lostroh *et al.*, 2000). SPI-1 encodes additional transcriptional regulators and different regulatory proteins. New sensory/regulatory mechanisms are discovered recently, making the regulation of SPI-1 gene expression a very complex process (Lostroh and Lee, 2001; Hansen-Wester and Hensel, 2001). It is proposed that the expression of SPI-1 TTSS is increased in the gut lumen, where it encounters a low oxygen tension and high osmolarity, while the expression of the SPI-2 TTSS, involved in macrophage survival, is down-regulated in this environment (Hansen-Wester and Hensel, 2001).

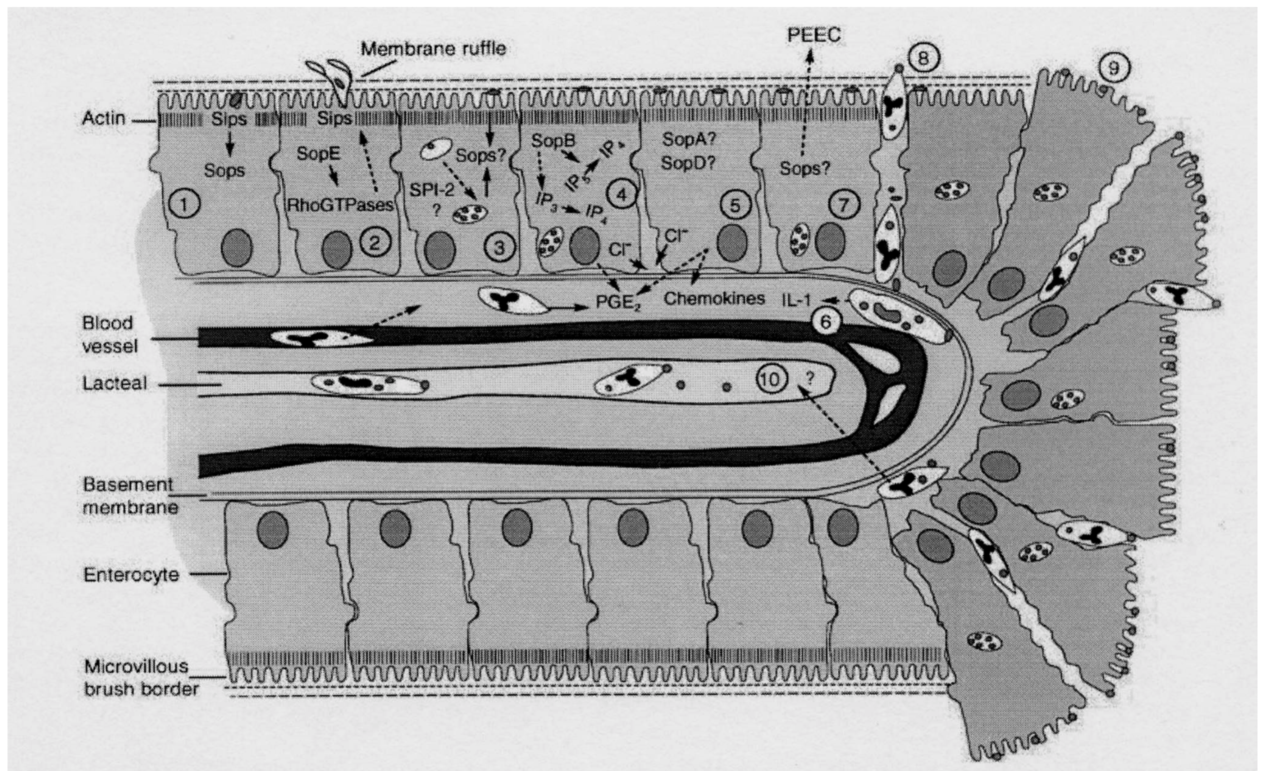

Figure 2. Schematic representation of proposed events affecting *Salmonella*-induced enteropathogenic responses in intestinal mucosa. (adapted from Wallis and Galyov, 2000)

1. *Salmonella* interacts with enterocytes and delivers Sop proteins into the cell cytoplasm via a SPI-1 and Sip-dependent pathway.
2. Sip proteins, SopE and possibly other Sops induce enterocyte membrane ruffling promoting bacterial invasion.
3. Intracellular bacteria reside within membrane-bound vesicles and possibly continue translocation of SPI-1 secreted effectors. The relative contribution of extracellular bacteria and intracellular bacteria to the translocation process remains to be elucidated. The replication of *Salmonella* within the vesicles is promoted by SPI-2.
4. The intracellular SopB protein affects inositol phosphate signalling events. One such event is a transient increase in concentration of Ins(1,4,5,6)P_4, which in turn can antagonize the closure of chloride channels influencing net electrolyte transport and thus fluid secretion.
5. *Salmonella*-infected epithelial cells secrete chemokines and prostaglandins (PGE) that act to recruit inflammatory to foci of infection. The release of at least some chemokines and prostaglandins is probably affected by the intracellular activity of Sops.
6. *Salmonella* interacts with inflammatory cells and stimulates the release of pro-inflammatory cytokines that enhance the inflammatory response.
7. *Salmonella*-infected epithelial cells release pathogen-elicited epithelial chemoattractant (PEEC) across the apical membrane. This substance acts to stimulate PMN transepithelial migration between the enterocytes.
8. Infiltrating inflammatory cells phagocytose *Salmonella* ; the fate of these cells is unclear.
9. *Salmonella*-infected enterocytes become extruded from the villus surface, leading to shedding of infected cells into the intestinal lumen and resulting in villus blunting and loss of absorptive surfaces.
10. Some of the infected cells migrate to the draining lymphatics, carrying *Salmonella* to systemic sites. The precise route of this process is not known.

The systemic phase of the infection (summarized in Figure 3)

Due to the pro-inflammatory cytokine production by epithelial cells, macrophages are attracted to the intestinal wall. *Salmonella* bacteria can be internalized by phagocytosis by these cells. *Salmonella* bacteria can survive within and replicate in the macrophage, and can thereby spread to the internal organs, such as liver and spleen, where the bacteria can be found in large numbers (Barrow, 1999). The replication in the macrophage is regulated by the *Salmonella* pathogenicity island 2 TTSS, which translocates bacterial proteins across the vacuolar membrane, where the bacteria reside (Hensel *et al.*, 2000).

Salmonella bacteria have developed different mechanisms for survival in macrophages. After internalization, *Salmonella* resides in a unique organelle, the so-called '*Salmonella* containing vacuole' (SCV). Early events in the biogenesis of the SCV are characterized by the acquisition of early endosome and recycling compartment markers, such as EEA1 and the transferrin receptor. The rapid acquisition of early endosomal markers suggests a rapid fusion of the nascent SCV with early endosomes. In contrast to early endosomes, direct interactions between SCVs and late endosomal compartments do not occur. *Salmonella* bacteria are capable of inhibiting the fusion between lysosomes and SCVs (Buchmeier and Heffron, 1991). This is documented by the exclusion of mannose-6-phosphate receptors and lysosomal enzymes from the SCV (Garcia-del Portillo and Finlay, 1995; Gorvel and Méresse, 2001). Bacteria start to replicate in the SCV from 3 hours after infection of the macrophages, although the maturation process of the SCV is completed at 1 hour post-entry. The possibility exists that the bacteria are able to sense certain cellular changes in the host cell. Co-incident with the bacterial intracellular replication, membrane tubules appear in the host cell which originate from the SCV and extend into the cell (Garcia-del Portillo *et al.*, 1993). These tubules are called *Salmonella* induced filaments (Sifs) and are induced by the bacterial sifA gene that is regulated by genes of SPI-2 ((Beuzón *et al.*, 2000). Since *Salmonella* replicates in the SCV the function of Sifs could be the maintenance and enlargement of the membrane of the vacuolar structure so that the bacteria can replicate (Stein *et al.*, 1996; Beuzon *et al.*, 2000; Brumell *et al.*, 2001 2002).

Most bacteria are killed by reactive oxygen and nitrogen species of the macrophage. The eukaryotic NADPH phagocyte oxidase is among the most effective antimicrobial

weapons employed by phagocytic cells. The enzymatic complex catalyses the univalent reduction of oxygen to superoxide, an antibacterial radical that serves as a precursor to more toxic reactive oxygen species as hydrogen peroxide and hydroxyl radicals (Babior, 1995). In resting cells, compartmentalization of membrane-bound and cytosolic components of the NADPH phagocyte oxidase insures that production of cytotoxic radicals is prevented. All subunits migrate to each other after stimulation of the macrophage and assemble to an active enzyme complex, either at the plasma or phagosomal membrane (Vazquez-Torres and Fang, 2001). *Salmonella* bacteria have developed strategies to avoid or inhibit this enzymatic complex. Besides the fact that *Salmonella* contains superoxide dismutases, catalases, scavengers and antioxidant defenses (Buchmeier *et al.*, 1995; Buchmeier *et al.*, 1997; Lundberg *et al.*, 1999), *Salmonella* inhibits trafficking of NADPH phagocyte oxidase-containing vesicles to the vicinity of the SCV, a process which is regulated by SPI-2. The exact mechanism is not known, but the SPI-2 protein SpiC is known to block fusion of vesicles harboring active NADPH phagocyte oxidase and the SCV membrane. Moreover, it is suggested that *Salmonella* promotes associations between the SCV and the actin cytoskeleton by a SPI-2 dependent process. This actin web could possibly hinder the targeting of incoming NADPH phagocyte oxidase-containing vesicles to SCV (Vazquez-Torres and Fang, 2001).

Salmonella bacteria exploit host cell death pathways and alter the viability of macrophages in their benefit. Important in this view is the induction of apoptosis and necrosis at particular stages of the infection. It is proposed that rapidly after *Salmonella* infection of macrophages a caspase-1 dependent cell death occurs, triggering IL-1 production by the macrophage (Knodler and Finlay, 2001). It is not yet known whether this cell death is apoptosis or necrosis. In the case of phagocytic macrophages, binding of the phosphatidylserine receptor with phosphatidylserine on the surface of the apoptotic cell triggers the release of anti-inflammatory cytokines (Fadok *et al.*, 2000). Necrosis is associated with the loss of membrane integrity and the release of intracellular contents, attracting immune cells in this way. In the case of the initial macrophage infiltration in the intestinal wall after *Salmonella* infection, the bacteria are proposed to induce cell death which attracts immune cells, so necrotic cell death should be induced (Knodler and Finlay, 2001). The caspase-1 dependent cell death is induced by the SPI-1 effector protein SipB (Hersh *et al.*, 1999). It seems to be the case that while SPI-2 is needed for intracellular replication and the systemic phase

of infection, SPI-1 genes are still involved in the interactions between immune cells and the bacteria in the intestinal wall. This induction of inflammation is expected to aid in the dispersal of *Salmonella* from the gastro-intestinal tract (Monack *et al.*, 2000). The bacteria are thought to sense the activation state of the macrophage by an unknown mechanism so that the way of inducing cell death can be altered. Indeed, once *Salmonella* has established a systemic infection, rapid killing of a host cell would not be advantageous for the bacteria. At this stage, the bacteria reside in an intracellular niche in which they can replicate, and delay the onset of apoptosis to allow sufficient time to replicate and reach deeper tissues (Jesenberger *et al.*, 2000; Van Der Velden *et al.*, 2000). The delayed type of cell death is thought to be apoptotic, not causing an inflammation.

Genes of SPI-2, involved in survival inside the macrophage, are known to be induced by nutritional starvation, low concentrations of Ca^{2+} , Mg^{2+} and phosphate and an acidic pH (Hensel, 2000; Garcia-del Portillo, 2001). These conditions seem to reflect the conditions inside the SCV of the macrophage. As an example, the SCV acidifies from pH 6 to about 4 in the first hours post-infection. This pH decrease triggers SPI-2 expression (Cirillo *et al.*, 1998; Holden, 2002). It is interesting that *Salmonella* is capable of regulating both SPI-1 and SPI-2 in a temporal and spatial way so that only that panel of virulence genes is expressed at those conditions when they are needed. Even more, *Salmonella* is capable of downregulating and upregulating gene expression to make sure the host response is influenced in such a way that it is beneficial for the bacteria.

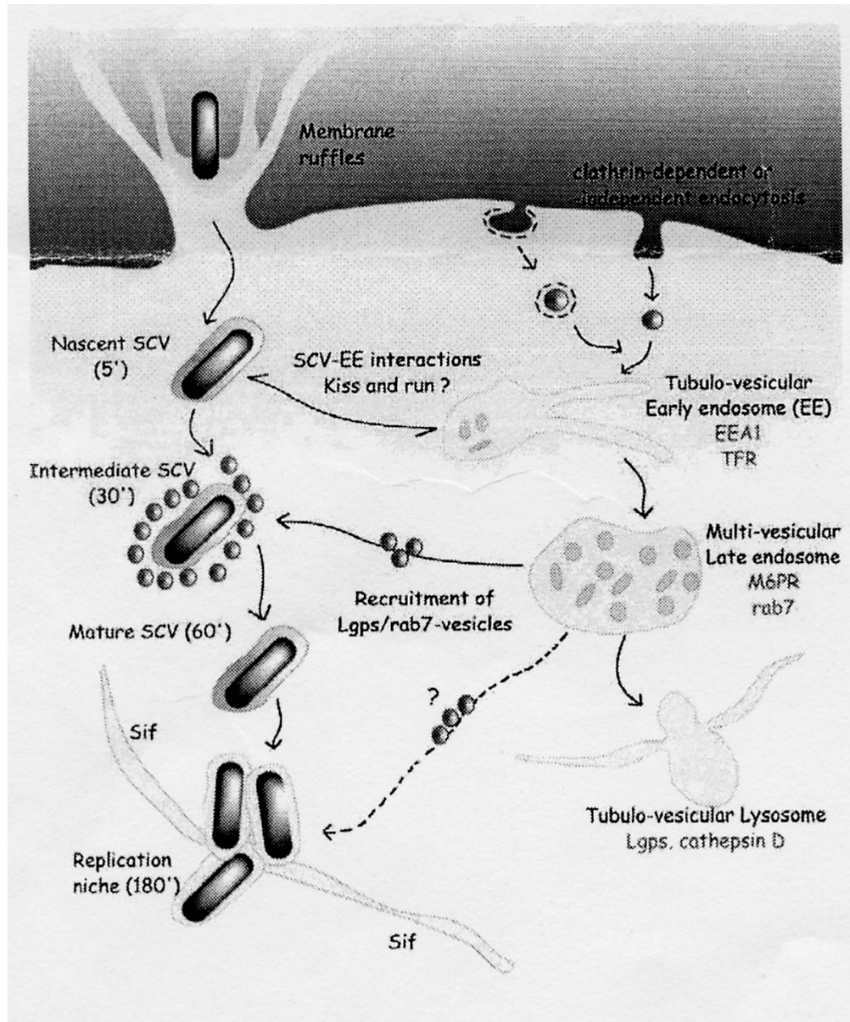

Figure 3. Intracellular pathway of *Salmonella*. (adapted from Gorvel and Méresse, 2001)

After internalisation the bacterium is found in a nascent *Salmonella* containing vacuole (SCV) interacting with early endosomes, probably by a 'kiss and run' mechanism, acquiring EEA1 and the transferring receptor. Then, the SCV matures to an intermediate stage characterized by the accumulation of Lgps/rab7-vesicles likely arising from late endosomes. This leads to the formation of a mature SCV in which the bacterium starts to replicate after a lag phase of 2-3h. Replication is concomitant with the formation of *Salmonella*-induced filaments (Sifs).

Control of *Salmonella* in poultry

In 1992, the Council of the European Community issued a directive (EU-92/117 and subsequent amendments) requiring member countries to monitor for zoonotic agents, to specify measures for reducing the risk of introduction of *Salmonella* on the farm and to control *Salmonella* in parent flocks. These instructions basically require a top – down approach, which means that measures are very strict in grand parent and parent flocks. These measures are expected to be of benefit to the general sanitary status of the whole production chain. This top-down concept implies that *Salmonella* is transmitted vertically in the poultry population, what is, to some extent, the case for invasive serotypes, such as Enteritidis, Typhimurium, Bertha, Thompson, Infantis and Hadar. For non-invasive serotypes, only egg-shell contamination can lead to vertical transmission. Since horizontal transmission is as important as vertical transmission in *Salmonella* infections in poultry, a top-down approach not including measures in the commercial broiler and layer flocks is doomed with limited succes. The original ambition of the EU of eradicating zoonotic agents from the animal production chain and thus producing animal products free from zoonotic agents has been considerably tempered. Plans now are designed to reduce the infection pressure of specified zoonotic agents at the different levels of the production chain.

Since poultry is an important source of infection for humans, protection measures are of utmost importance. *Salmonella* control schemes have been put in place in most EU countries in recent years. These include pre-harvest, harvest and post-harvest measures. All of these are equally important and each type of measure has a more or less important effect on reducing the *Salmonella* incidence, but no measure is successful on its own. Harvest measures are essentially hygienic measures during catching and transport, while post-harvest measures include both hygienic measures and the application of decontaminating treatments on the meat or eggs. However, carcass disinfectants are prohibited in the European Community and decontamination of fresh table eggs is difficult. Therefore, prevention and monitoring/eradication during the live phase (pre-harvest) is of great importance in Europe. Pre-harvest prevention and control measures include preventive hygienic measures as well as physical and chemical decontamination treatments of feed, drinking water and the environment of the birds (Campbell *et al.*, 1986; Rouse *et al.*, 1988, Screenivas 1998,

Farkas, 1998; Davies and Hinton, 2000). Eradication of contaminated flocks has not been shown to be an effective measure.

The use of genetically more resistant chicken lines can also help in controlling *Salmonella* (Kramer *et al.*, 2001). Feed additives to control *Salmonella* in poultry are widely used, with the rate of success dependent on the additive used. Also undefined mixtures of bacteria are used to compete with *Salmonella* in the chicken intestinal environment. Finally, vaccination with dead and live *Salmonella* bacteria is probably the most widely used control measure against *Salmonella*. In this literature review only pre-harvest control measures will be discussed.

A) Feed additives to control *Salmonella*

1) Antibiotics

Antibiotics can be given to the animals to clear a flock from *Salmonella*. Fluoroquinolones, such as enrofloxacin, can eliminate *Salmonella* Enteritidis from poultry flocks, especially when competitive exclusion cultures are used directly after the antibiotic treatment (Humbert et al., 1997; Seo et al., 2000). Care should be taken however when using antibiotics since stopping antibiotic treatment can result in serious increases in *Salmonella* infections in poultry flocks, due to the killing of the anaerobic microflora by the used antibiotics (Manning et al., 1992). Also trimethoprim and polymyxin B have been shown to eliminate *Salmonella* Enteritidis from infected flocks (Goodnouch and Johnson, 1991). Next to their prophylactic and curative use, antibiotics have also been given in feed to poultry for their growth promoting effects. Most antibiotic growth promoters act by modifying the intestinal flora, especially targeting gram-positive bacteria which are associated with poorer health and performance of the animal (Bedford, 2000). Antibiotics have been used for many years as growth promoting agents. In recent years, concerns arose about the use of antibiotics in livestock. Antibiotic feed additives were linked to the emergence of multiple drug resistant bacteria (Wray and Davies, 2000). The presence of undesired antibiotic residues in meat and environmental contamination has largely added to the public concerns regarding the use of antibiotics in the feed. As a consequence at the end of June 1999 the majority of antibiotics as growth promoters in monogastric diets

have been banned within the E.U.. Exceptions are the anticoccidial products with antibiotic activity salinomycin, monensin and narasin and two antibiotics unrelated to therapeutically used products in humans, being flavomycin and aviliamycin. Effects of these antibiotics on *Salmonella* shedding and intestinal colonization are variable (Ford *et al.*, 1981; Hinton, 1988; Humpert *et al.*, 1991; Manning *et al.*, 1994; Bolder *et al.*, 1999).

2) Prebiotics

Prebiotics have been defined by Gibson and Roberfroid (1995) as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health". Based on this definition, Russell (1998) formulated the criteria according to which a substance can be a prebiotic. First, prebiotics are always feed ingredients that are not digested by the host, not or little used and / or metabolized as they pass through the upper portion of the intestinal tract, so they can reach the flora of the large intestine. Second, they have to be able to serve as a substrate for one or more bacterial species with a potentially beneficial effect on the host. Finally they have to be able to cause a shift in the microflora that improves the health of the host. In principle only non-digestible, fermentable feed components are prebiotics. These carbohydrates are divided in groups based on their molecular length: mono-, di-, oligo- and polysaccharides (Iji and Tivey, 1998).
Mannose is by far the most commonly used monosaccharide feed additive (Allen *et al.*, 1997). Mannose has been shown to block *S.* Typhimurium adherence to chicken caecal mucus and intestinal epithelial cells in vitro (Oyofo *et al.*, 1989a, Craven *et al.*, 1997). Moreover, supplementation of 2.5% mannose in broiler diets resulted in a strong decrease in caecal colonization one week after infection of 3-days old chickens with a *S.* Typhimurium strain (Oyofo *et al.*, 1989b). According to the definition of Russell (1998), mannose is not a prebiotic product since it can be absorbed and metabolized by the host. Therefore effects of mannose on the intestinal flora will probably be very limited, if existent.
The most important natural disaccharides are sucrose, lactose and maltose. Isomerization products of these compounds can be used as prebiotics, e.g. lactulose

(based on lactose). Lactose (Corrier *et al.*, 1993), lactulose (Iji and Tivey, 1998) and lactosucrose (Terada *et al.*, 1994) reportedly have prebiotic effects in chickens. A reduction in the total number of positive *Salmonella* Enteritidis organ invasions was observed 14 days and 19 days after infection in chicks fed with lactose (Corrier *et al.*, 1993). Lactose is not hydrolyzed or absorbed intact from the intestinal tract of chicks, and as much as 50% of ingested lactose in poultry diets may be excreted unchanged. Because of its lack of digestion and absorption, lactose passes into the lower segments of the intestine and caeca. The hydrolization of lactose that does occur is primarily the result of the microflora utilization of the disaccharide (Tellez *et al.*, 1993a).
Oligosaccharides are usually defined as glycosides that contain a limited number of hexose or pentose units. Mostly they are obtained through enzymatic synthesis or hydrolysis. Fructo-oligosaccharides (FOS) are short-chain polymers of β 1-2-linked fructose units, which are produced commercially by hydrolysis of inulin or by enzymatic synthesis from sucrose or lactose (Le Blay *et al.*, 1999). FOS are the oligosaccharides most extensively studied in chickens with respect to their prebiotic effect and their activity against *Salmonella* (Fukata *et al.*, 1999) and *Campylobacter* (Schoeni and Wong, 1994). FOS lead to a reduction of colonization of the intestine by *Salmonella* in chickens, especially when the animals also received competitive exclusion flora in addition to FOS (Bailey *et al.*, 1991). Mannan-oligosaccharides (MOS) or mannose-based carbohydrates occur naturally in many products such as yeast cell walls and gums (Ishihara *et al.*, 2000). Commercial products are available for poultry which contain yeast cell wall fragments derived from *Saccharomyces cerevisiae* after centrifugation of lysed yeast cultures (Spring *et al.*, 2000). When 3-days-old chicks were orally challenged with 10^4 cfu *S*. Typhimurium, chickens receiving 4000 ppm of dietary MOS had reduced caecal *S*. Typhimurium concentrations compared with birds with no MOS in their feed. Also when *S*. Dublin was used as challenge organism, the number of birds that was positive in their caeca was less when MOS was part of the diet (Spring *et al.*, 2000).

Prebiotics can have a direct effect by binding of pathogens in the intestinal lumen. Indigestible carbohydrates with mannose residues may bind the type 1 fimbriae of *Salmonella* and therefore block the adhesion of the bacteria to the epithelial cells (Finucane *et al.*, 1999). More often, however, their effects are indirect, mediated by

metabolites which are generated by the intestinal flora that uses the prebiotics for their own metabolism and for fermentation (Cummings, 1981). Such metabolites include short-chain fatty acids, lactate, polyamines and bacteriocins. Prebiotics may constitute a substrate for the growth of intestinal flora. This multiplication of normal flora may inhibit the colonization with pathogenic bacteria. This phenomenon of inhibition is called "competitive exclusion" (see further).

3) Probiotics

A probiotic is defined as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 1989). The use of probiotics for farm animals was stimulated by the Schwann Committee in 1969 which recommended that antibiotics in animal feeds should be restricted, hereby creating a void which probiotics began to fill (Fuller, 1999). A very crucial event in the development of probiotics was the finding that newly hatched chickens could be protected against colonization by *Salmonella* Enteritidis by dosing a suspension of gut contents derived from healthy adult chickens (Nurmi and Rantala, 1973). This concept is called competitive exclusion (see below).

Many research efforts have focused on the probiotic use of *Lactobacillus* species, isolated from poultry (Gusils *et al.*, 1999). It was shown that *Lactobacillus plantarum*, *reuteri* and *salivarius* can protect chickens against *Salmonella* challenge. This is thought to be due to the 'exclusion effect' of these bacteria (see further) and the growth inhibiting effects of these lactobacilli against *Salmonella*, e.g. production of antimicrobial metabolite reuterin by *L. reuteri* (Jin *et al.*, 1996a,b; Mulder *et al.*, 1997; Bengmark, 1998; Pascual *et al.*, 1999).
Also inoculation of *Enterococcus* spp. protected chickens against *Salmonella* challenge, due to the combined effects of lactic acid production and bacteriocins (Audisio *et al.*, 1999, 2000) . Finally, supplementation of live yeast cells reduced caecal colonization in broiler chickens when they were infected at their first day of life (Line *et al.*, 1998).

4) Synbiotics

A synbiotic is, in its simplest definition, a combination of probiotics and prebiotics (Collins and Gibson, 1999; Schrezenmeir and de Vrese, 2001). This combination could improve the survival of the probiotic organism, because its specific substrate is available for fermentation. This could result in advantages to the host offered by the live microorganism and the prebiotic. Bengmark (2001) defines synbiotics as products produced by fermentation. Since in mixtures of pre- and probiotics the prebiotics will be fermented when the appropriate choice of products is made, also this definition is plausible. Fermentation can lead to the production of short-chain fatty acids, which are discussed below. Examples of synbiotics are FOS and *bifidobacteria*, and lactitol and *lactobacilli* (Collins and Gibson, 1999). Bailey *et al.* (1991) used a combination of FOS and competitive exclusion flora to reduce *Salmonella* colonization in chickens. The combination was more effective in reducing *Salmonella* colonization than FOS or competitive flora alone.

5) Short chain organic acids

Volatile fatty acids (VFA) (mainly acetic, propionic and butyric acid) are produced by the normal anaerobic intestinal flora as end products of their metabolism (Mead, 2000). The production of these VFA by the intestinal flora can be stimulated by adding fermentable prebiotics to the feed (Cummings, 1981). It is clear that the use of probiotics can also stimulate fermentation and therefore the production of the VFA. VFA or short chain fatty acids (SCFA) can also be added directly to the feed. Commercial products consisting of propionic and formic acid, either in powder form or encapsulated in silica beads, are applied in the field. Encapsulation allows slow release of the acids, so that a sufficient amount of product reaches the lower intestinal tract. The acids not only exert an antibacterial effect in the intestine, but also in the crop (Hinton and Linton, 1988). In most experiments where single VFA were added to the feed, no protection against *Salmonella* was found (Izat *et al.*, 1990; Hume *et al.*, 1993). Commercial products however contain mixtures of volatile fatty acids, and proved to decrease shedding of *Salmonella* (Thompson and Hinton, 1997).

SCFA are bacteriostatic or bactericidal in vitro for gram-negative bacteria, provided that there are sufficient undissociated acid molecules present and that they are in contact with the bacteria for sufficiently long time (Thompson and Hinton, 1997). Within practical limits, lowering the pH increases the concentration of undissociated molecules. Therefore the products will be more efficient at low pH. This was proved in in vitro experiments by a decreased growth rate of *Salmonella* Typhimurium at decreasing pH levels in the presence of VFA (Durant *et al.*, 2000). Formic acid/propionic acid 1% in the feed reduces the cecal pH (Waldroup *et al.*, 1995), which increases the proportion of undissociated acids. The SCFA diffuse into the bacterial cell in undissociated form. Inside the bacterial cell, the acid dissociates, resulting in reduction of intracellular pH and anion accumulation (van der Wielen *et al.*, 2000). Concentrations of VFA sufficient to cause growth inhibition of *E. coli in vitro* immediately slowed the rates of RNA, DNA, protein, lipid and cell wall synthesis (Cherrington *et al.*, 1990). Since synthesis of macromolecules other than DNA was not completely inhibited, and since there was no loss in membrane integrity, bacterial mass increased without cell division in the presence of VFA (Cherrington *et al.*, 1990;1991).

VFA also have undesirable effects, which are thought to be beneficial for bacteria. The acid resistance of *Salmonella* Typhimurium was enhanced upon exposure to VFA (Kwon and Ricke, 1998). While *Salmonella* bacteria normally do not survive at pH 3.0 in culture, a high number of bacteria survived after exposure to any VFA for some hours. Since bacteria can already be in contact with the VFA before ingestion, these bacteria could be protected against the gastric acidity (pH 3.0). Also the acidification of the SCV which *Salmonella* would encounter when phagocytosed by macrophages would be no problem for bacteria that are pre-exposed to VFA, either in the feed or in the intestinal tract (Kwon and Ricke, 1998). In vitro studies suggest that VFA might have an undesirable effect possibly favoring the invasiveness of *Salmonella* and the development of the carrier state. *In vitro* data demonstrate that exposure of *Salmonella* Typhimurium to VFA affect cell-association and invasion of cultured Hep-2 cells, an epidermal carcinoma cell line derived from the human larynx (Durant *et al.*, 1999; 2000). Indeed at pH 6, all VFA induce hilA and invF expression (Durant *et al.*, 2000). These genes are transcriptional regulators of the *Salmonella* pathogenicity island I, needed for invasion of host tissues. Moreover SCFA also

induce spv expression in *Salmonella* Dublin (El-Gedailly *et al.*, 1997). Spv genes are common in most *Salmonella* serovars and are involved in virulence, inducing systemic disease and macrophage survival (Libby *et al.*, 2000).

Above described studies indicate that care should be taken when VFA are used to control *Salmonella* in poultry. Until now, the choice of type of VFA used in commercial products is rather empirical and little information is available concerning the best combination of VFA in these products. If the purpose is only to reduce *Salmonella* bacteria in faeces and litter and to lower the infection pressure in the farm, VFA are effective products. Based on the expression of virulence genes, it seems not unlikely that *Salmonella* bacteria are driven to intracellular compartments of the intestine after exposure to VFA. In this case bacteriological examination of cloacal swabs and litter samples could be negative while the animals are carrying the bacteria inside their tissues. It is known that carrier animals can excrete the bacteria intermittently under certain stress conditions and can contaminate unexpectedly the other birds in the flock (Ducatelle *et al.*, 2000). VFA can be effective products since there is a good chance that these acids act fast and can also be active in young chickens, in contrast to many other types of feed additives. The effects of the different single VFA on the virulence of the bacteria, however, is not yet known.

B) Competitive exclusion

Nurmi and Rantala (1973) originally developed the concept of CE. They stated that newly hatched chicks have little opportunity for rapid development of a normal intestinal microflora, due to the clean housing conditions in which the chicks are reared. Rapid transfer of normal flora from the hen to the chick is impossible in modern mass production systems and development of microflora is considerably delayed (Schneitz *et al.*, 1992). By oral administration of a suspension of alimentary tract contents from adult birds to newly hatched chicks, an adult-type microflora is established, which protects from *Salmonella* infection (Nurmi and Rantala, 1973).
This treatment is a prophylactic measure that aims at increasing the resistance of young chicks to *Salmonella* or other infections by fastening the development of normal gut microflora (Mead, 2000). It may also be used after curative antibiotic therapy to eliminate an existing (*Salmonella*) infection and, in this case, the CE flora

restores the microflora (Seo *et al.*, 2000). The first method of application was to incorporate the preparation in the drinking water. Uptake of drinking water in the first 24h after hatch however is very variable and the viability of the anaerobic strains in the water may be reduced. Moreover there is a delay between hatch and placement in rearing houses (Mead, 2000). Spray application in the hatchery has been designed to overcome these problems (Goren *et al.*, 1984). The birds naturally preen themselves after being sprayed and thereby ingest the microflora. This is commonly enhanced by using a bright light (Schneitz *et al.*, 1992).

The mechanism of action of CE flora may be explained in part by a bacteriostatic effect on *Salmonella* in the caeca (Impey and Mead, 1989). This may be due to the production of VFA by the bacteria (see above). Competition for receptor sites in the intestinal tract and competition between pathogens and native microflora for nutrients (Ha *et al.*, 1994) also may play a role.

C) Vaccination

1) Immunity to *Salmonella*

Discussion of vaccination requires knowledge of mechanisms of immunity against the pathogen. It is widely accepted that cell-mediated immunity is more important than humoral responses in the protection against *Salmonella*. In mice, cytokines of the Th1 axis, enhancing cell-mediated responses, are crucial for protective immunity against *Salmonella* and other intracellular pathogens, in contrast to Th2 cytokines (Raupach and Kaufmann, 2001; Eckmann and Kagnoff, 2001). Evidence for the importance of Th1 responses comes from experiments using IFN-γ receptor knockout mice and mice with neutralizing antibodies to IL-12, which were unable to resolve infection with an attenuated strain, in contrast to mice lacking class-I-restricted T cells, γδ T cells or Ig producing B-cells, that were able to clear the infection (Hess *et al.*, 1996; Sinha *et al.*, 1997; Mastroeni *et al.*, 1998). Moreover, in animal models, protective roles have been shown for IL-1α, TNFα, IFN-γ, Il-12, IL-18 and IL-15, whereas IL-4 and IL-10 inhibit host defenses against *Salmonella*, again indicating the importance of the Th1 response in control of *Salmonella* (Eckmann and Kagnoff, 2001). An important distinction has to be made for protection against virulent *Salmonella* strains after

vaccination with avirulent strains. Although studies with Igμ$^{-/-}$ knockout mice have shown that control of primary infection with avirulent *Salmonella* vaccine strains strictly depends on IFN-γ producing CD4^{+} T-cells, vaccine induced protection against systemic infection involved both cell-mediated and humoral responses, the latter in the later stages of infection (McSorley and Jenkins, 2000; Mastroeni *et al.*, 2000; Mittrücker *et al.*, 2000; Mastroeni and Ménager, 2003).

Concerning poultry, little is known about immune responses to virulent and attenuated *Salmonella* strains. It is not possible to assess the role of Th1 relative to Th2 immune responses, since cytokines of the Th2-axis have not yet been found. It is proposed that cell-mediated immunity is more important than humoral responses for tissue clearance of virulent strains, while IgA responses and polymorphonuclear leukocytes seem to be the key players in intestinal clearance of *Salmonella* (Nagaraja and Rajashekara, 1999; Barrow and Wallis, 2000). Clearance of *S.* Typhimurium infection in chickens correlated with high cell-mediated responses (delayed type hypersensitivity reaction) and not with high antibody levels (Lee *et al.*, 1981, 1983). A study of Desmidt *et al.* (1998) with *S.* Enteritidis infected bursectomized chickens showed that B-cell depleted chickens had increased faecal excretion and higher caecal *Salmonella* counts, while having normal counts in internal organs, indicative for a protective effect of IgA. Colonization of liver and spleen decreased over time in control as well as in bursectomized animals, indicating that other immune mechanisms play a role in systemic clearance of *S.* Enteritidis in chickens (Desmidt *et al.*, 1998). The importance of cell-mediated immune mechanisms in systemic clearance of *S.* Enteritidis in chickens was recently investigated by Farnell *et al.* (2001). In this study, intraperitoneal administration of recombinant IFN-γ resulted in a decrease in organ colonization after oral *S.* Enteritidis infection, hereby proving the importance of cell-mediated immune responses in control of systemic *Salmonella* infection in chickens. Finally, in mice as well as in chickens, polymorphonuclear cells certainly play a role in resistance to *Salmonella* infections (Fierer, 2001; Hargis *et al.*, 1999). In a granulocytopenic chicken model, it was shown that heterophil-depleted animals were much more susceptible to *S.* Enteritidis organ invasion, with the increase in bacterial number in the internal organs being proportionally related to the decrease in number of circulating polymorphonuclear cells (Kogut *et al.*, 1993, 1994). Another experimental proof for the importance of chicken heterophils in protection against

Salmonella organ invasion was the finding that intraperitoneal administration of *S.* Enteritidis-immune lymphokines (SE-ILK) to 18-week old chickens protected the animals from organ invasion by *S.* Enteritidis (Tellez *et al.*, 1993b; Hargis *et al.*, 1999). SE-ILK are soluble products produced by T-lymphocytes, derived from *S.* Enteritidis-immune hens, cultured in the presence of concanavalin A. Intraperitoneal administration of SE-ILK in chickens resulted in a dramatic increase in the number of heterophilic granulocytes into the peritoneum without changing the numbers of other leukocytes (Kogut *et al.*, 1995a,b). It is known that mice polymorphonuclear cells can be attracted rapidly to a site of infection, within a few hours after *Salmonella* infection (Fierer, 2001). In chickens, studies with heterophil-depleted animals showed a severe morbidity and mortality when a normally sublethal dose of *S.* Enteritidis was orally inoculated, indicating the importance of heterophilic granulocytes (Kogut *et al.*, 1993). Heterophilic granulocytes of one-day old chicks are known to have killing activities for *S.* Enteritidis to the same level as heterophilic granulocytes of adult animals, in contrast to macrophages (Kodama at al., 1976; Wells *et al.*, 1998).

2) Vaccination of chickens

It is clear that *Salmonella* vaccines have to induce cell-mediated immunity as well as humoral responses, due to the above mentioned immunological data. Live vaccines confer better protection against *Salmonella* than killed vaccines, because the former stimulate both cell-mediated and humoral immune responses, while the latter stimulate mainly antibody production. The poor protective immunity with killed vaccines may also be due to the rapid destruction and elimination of the organism from the host system and the destruction of relevant antigens during vaccine preparation (Barrow, 1991). Killed vaccines only contain those antigens that were induced by the environmental conditions in which they were grown (Barrow and Wallis, 2000). The protective efficacy of inactivated vaccines is further limited by their low immunogenicity in unprimed hosts and their inability to induce cytotoxic T cells (Nagaraja and Rajashekara, 1999). Finally, killed vaccines generally fail to elicit secretory IgA responses, which are potentially important in protecting mucosal surfaces (Barrow and Wallis, 2000). For the reasons discussed above, vaccination trials with killed vaccines have variable success rates, dependent on the nature of the

killed vaccine (Timms *et al.*, 1990; Gast *et al.*, 1992, 1993; Woodward *et al.*, 2002). Live vaccines are known to be more effective in reducing colonization of the gut and internal organs of chickens. The efficacy of live vaccines is due to their distribution within the animal and therefore the stimulation of cell-mediated immune responses (Nagaraja and Rajashekara, 1999; Babu *et al.*, 2003). Besides being efficacious against intestinal and organ colonization, live vaccines should also be sufficiently attenuated to be cleared from the animal within a certain time period and should not revert to virulence.

Recently developed *Salmonella* vaccine strains carry defined nonreverting mutations which fall into two categories : metabolic functions and virulence factors. The most widely studied metabolically attenuated strains include mutants in the biosynthesis of aromatic amino acids (aroA, aroC, aroD), purines (purA, purE), adenylate cyclase (cya) and the cyclic AMP receptor protein (crp) (McFarland and Stocker, 1987; Cooper *et al.*, 1990, 1992, 1994a,b; Hassan and Curtiss, 1990). Virulence-attenuated vaccine strains typically have mutations in the phoP/phoQ two-component regulatory system or in genes of the SPI-2 (Medina *et al.*, 1999; Raupach and Kaufmann, 2001). Protection of chickens against colonization with virulent *Salmonella* strains by live oral vaccination with any of the above mentioned types of vaccine strains is well documented (Curtiss and Kelly, 1987; Barrow *et al.*, 1990; Cooper *et al.*, 1990, 1993, 1994a,b; Hassan and Curtiss, 1994, 1996, 1997). However, experimental setups usually use models in which challenge infections are done weeks after vaccination, while infections in chickens are also expected to take place early after hatching. Especially in broiler houses, there is need for early protection since the animals only reach the age of 6 weeks and humoral responses only appear a few weeks after vaccination of newly-hatched chickens.

Chickens are very susceptible to *Salmonella* infection the first days of life. When chicks are infected at the early stages of life they can carry *Salmonella* and shed the bacteria intermittently, being a threat for spreading of the disease (Duchet-Suchaux *et al.*, 1997; Asakura *et al.*, 2001). Oral administration of an avirulent *Salmonella* Typhimurium to day-old chicks however can confer protection against oral infection by a virulent strain one day later. This remarkable process is called colonization-inhibition (Barrow *et al.*, 1987; Berchieri and Barrow, 1990). Colonization-inhibition is genus-specific and not caused by bacteriocin activity (Barrow *et al.*, 1987). Analogous phenomena have been observed *in vitro* with 24-h-old stationary-phase

nutrient broth cultures of *S.* Typhimurium, when these cultures were inoculated with other *Salmonella* strains (Berchieri and Barrow, 1991). The phenomena were genus-specific, but dependent on the *Salmonella* serotype and strains used. Growth suppression of the inoculated strains is thought to operate through of the absence of utilizable carbon sources and electron acceptors (Zhang-Barber *et al.*, 1997). The colonization-inhibition phenomena are not thought to depend on host-immunity (Barrow *et al.*, 1987; Zhang-Barber *et al.*, 1997). The mechanisms of *in vivo* colonization-inhibition, however, are still not known.

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AIMS

Food of poultry origin is the most important source of *Salmonella* Enteritidis in men. Therefore control measures for *Salmonella* infections in poultry have been mandatory in the EU since the introduction of the EU directive 92/117 in 1992. These control measures have hitherto been introduced with relatively limited success. One major reason is that most of these measures, applied to the birds themselves, only reduce *Salmonella* excretion and leave critical periods in the production cycle uncovered. One of these periods is the early post-hatch period. Newly hatched chickens are extremely susceptible to *Salmonella* infection. Infection in this early post-hatch period with *Salmonella* often results in persistent or possibly life-long infection. Once infection is established, it is difficult to clear and eliminate. Therefore interrupting the initial steps of the infection is important for control of *Salmonella* infections in the live birds.

There are a limited number of tools that can target this early stage. One well established tool is the administration of a competitive exclusion flora. The general purpose of the present study was to explore two other tools that could possibly be applied for early protection, i.e. the colonization-inhibition phenomenon, and the effects of short-chain fatty acids.

Colonization-inhibition is a phenomenon in which inoculation of a *Salmonella* strain to one-day old chickens induces protection against a virulent strain administered one day later. The mechanism that governs this effect *in vivo* is not fully understood. Competition between the strains for receptors on epithelial cells and competition for nutrients and electron acceptors have been proposed as an explanation. No information is available on the possible involvement of the host reponse. The first specific purpose of this work was to investigate whether the host response could play a role in this colonization-inhibition phenomenon. In chapter one, basic data are collected concerning the dynamics of immune cells infiltrating in the caecal wall after neonatal *Salmonella* infection. In chapter two, colonization of the intestine of neonatal chickens by a virulent *Salmonella* Enteritidis strain was inhibited by an avirulent *Salmonella* strain and the caecal immune cell infiltration, attracted by the avirulent strain, was analyzed. In chapter three, the importance of heterophilic granulocytes in the colonization-inhibition phenomenon was investigated by the use of heterophil-depleted chickens.

Short-chain fatty acids are used as feed additives to reduce *Salmonella* in poultry. The choice of the acids is empirical and data derived from studies with mammalian cell lines suggest differences in virulence of *Salmonella* after contact with the different short chain fatty acids. The second specific purpose of this thesis was to examine the effects of different short-chain fatty acids on the early interactions between *Salmonella* and the host, i.e. the chicken. Therefore in chapter 4 the effects of the short-chain fatty acids formic, acetic, propionic and butyric acid on invasion of *Salmonella* Enteritidis in intestinal epithelial cells was analyzed *in vitro*. In chapter 5, *in vivo* colonization of caeca and internal organs was studied in young chickens in the early phase after *Salmonella* infection, when the animals were given feed supplemented with the respective short-chain fatty acids in microencapsulated form, ensuring the release of the acids in the intestinal tract.

CHAPTER 1

Dynamics of immune cell infiltration in the caecal lamina propria of chickens after neonatal infection with a *Salmonella* Enteritidis strain

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ABSTRACT

Dynamics of leukocyte infiltration and bacterial invasion in the caecal wall were studied after oral infection of 2-day-old chicks with *Salmonella enterica* ser. Enteritidis. Bacteria invaded the lamina propria of the caecal wall from 12 hours post-challenge onwards. Bacteriological examination of internal organs (liver, spleen) showed a peak in *Salmonella* bacteria at 3 days post-infection, whereafter the number of bacteria decreased. Immunohistochemistry revealed macrophages and T-lymphocytes invading the caecal propria mucosae from 24 hours after challenge onwards, while B-lymphocytes came somewhat later, subsequently organising into follicular aggregates. An early increase in granulocytes was partly masked by the response to natural flora. While the B-lymphocyte and granulocyte populations were maintained, T-lymphocyte and macrophage populations were already reducing by 10 days post-challenge. The infiltration of macrophages and T-lymphocytes in the caecal wall, followed by B-lymphocytes, is the result of an inflammatory response, caused by invading bacteria at this site. The structural maturation of the mucosa-associated lymphoid tissues is antigen driven, since B-cells organized in a follicular pattern.

INTRODUCTION

Salmonella enterica ser. Enteritidis is a major cause of gastrointestinal disease in humans all over the world, mainly due to the consumption of chicken meat and egg products (Poppe, 1999). In poultry infected with *S*. Enteritidis symptoms of disease are mostly seen in young chicks, which have not yet fully developed lymphoid organs (Jeurissen and Janse, 1996). Young chickens are indeed very susceptible to *S*. Enteritidis infection, which can lead to death. The cellular immune response is considered more important for the development of a protective immune response to *S*. Enteritidis than antibodies (Desmidt *et al*., 1998).

On the basis of experimental studies it is thought that infections at an early age include a greater risk of evolving into a carrier state, with birds becoming life-long infected. These birds may excrete the bacteria intermittently and constitute an important source of infection (Ducatelle *et al*., 2000). Although many studies have been performed on the bacteriology and serology of the infection (Cooper *et al*., 1989; Chart *et al*., 1992; Poppe, 2000), information about early cellular immune responses after infection in young chickens is scarce.

The aim of this study was to gain insight into the dynamics of immune cell infiltration in the caecal lamina propria of the chicken during the first ten days after infection of newly hatched chickens and to correlate these data with bacterial invasion and colonization.

MATERIALS AND METHODS

Animals

Specific pathogen free (SPF) chicken eggs (Lohmann Tierzucht GmbH, Cuxhaven, Germany) were hatched in isolation. After hatching and before challenge infection cloacal swabs were taken from the chicks and checked for *Salmonella.* Also serum samples were taken to detect maternal antibodies against *S.* Enteritidis by means of an indirect ELISA, based on the lipopolysaccharide of *S.* Enteritidis (Desmidt *et al.*, 1996). Both tests gave negative results.
The chickens were divided into two groups of 30 chickens each. Both groups were housed under the same conditions in different isolation units. The feed (Versele-Laga, Deinze, Belgium) was treated with 25 kGy (kilogray = Joule/kg) of γ-irradiation (Griffith-Mediris, Fleurus, Belgium) and given ad libitum from the second day after hatching. Sterile water was given ad libitum.

Experimental design

One group of 30 animals was inoculated about 48 hours post-hatch with $5x10^3$ cfu of a nalidixic acid resistant strain of *S.* Enteritidis (NIDO76Sa88 Nal^R) in 0.5 ml BHI (Brain Heart Infusion, Oxoid, Basingstoke, England) broth. The animals of the control group (30 animals) were sham-inoculated with BHI broth. Inoculation was performed by the oral route in the crop using a plastic tube. At 1, 3, 6, 9 and 12 hours and 1, 2, 4, 6 and 10 days after inoculation, from each group 3 chickens were euthanised. Caecal content, liver and spleen were bacteriologically examined. Samples of proximal and central part of the caecum were snap-frozen at –70°C and used for immunohistochemical analysis.

Bacteriological examination

Bacteriological examinations were done by direct inoculation as well as by pre-enrichment/enrichment. Caecal content was inoculated on BGA (Brilliant Green Agar, Oxoid, Basingstoke, England) with 20 mg/l of nalidixic acid. Liver and spleen

were homogenised and 10-fold dilutions were made in buffered peptone water starting from a 10- and 20-fold dilution for liver and spleen, respectively. For each dilution 6x20 μl were inoculated on a BGA plate with nalidixic acid. After incubation overnight (37°C) the number of cfu/g tissue was determined (direct inoculation). The detection limit after direct inoculation was $8.3x10^1$ cfu/g for the liver and $1.7x10^2$ cfu/g for the spleen. For all samples pre-enrichment was done in buffered peptone water overnight at 37°C, whereafter enrichment (overnight, 37°C) was done in tetrathionate brilliant green broth (Oxoid, Basingstoke, England). Thereafter, a drop of the enrichment broth was plated on BGA. Samples that were negative after direct inoculation but positive after *Salmonella* enrichment, were presumed to have 5.10^0 (liver) or 10^1 cfu/g (spleen). Samples that were negative after enrichment were presumed to have 0 cfu/g. The mean cfu/g tissue of liver or spleen was calculated for the 3 chickens for each time point.

Immunohistochemistry

Cryostat sections of 6 μm thick were cut from the snap frozen caecal specimens and fixated in acetone. Immunohistochemical labelling of leukocytes was done as described by Mast (1998a). Briefly, tissue sections were incubated for 45 min with monoclonal antibodies specific for the different leukocyte subpopulations (see below) in an appropriate dilution, in phosphate buffered saline (PBS, pH = 7.85) containing 2% skimmed milk powder at room temperature. After rinsing thoroughly with PBS, a goat anti-mouse IgG1 conjugate, labelled with peroxidase (Dako, Glostrup, Denmark, 1/200 in PBS pH = 7.85 with 2 % skimmed milk powder) was added to the tissue sections (45 min, room temperature). After rinsing again, positive cells were stained brown after conversion of the substrate (3,3' diaminobenzidine tetrahydrochloride, Sigma, St.Louis, USA) in the presence of H_2O_2. The monoclonal antibodies used were KUL01, KUL05, AV20 and IC3. KUL01 is a marker for chicken macrophages, monocytes and interdigitating cells (Mast *et al*., 1998), while KUL05 recognises T-lymphocytes (Mast, 1998b). AV20 recognises a monomorphic determinant on the antigen Bu-1 and identifies both bursal and peripheral B-lymphocytes (Rothwell *et al*., 1996). IC3 recognises 90% of chicken granulocytes (Mándi *et al*., 1987).

A polyclonal rabbit anti-*Salmonella* Enteritidis serum, produced in our laboratory, was used for the detection of *S*. Enteritidis bacteria in the caecal wall. In this case, a goat anti-rabbit IgG conjugate, labelled with peroxidase, was used as secondary antibody in the above described protocol.
The number of cells or bacteria was scored with an automatic image analysis system (Optimas 6.5., Media Cybernetics, Silver Spring, USA), measuring the percent area occupied by the labelled cells for the leukocytes and counting the number of bacteria per unit surface area for the *Salmonella* bacteria. These measurements were made in the lamina propria of the caecal wall. Leukocytes were scarce in the submucosa and muscular layer. For each time point and for each chicken, 2 locations (proximal and central) of the caecum were analysed. For each location, 6 to 8 microscopic fields (multiplication 400x) were taken at random and analysed by the image analysis program. For each time point, the mean of the percent area or counts was calculated for the 3 chickens of the same treatment. In all figures, these mean values are shown.

Sandwich ELISA for total IgA in caecal contents

To extract Ig from the caecal contents, one part of each sample was diluted 10 times with PBS (w/v) and centrifuged at 10000 x g for 10 min. Subsequently, supernatants were collected and stored at –20°C until analysis.
Maxisorp ELISA plates (Nunc, Wiesbaden, Germany) were coated overnight at 4°C with 0.5 µg/ml of the chicken IgA-specific MAb A1 (Erhard *et al*., 1992) in coating buffer (carbonate - bicarbonate buffer, 50 µM, pH 9.6). After three washes with PBS containing 0.05% Tween 20 (washing buffer), the plates were incubated for one hour at RT with the samples diluted 1/200 in sample buffer (washing buffer containing 1% BSA). Duplicates of each sample were analyzed.
The bound chicken IgA were detected using a peroxidase-labelled, chicken Ig-specific conjugate produced in rabbit (Sigma, St. Louis, USA) diluted in washing buffer. After one hour of incubation and three subsequent washes, peroxidase activity was developed using the TMB peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersbury, Maryland, USA). After 10 min, the reaction was stopped with 100 µl peroxidase stop solution per well. The absorbance at 510 nm was recorded with a Multiscan MCC ELISA reader and could be considered as a measure

for total IgA as, in a preliminary experiment, it was verified that for representative samples the applied sample dilution was always situated in the linear part of the serum dilution curve.

Statistical analysis

The data were analysed with SPSS 9.0 software using general analysis of variance methods. Treatment (control or challenged group), location in caecum (proximal or central) and time after inoculation were used as crossed factors (Maxwell and Delaney, 1990). To the response variable, percent area occupied by the labelled cells, an arcsine transformation was applied in order to comply to the assumption of normality of the statistic. As significant interactions occurred in the effect of the challenge infection with time, one-way analysis of variance with treatment as factor was repeated for each combination of caecum location and time. The effect of the challenge infection at a specific time after infection was considered significant if this was effectively so at both caecum levels.

RESULTS

Bacteriology

Bacteria in liver and spleen peaked at about 4 days post-challenge to reach about 10^4 cfu/g in spleen and 10^3 cfu/g in liver, in animals inoculated with 5.10^3 cfu *S.* Enteritidis. Afterwards bacterial cfu gradually decreased to reach 5.10^3 and 10^2 cfu/g at 10 days post-challenge in spleen and liver respectively (Figure 1). Bacteriological analysis of caecal contents yielded more than 1000 colonies in direct plating at all time points. Control animals were negative for *S.* Enteritidis during the experiment.

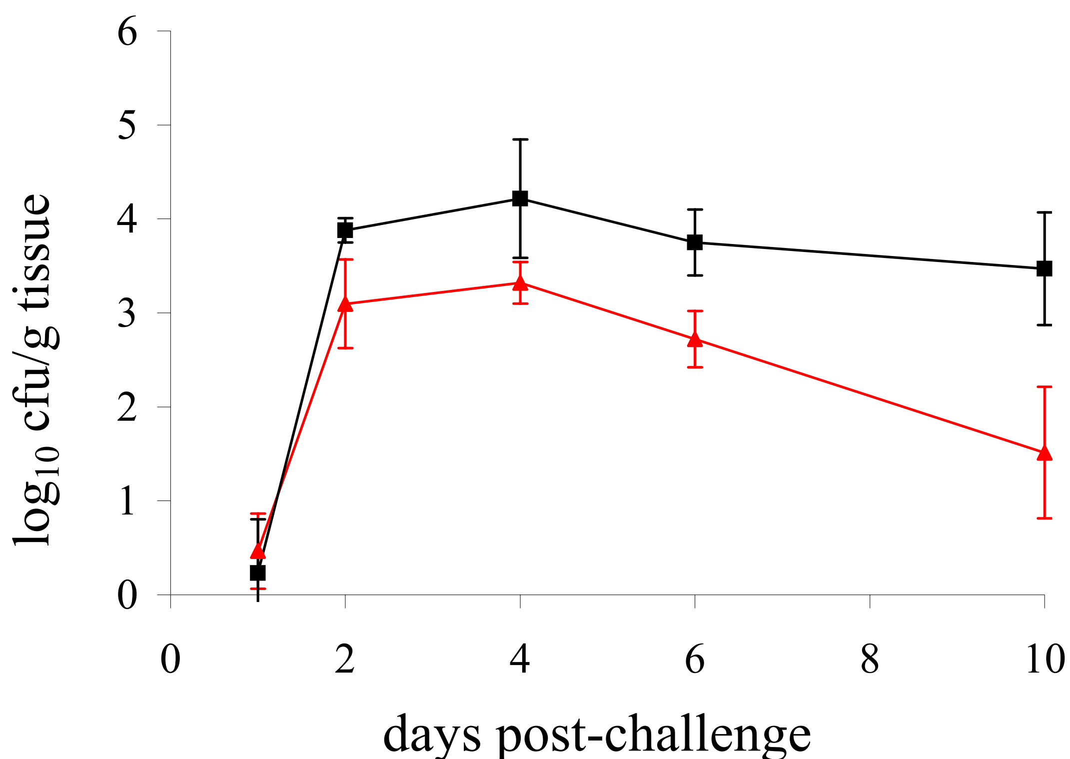


Figure 1. *Salmonella* bacteria present in spleen (■) and liver (▲) after oral challenge of 2 day-old chicks.

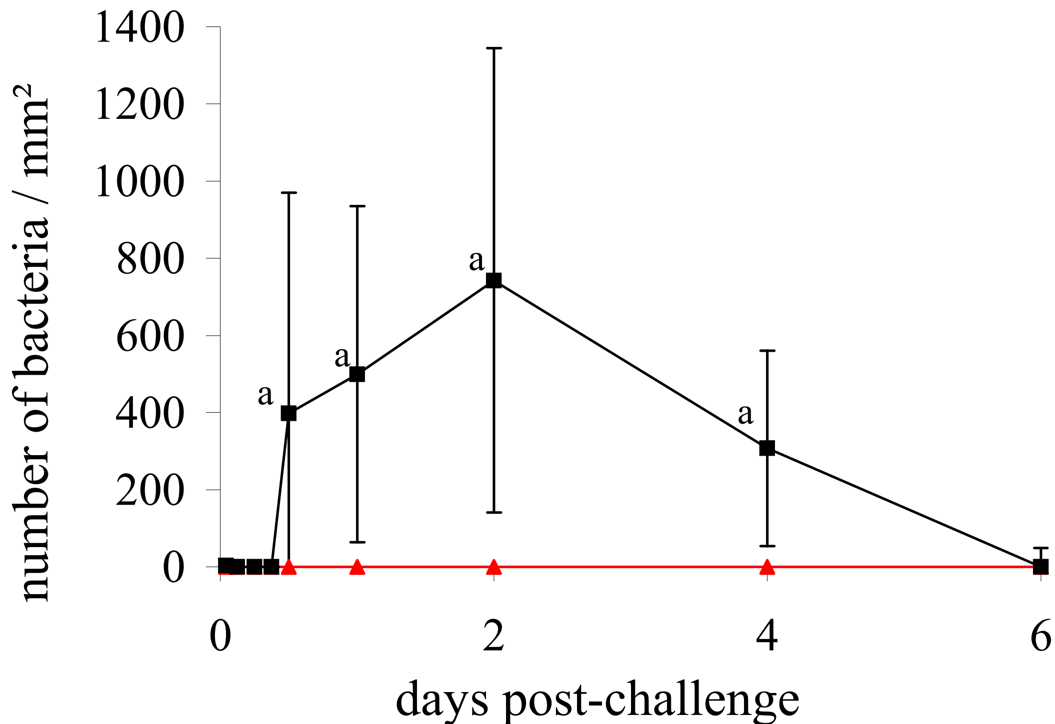


Figure 2. *Salmonella* bacteria in the caecal lamina propria in control (▲) and challenged (■) chicks. 'a' denotes statistically significant differences ($p < 0.01$) in number of bacteria / mm².

Immunohistochemistry

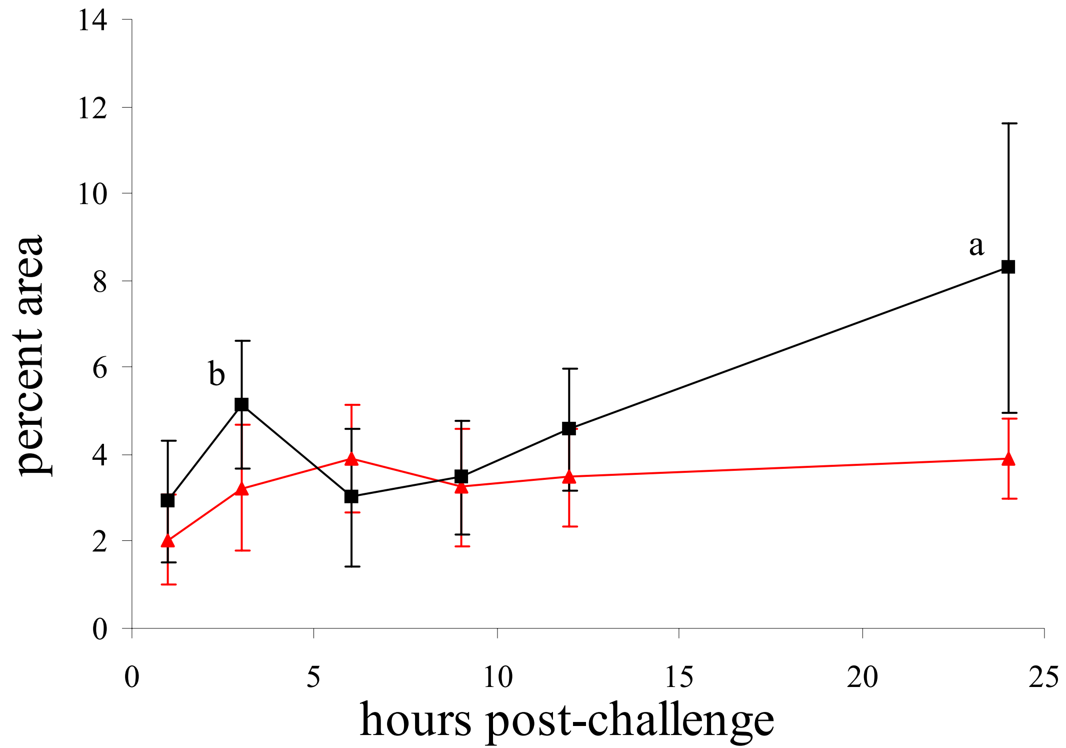
In the caecal lamina propria, invading S*almonella* bacteria were detectable from 12 hours post-challenge onwards (Figure 2). Thereafter the number of bacteria in the lamina propria increased to reach a peak level at 2 days post-challenge. At that moment bacterial concentration in the lamina propria of the caecal wall amounted to 750/mm^2. Bacteria were no longer detectable 6 days post-challenge (Figure 2).

In the control group, the area of the caecal lamina propria occupied by resident macrophages consistently varied between 2 and 4%. In the challenged chicks, a first, transient increase of the area percentage of the lamina propria occupied by macrophages was observed at 3 hours post-challenge, with macrophages occupying more than 5% of the area. After a brief decline, the area occupied by the macrophages increased steadily to reach a peak of 12% at 2 days post-challenge (Figure 3). By 10 days post challenge the macrophage occupied area returned to 6%, the level seen in control animals.

T-lymphocytes in control chicks occupied between 1 and 4% of the propria mucosae. In the challenged birds, T-lymphocytes started increasing at 24 hours post-challenge and reached a plateau level from 2 to 6 days, occupying approximately 12% of the propria mucosae (Figure 4). Afterwards, the percent area declined.

The B-lymphocyte population in the caecal lamina propria in control chicks continuously occupied less than 0.2 % of the tissue. In the challenged chicks this population began to increase from 2 days after challenge onwards, to reach a fairly constant level of approximately 6% of the area from day 4 till the end of the experiment on day 10 (Figure 5). During this period B-lymphocytes were seen organising in a focal or even follicular pattern (figure 6).

Granulocytes in the caecal lamina propria in control chicks occupied approximately 2 to 7 percent of the area in the first 24 hours, with a small initial peak at 6 hours and a gradual increase thereafter. Later on granulocytes continued to increase, to reach peak levels of 9% at 2 days after sham-inoculation. In the challenged chicks, this cell population followed a fairly similar pattern for 2 days. From 3 to 10 days post challenge however, the granulocyte population in the caecal lamina propria increased to occupy between 10 and 14 % of the area (Figure 7).



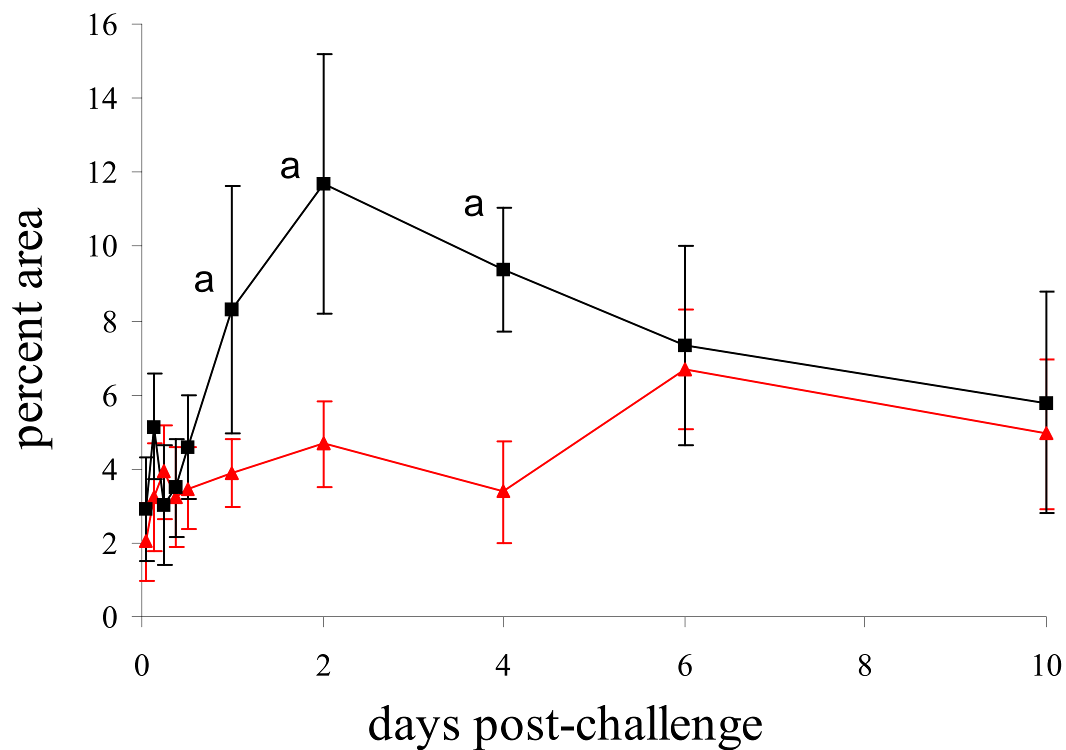
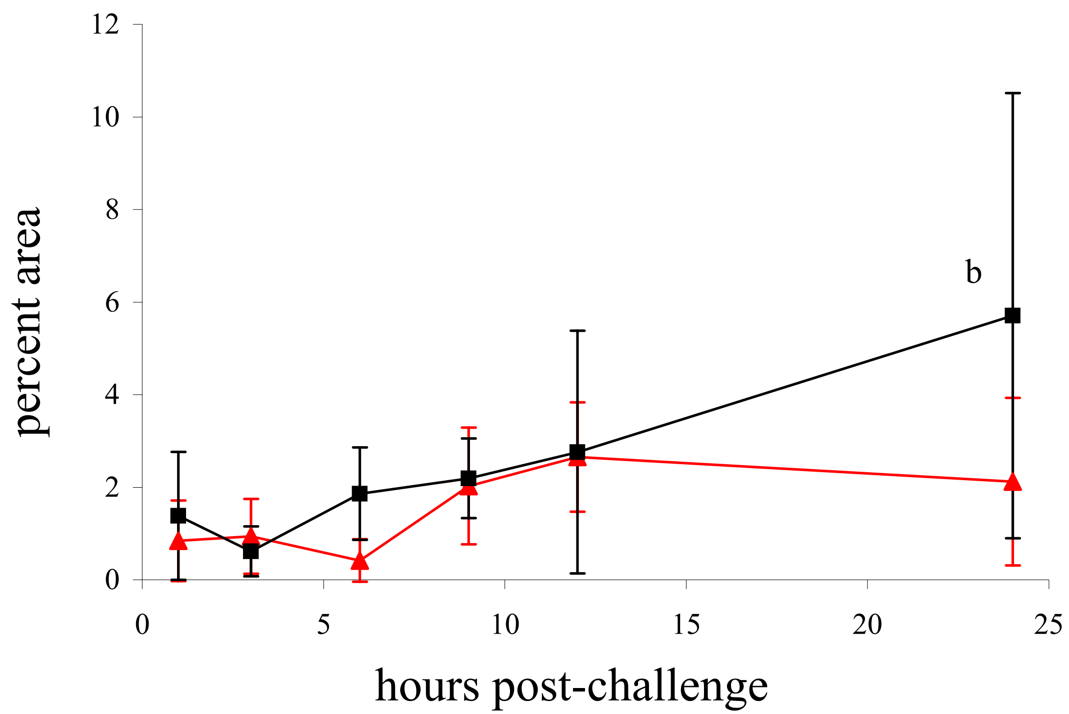


Figure 3. Area percentage of caecal lamina propria occupied by macrophages in control (▲) and challenged (■) chicks. 'a' ($p < 0.01$) and 'b' ($p < 0.05$) denote statistically significant differences in percent area between treatments at a given moment.



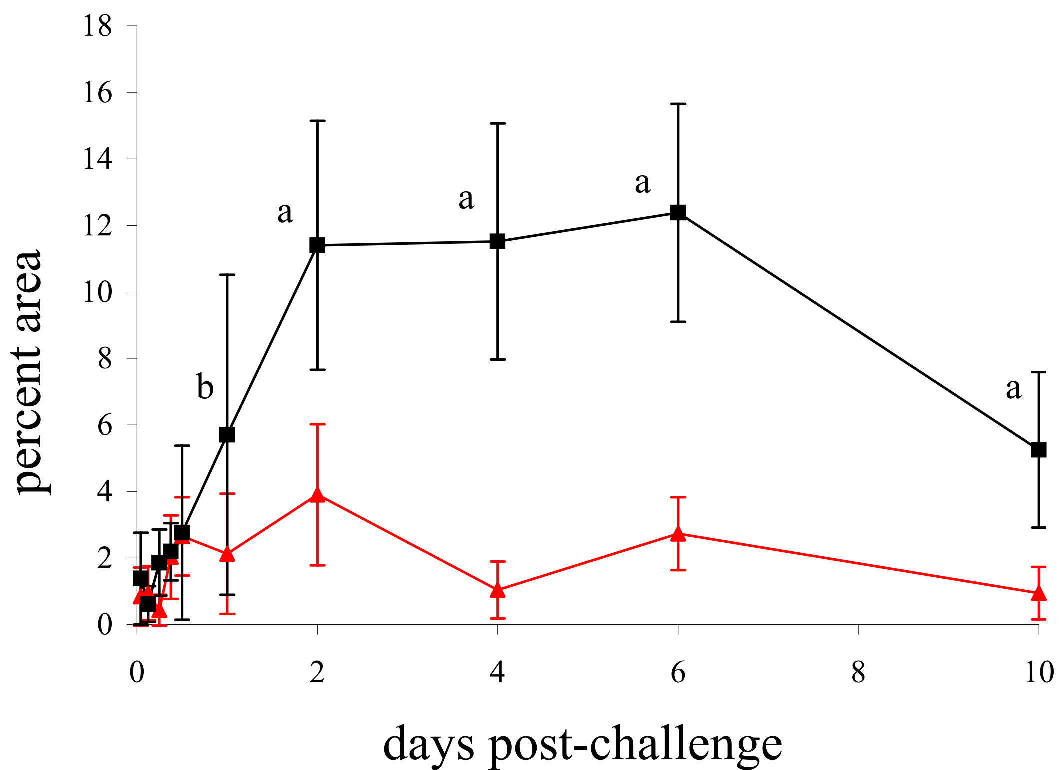
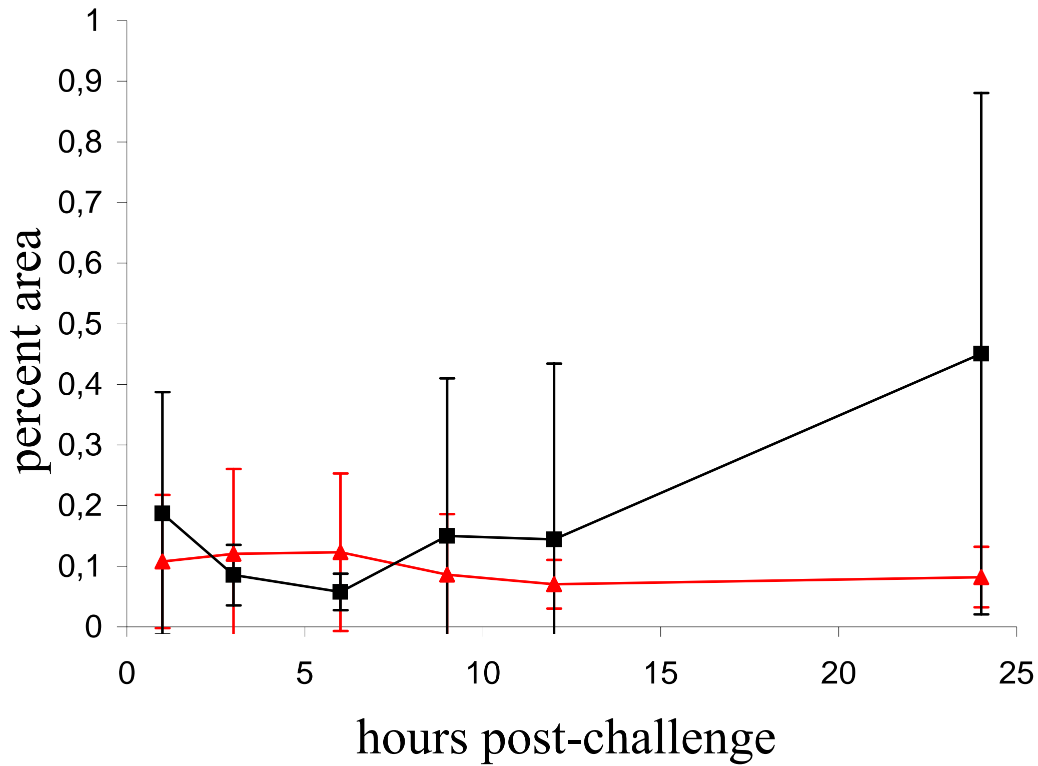


Figure 4. Area percentage of caecal propria mucosae occupied by T-lymphocytes in control (▲) and challenged (■) chicks. 'a' ($p < 0.01$) and 'b' ($p < 0.05$) denote statistically significant differences in percent area between treatments.



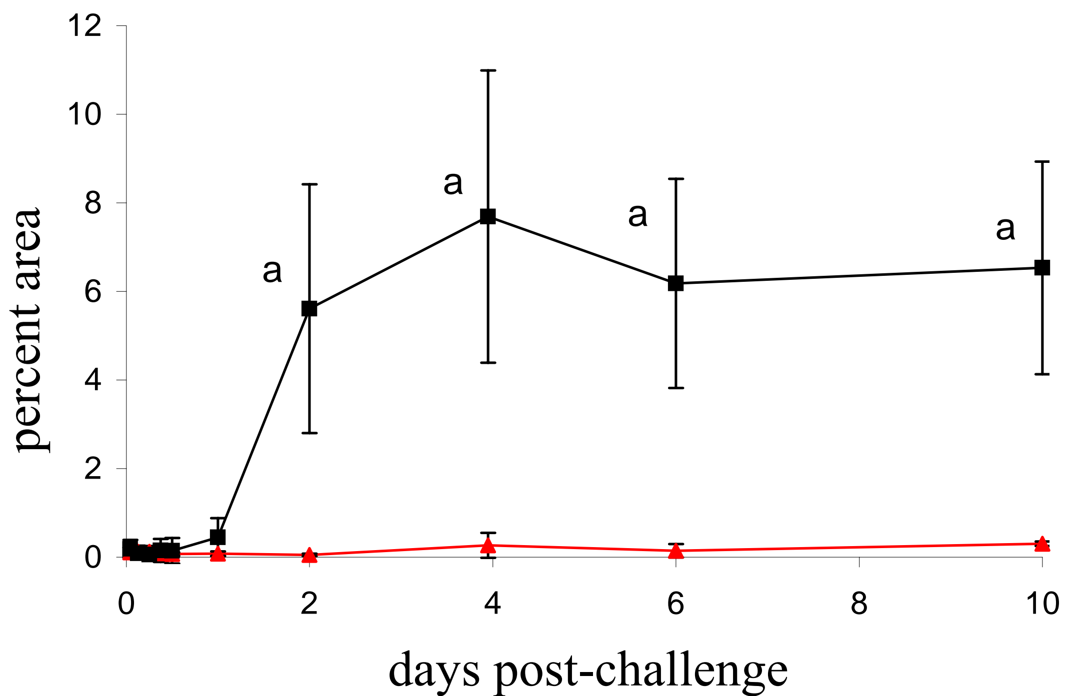


Figure 5. Area percentage of caecal propria mucosae occupied by B-lymphocytes in control (▲) and challenged (■) chicks. 'a' denotes statistically significant differences ($p < 0.01$) in percent area between treatments at a given moment.

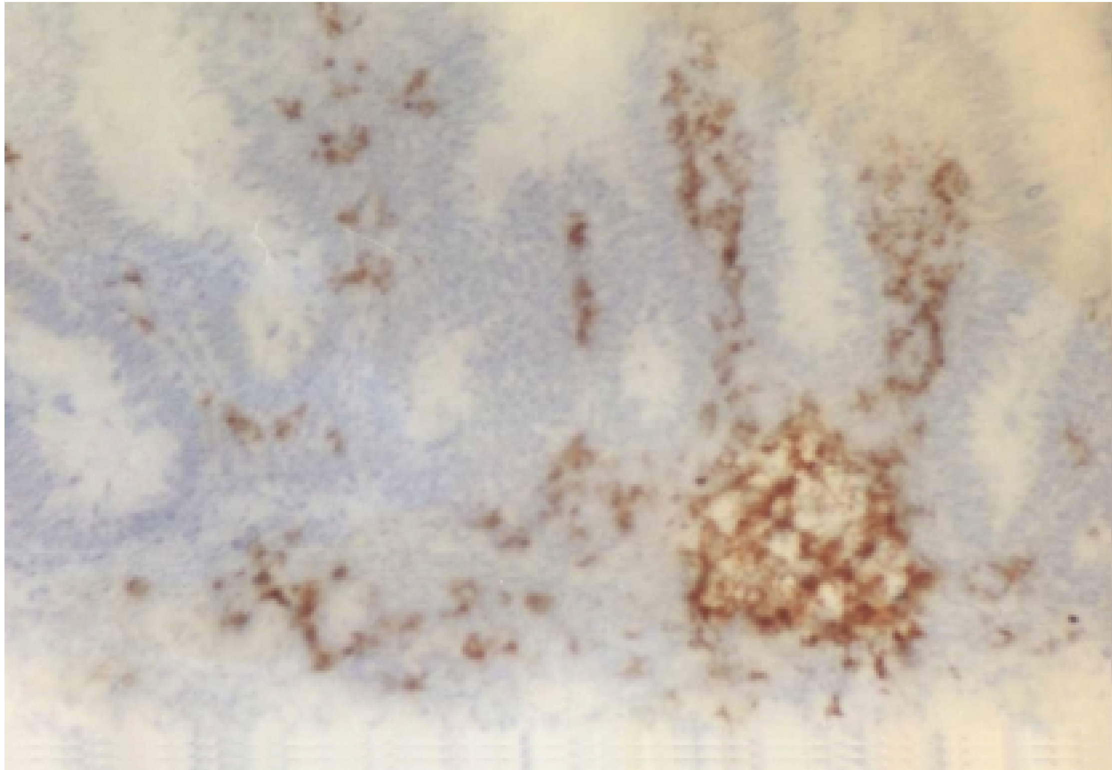
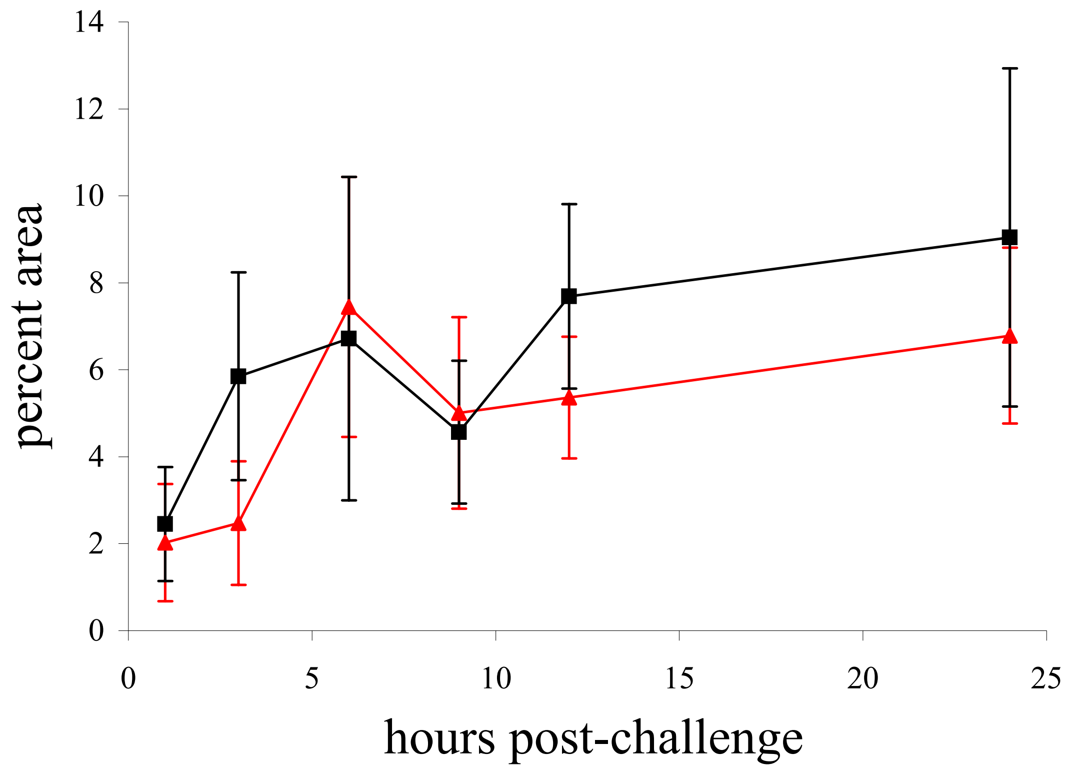


Figure 6. Follicular pattern of B-lymphocyte ($AV20^{+}$) distribution in the caecal lamina propria at 6 days post-challenge.



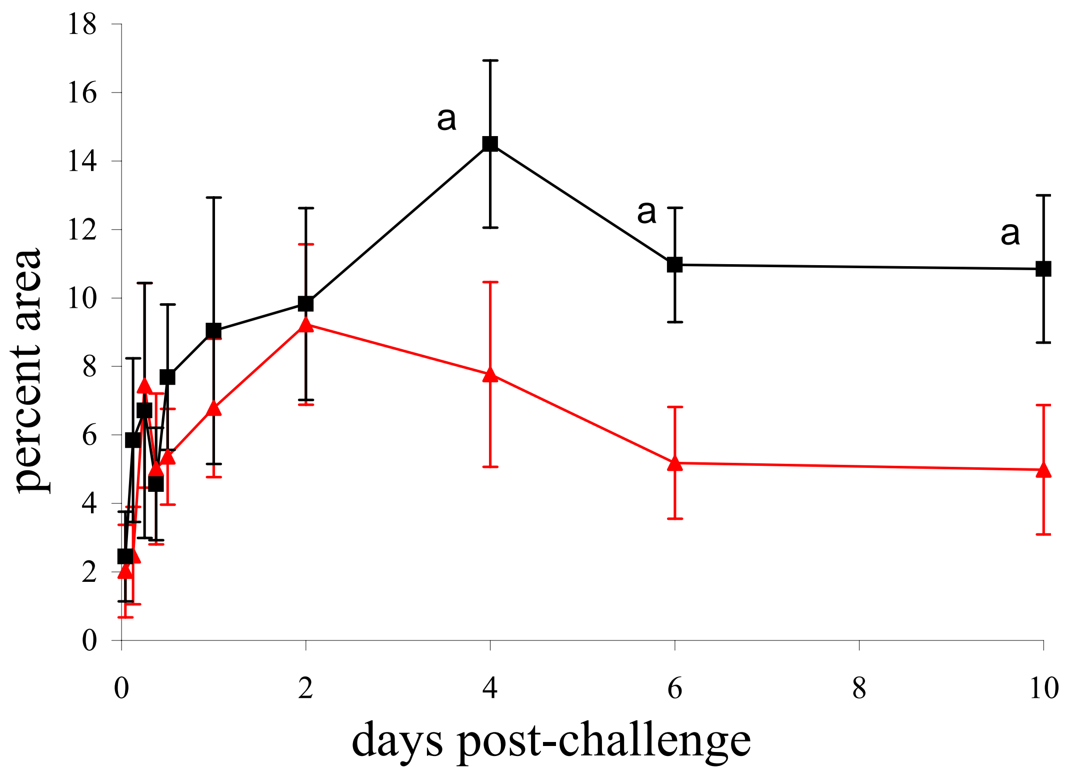


Figure 7. Area percentage of caecal lamina propria occupied by granulocytes in control (▲) and challenged (■) chicks. 'a' denotes statistically significant differences ($p < 0.01$) in percent area between treatments at a given moment.

Sandwich ELISA for total IgA in caecal contents

The mean total IgA in challenged animals increased from 4 days post-infection onwards (Figure 8). The control animals still had a basal level of mean total IgA 6 days after sham-challenge.

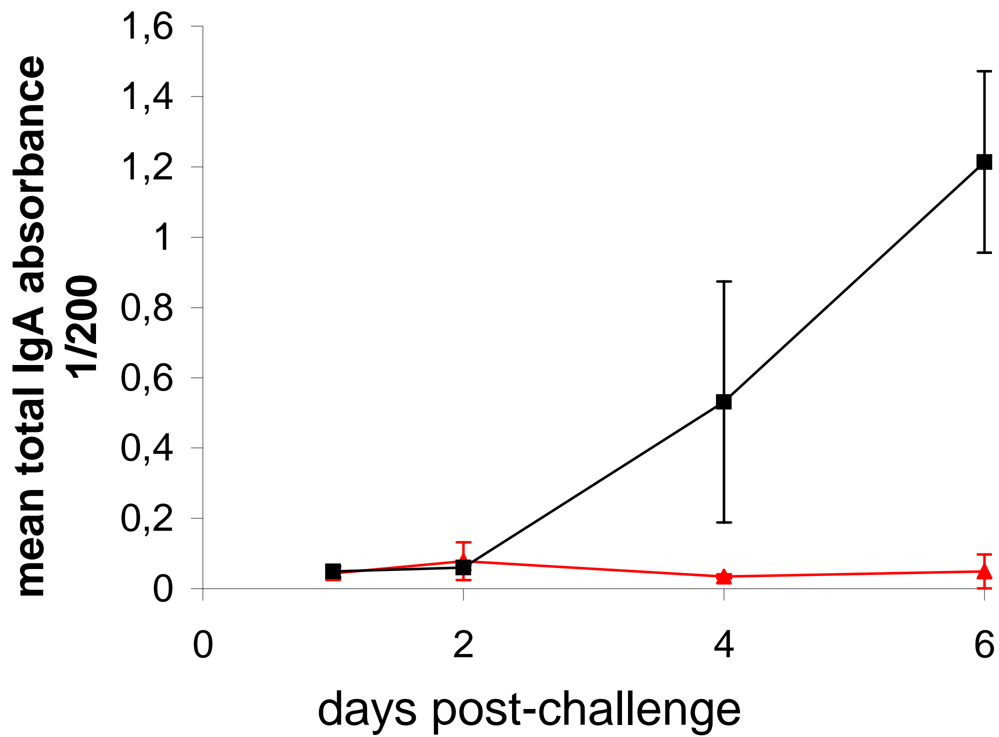


Figure 8. Mean total IgA (absorbance 1/200) of caecal samples in control (▲) and challenged animals (■).

DISCUSSION

Salmonella bacteria that reach the intestinal tract can cross the intestinal epithelium after attachment to the mucosa. From there they can reach the lamina propria, where they replicate, or proceed to deeper tissues, presumably carried within non-activated macrophages. After reaching the blood stream they infect organs, such as liver and spleen (Phalipon and Sansonetti, 1999). In our experiment bacteria were first detected 3 hours post-infection in the caecal lumen and 12 hours post-infection in the caecal lamina propria. Colonization of liver and spleen started more than one day post-inoculation.

Our analyses of the dynamics of leukocyte infiltration in the lamina propria show that upon oral challenge with *Salmonella*, neonatal SPF chicks raise an active biphasic immune response against these bacteria. The initial transient increase of the area occupied by macrophages, and to a lesser extent by granulocytes, can be interpreted as the result of changes in shape and size of the resident cells. For macrophages, these changes are known to be influenced by bacterial LPS and they are indicative for the macrophage activation status. Indeed, Qureshi (1994) demonstrated by electron microscopy that the uptake of *S.* Enteritidis by macrophages resulted in changes of macrophage morphology. These changes are accompanied by an increase of endogenous enzymatic activities, like acid phosphatase and inducible nitric oxide synthase, and the production of pro-inflammatory cytokines, like IL-1, IL-6 and TNF-α (Kaiser, 1994; Klasing, 1998). These, and other macrophage-derived cytokines and immune mediators initiate local and systemic inflammatory responses by their chemotactic properties and induction of adhesins on the surrounding tissue. The strong influx and continued presence of macrophages, T-cells, B-cells and granulocytes in the caecal lamina propria from 24 hours post-challenge onwards up to 10 days most likely can be explained by this local inflammation.

In the caecal lamina propria, the observed inflammatory response reduces bacterial numbers to undetectable levels within 6 days, and this while high numbers of bacteria remain present in the caecal contents.

Our results suggest that initially an antigen non-specific inflammatory response mediates the clearance of bacteria in the lamina propria of neonatal chicks, thereby reducing entrance of bacteria in the blood stream. Macrophages (and granulocytes)

are the primary effector cells in this process. Attraction and activation of T-cells will further reduce bacterial invasion. Genovese (1999, 2000) demonstrated a reduced organ invasion of *S*. Enteritidis after administration of *S*. Enteritidis immune lymphokines to young chickens. Further, T cells will contribute to an antigen-specific B-cell response: B-cells are already organised in follicular aggregates within the next 3 days following their influx on day 3 post-challenge. In a study of Desmidt (1998), a peak by intestinal IgA against *S*. Enteritidis paralleled the reduction of faecal excretion, indicating that the IgA response might play a role in the clearance of the bacteria from the gut. During the first 6 days post-infection, no S*almonella*-specific IgA antibodies were detected in our experiment (data not shown) , but the mean total caecal IgA increased a few days after infection.

Comparison of leukocyte infiltration in the caecal lamina propria of challenged and control chicks, where no infiltration is observed, clearly illustrates earlier observations of Hegde (1982) that the structural maturation of the mucosa associated lymphoid tissues (MALT) is antigen-driven. Indeed, any differences between control and challenged chicks seem to be related to the massive invasion of *Salmonella* bacteria from 12 hours post-infection onwards. This opens possibilities for the priming of at least the non-antigen-specific immunity of the MALT using non-pathogenic bacterial strains to accelerate future responses against pathogenic strains.

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CHAPTER 2

The effect of vaccination with a *Salmonella* Enteritidis *aroA* mutant on early cellular responses in caecal lamina propria of newly-hatched chickens

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ABSTRACT

When newly hatched chicks are inoculated with a *Salmonella* strain, they induce a rapid onset of resistance to intestinal colonization by other *Salmonella* strains. The exact mechanism of this early colonization-inhibition is not known. To study host-related contributions to this phenomenon, the kinetics of immune cell infiltration in the caecal wall was analyzed during the first ten days after vaccination of newly hatched chickens with a *Salmonella enterica* ser. Enteritidis *aroA* mutant, and infection one day later with a virulent *Salmonella enterica* ser. Enteritidis strain. These data were correlated with bacterial colonization and clearance of the *Salmonella* Enteritidis challenge strain. Bacteriological data showed that vaccinated animals had a much lower number of challenge bacteria in their organs and caecal contents the first days post-challenge, relative to unvaccinated animals. Immunohistochemical analysis of the caecal lamina propria revealed that heterophils started infiltrating the caecal lamina propria from 12 hours post-vaccination. Macrophages and T-lymphocytes started infiltrating from 20 hours and B-lymphocytes from 24 hours post-vaccination. These data imply that immune cells already colonized the caecal wall at the time of challenge in vaccinated animals. The presence and activity of these cells in the caecal wall shortly after administration of a *Salmonella* Enteritidis *aroA* mutant might contribute to the inhibition of colonization of a virulent *Salmonella* strain, subsequently administered.

INTRODUCTION

Salmonella enterica ser. Enteritidis is a facultative intracellular pathogen that can cause disease in man and animals. Currently a worldwide epidemic of *Salmonella* occurs that has started in the late 1960s (Baümler *et al*., 2000). Poultry meat and eggs are considered to be the major source of infection for humans.

Control programs are currently being developed to combat *Salmonella* in poultry. In these programs, vaccination of poultry is becoming one of the most important control measures, because of the cost and impracticability of improvements in hygiene and the increasing antibiotic resistance of bacteria (Zhang-Barber *et al*., 1999). Killed vaccines have been tested with varying results. Live vaccines confer better protection, probably because the latter stimulate both cellular and humoral responses, while the former stimulate antibody production only (Chatfield *et al*., 1993; Barrow, 1996). During the last decade, many attenuated vaccines against *Salmonella* serovars have been constructed and used in experimental conditions. Among these attenuations, *aroA* mutations, which make the bacteria unable to grow in host tissue, are very promising (Chatfield *et al*., 1993; Nagaraja and Rajashekara, 2000). The vaccinations should confer protection as soon as possible because newly hatched chicks are very susceptible to *Salmonella* infection (Cooper *et al*., 1994a).

Administration of live *Salmonella* to one-day old chickens elicits antibody responses to the LPS antigen starting from 18 days post-infection (Desmidt *et al*., 1997). Little is known about the time of onset of a specific cellular immune response after administration of live *Salmonella* in the chicken. As a result of the immunological immaturity of newly hatched chickens, there is an immunity gap during the first days of their life. However, it has been shown that when live *Salmonella* are administered to chickens in the first hours post-hatch, they induce a rapid onset of resistance to intestinal colonization by a number of *Salmonella* challenge strains, depending on the serovar used (Barrow *et al*., 1987; Berchieri and Barrow, 1990). The exact mechanism of this early colonization-inhibition is not known, but microbiological and/or host-related phenomena are thought to be involved. *In vitro* studies support the microbiological hypothesis suggesting that growth suppression of a challenge strain operates through the depletion of utilizable carbon sources or electron acceptors (Zhang-Barber *et al*., 1997).

To study host-related contributions to the colonization-inhibition phenomenon, the kinetics of immune cell infiltration in the caecal wall were analyzed during the first ten days after vaccination of newly hatched chickens with a *Salmonella* Enteritidis *aroA* mutant. These data were correlated with bacterial colonization and clearance of a *Salmonella* Enteritidis challenge strain, subsequently administered.

MATERIALS AND METHODS

Salmonella strains

An *AroA* mutant of *Salmonella* Enteritidis was used as vaccine strain : *Salmonella* Enteritidis, strain LA5, phage type 4, CVL30. CVL30 is a well-defined stable mutant, created by a site-directed insertion mutation (Cooper *et al*., 1994b). The *aroA* gene encodes for 5-enolpyruvylshikimate 3-phosphate synthase, an enzyme used in the pathway for chorismate synthesis in bacteria. Chorismate is a precursor for aromatic acid biosynthesis, for production of tetrahydrofolate (via p-aminobutyric acid, PABA), ubiquinones and enterocholin (via 2,3 dihydrobenzoate, DHB), a siderophore molecule. *AroA* mutant bacteria are auxotrophic for molecules such as PABA and DHB, that are lacking in vertebrate tissues. Therefore the *aroA* mutant bacteria are not able to survive in tissues of the chicken (Hoiseth ans Stocker, 1981). As challenge strain *Salmonella* Enteritidis NIDO76Sa88 Nal^R PT4 was used. A brain heart infusion (BHI) (Oxoid, Basingstoke, Hampshire, England) broth culture was made for both *Salmonella* strains. After inoculation on ISO-sensitest-agar (Oxoid, Basingstoke, Hampshire, England), ten colonies were transferred into BHI broth (20 hours, 37°C, shaking). Purity was checked by inoculation on blood agar and the number of colony-forming units (cfu) was determined by inoculation of 10-fold dilutions on brilliant green agar (BGA) (Oxoid, Basingstoke, Hampshire, England). The suspension was stored at 4°C before usage in the experiment. The appropriate inoculation dose was obtained by diluting the culture with phosphate buffered saline (PBS).

Chickens

Specific pathogen free (SPF) eggs (Lohmann, Cuxhaven, Germany) were hatched in isolation. After hatching and before the start of the experiment cloacal swabs were taken from 10 chickens/group and checked for *Salmonella*, to confirm that the animals were *Salmonella*-free. Also serum samples were taken to detect maternal antibodies against *Salmonella* Enteritidis by means of an indirect ELISA, based on lipopolysaccharides of *Salmonella* Enteritidis (Desmidt *et al*., 1996).
The chickens were divided into two groups of 30 and two groups of 45 chickens. Each group was housed in a different isolation unit but housed under the same conditions.

The feed (Versele-Laga, Deinze, Belgium) was treated with 25 kilogray (kGy) of γ-radiation (Griffith-Mediris, Fleurus, Belgium) and given ad libitum from day 2 on. Autoclaved water was available ad libitum.

Experimental design

The chickens were divided into four groups : VC (30 animals), V (45 animals), C (30 animals) and O (45 animals). The animals of group VC were vaccinated on the first day of life with 10^8 cfu *Salmonella* Enteritidis CVL30 and challenged 24 hours later with 5.10^3 cfu *Salmonella* Enteritidis NIDO76Sa88 Nal^R. The animals of group V were vaccinated with 10^8 cfu *Salmonella* Enteritidis CVL30 on their first day of life and sham-challenged with BHI broth one day later. The animals of group C were sham-vaccinated with BHI broth on their first day of life and challenged with 5.10^3 cfu *Salmonella* Enteritidis NIDO76Sa88 Nal^R one day later. Group O was the negative control group : these animals were sham inoculated on day 1 as well as on day 2. The vaccination and challenge (sham or not) was done by oral inoculation in the crop using a plastic tube.

At 1, 12, 16, 20 and 24 hours after vaccination and after 1, 3, 6, 9 and 12 hours and 1, 2, 4, 6 and 10 days following infection or sham inoculation on day 2, for each group and for each time, 3 chickens were euthanised. Samples of caecal content, liver and spleen were taken for bacteriological analysis. At all time points, samples were also taken for immunohistochemical analysis : small parts of caecum (proximal and central) were snap frozen at –70°C.

Bacteriological examination

Bacteriological examinations were done by direct inoculation as well as by pre-enrichment/enrichment. Direct inoculation was done on brilliant green agar (BGA). Growth of the challenge strain (Nal^R) was evaluated on BGA with nalidixic acid. The vaccine strain, CVL30, is nalidixic acid sensitive. When, in direct inoculation, no growth was visible, additional samples were pre-enriched in buffered peptone water (Oxoid, Basingstoke, Hampshire, England) and afterwards enriched in tetrathionate-brilliant green broth. Suspicious colonies were biochemically identified (Kligler,

Lysine, Ureum) and identified by a rapid plate agglutination test. The difference between vaccine and challenge strain was also made by inoculation on xylose lysine decarboxylase (XLD) (Amersham, Topley House, Bury, England) agar. The challenge strain produced black colonies, in contrast to the vaccine strain.
The numbers of cfu of *Salmonella* bacteria per gram liver or spleen tissue was determined by plating 10-fold dilutions of 5% (spleen) or 10% (liver) weight/volume dilutions in buffered peptone water (BPW) on BGA, with and without nalidixic acid.
Samples that were negative after direct inoculation were pre-enriched in BPW and enriched in tetrathionate - brilliant green broth. Samples that were positive after *Salmonella* enrichment, were presumed to have 10^1 (liver) or $2x10^1$ cfu/g (spleen) tissue. Samples that were negative after enrichment were presumed to have 0 cfu/ml. The mean number of cfu/g tissue liver or spleen was calculated for 3 chickens for each time point.

Immunohistochemistry

Cryostat sections of 6 μm thick were cut from the snap frozen caecal specimens and fixed in acetone. Immunohistochemical labelling of leukocytes was done as described before (Mast, 1998). Briefly, tissue sections were incubated for 45 minutes with monoclonal antibodies specific for the different leukocyte classes (see below) in an appropriate dilution, in phosphate buffered saline (PBS, pH = 7.85) with 2 % skimmed milk powder at room temperature. After rinsing thoroughly with PBS, a goat anti-mouse IgG1 conjugate, labelled with peroxidase (Dako, Glostrup, Denmark, 1/200 in PBS pH = 7.85 with 2 % skimmed milk powder) was added to the tissue sections (45 minutes, room temperature). After rinsing again, positive cells stained brown after conversion of the substrate (3,3' diaminobenzidine tetrahydrochloride, Sigma, St.Louis, USA) in the presence of H_2O_2. The monoclonal antibodies used were KUL01, KUL05, Av-20 and IC3. KUL01 is a marker for chicken macrophages, monocytes and interdigitating cells (Mast *et al*., 1998), while KUL05 recognises T-lymphocytes (Mast, 1998). The Av-20 monoclonal antibody recognises a monomorphic determinant on the antigen Bu-1 and identifies a marker on both bursal and peripheral B-lymphocytes (Rothwell *et al*., 1996). IC3 is a monoclonal antibody recognising 90% of chicken granulocytes (Mándi *et a*l., 1987). A polyclonal rabbit

anti-*Salmonella* Enteritidis serum was used for the detection of *Salmonella* Enteritidis bacteria in the caecal wall. In this case, a goat anti-rabbit IgG conjugate, labelled with peroxidase, was used as secondary antibody in the above described protocol.
The cells or bacteria were scored with an automatic image analysis system (Optimas 6.5., Media Cybernetics, Silver Spring, USA), measuring the percent area occupied by the labelled cells for the leukocytes and counting the number of bacteria per unit surface area for the *Salmonella* bacteria. These measurements were made in the lamina propria of the caecal wall. Leukocytes in submucosa and muscular layer were scarce. For each time point and for each chicken, 2 locations (proximal and central) of the caecum were analysed. For each location, 6 to 8 high power microscopic fields (magnification 400x) were taken at random and analysed by the image analysis program. For each time point, the mean of the percent area or counts was calculated for the 3 chickens of the same treatment. In all figures, these mean values are shown.

Sandwich ELISA for total IgA in caecal contents

To extract Ig from the caecal contents, one part of each sample was diluted 10 times with PBS (w/v) and centrifuged at 10000 x g for 10 minutes. Subsequently, supernates were collected and stored at -20ºC until analysis.
Maxisorp ELISA plates (Nunc, Wiesbaden, Germany) were coated overnight at 4ºC with 0.5µg/ml of the chicken IgA-specific MAB A1 (Erhard *et al*., 1992) in coating buffer (carbonate - bicarbonate buffer, 50µM, pH 9.6). After three washes with PBS containing 0.05% Tween 20 (washing buffer), the plates were incubated for one hour at RT with the samples diluted 1/200 in sample buffer (washing buffer containing 1% BSA). Duplicates of each sample were analyzed.
The bound chicken IgA were detected using a peroxidase-labelled, chicken Ig-specific conjugate produced in rabbit (Sigma) diluted in washing buffer. After one hour of incubation and three subsequent washes, peroxidase activity was developed using the TMB peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersbury, Maryland, USA). After 10 minutes, the reaction was stopped with 100µl peroxidase stop solution per well. The absorbance at 510 nm was recorded with a Multiscan MCC ELISA reader and could be considered as a measure for total IgA, as in a preliminary experiment, it was verified that for representative samples the applied sample dilution was always situated in the linear part of the serum dilution curve.

Statistical analysis

The data were analysed with SPSS 9.0 software using general analysis of variance methods. Treatment (O, V, C and VC), location in caecum (proximal or central) and time after inoculation were used as crossed factors (Maxwell and Delaney, 1990). To the response variable, percent area occupied by the labelled cells, an arcsine transformation was applied in order to comply to the assumption of normality of the statistic. As significant interactions occurred in the effect of the treatments with time, one-way analysis of variance with treatment as factor was repeated for each combination of caecum location and time. The effect of the treatments at a specific time after infection was considered significant if this was effectively so at both caecum levels. The effect of the treatment versus the control treatment was evaluated with the Least Significant Difference-test and the inter-treatment effects were evaluated with the Scheffé test (Maxwell and Delaney, 1990).

RESULTS

Bacteriological data

As shown in Figure 1, the *aroA* mutant strain efficiently reached liver and spleen, with peak values of about 10^5 cfu/g organ at 3 days post-infection. During the whole experiment, the number of vaccine bacteria was higher in spleen compared to liver. At the end of the experiment (11 days after delivery of the vaccine strain), the vaccine strain was decreasing in both organs to reach values of about 5.10^3 and 5.10^2 cfu/g for spleen and liver, respectively.

The number of challenge bacteria in internal organs reached peak values of about 10^4 cfu/g for spleen and 5.10^3 cfu/g for liver at 4 days post-infection in non-vaccinated, challenged animals (Figure 2). Afterwards, their number decreased in time to reach titers of 5.10^2 cfu/g for spleen and 5.10^1 cfu/g for liver at 11 days post-vaccination. The vaccinated animals had less than 10cfu/g challenge bacteria in their internal organs the first six days post-challenge. At ten days post-challenge, no differences in cfu/g organ between vaccinated and non-vaccinated animals could be detected anymore.

The titer of the challenge strain was about 10 times lower in caecal contents of the vaccinated animals during the first 6-days after challenge, compared to the unvaccinated animals (data not shown).

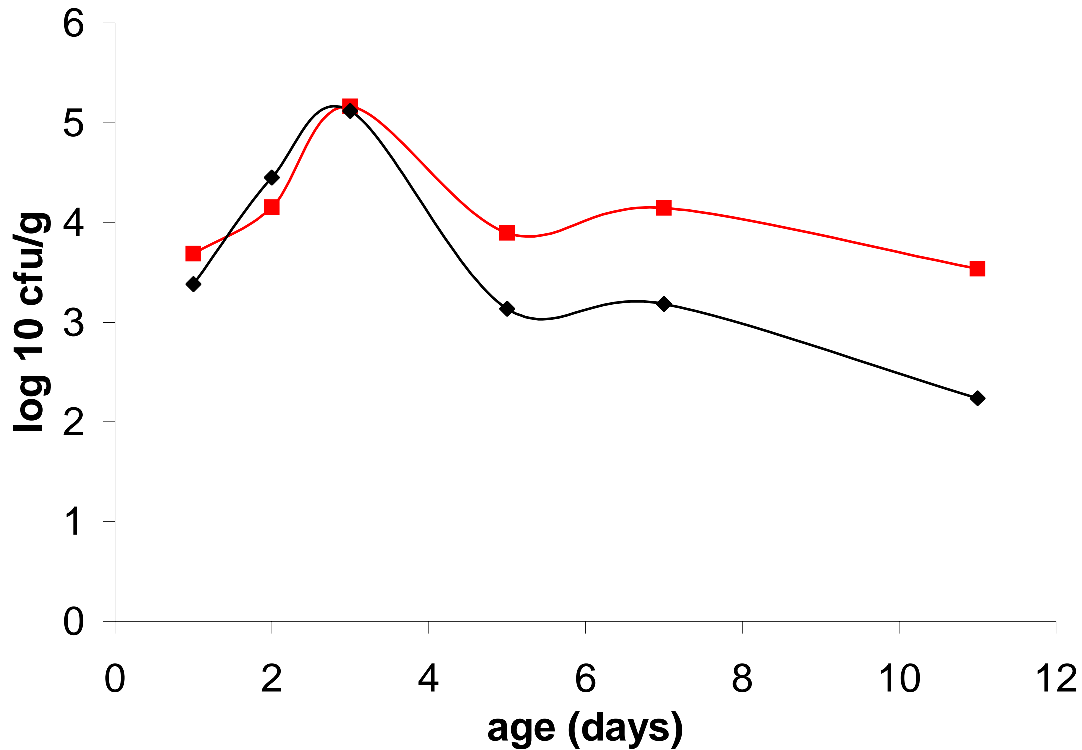


Figure 1. Mean $\log_{10}$ cfu *Salmonella* Enteritidis CVL30 bacteria present per gram spleen (■) and liver (◆) in chickens that were vaccinated at their first day of life with 10^8 cfu *Salmonella* Enteritidis CVL30 and sham-treated one day later.

Immunohistochemical data

Bacteria were first detected in the caecal lamina propria of non-vaccinated challenged animals at 16 hours post-challenge (Figure 3). In these animals, no bacteria could be detected anymore after 6 days post-challenge. Bacteria were present in the caecal wall 24 hours post-vaccination in vaccinated non-challenged animals. From 3 days post-vaccination onwards, their number decreased to reach less than 60 bacteria/mm² caecal lamina propria at 11 days post-vaccination. The number of bacteria in the caecal wall in vaccinated challenged animals was similar as for the vaccinated non-challenged animals from 2 days post-(sham-) infection on. No bacteria were detected in control animals.

Differences in infiltration of leukocytes between vaccinated non-challenged and vacinated challenged animals were small (Figures 4 to 7). Therefore both groups are named vaccinated below.

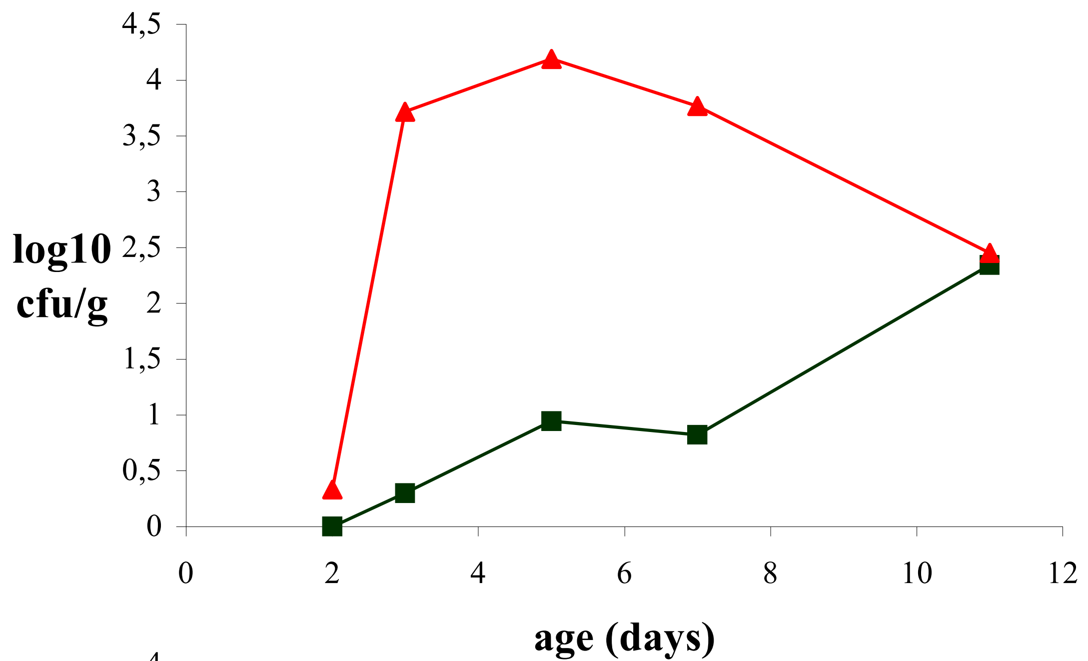
In vaccinated animals, granulocytes started infiltrating the caecal wall lamina propria from 12 hours post-vaccination on, to occupy around 6% of the area at 24 hours post-vaccination (time of challenge). From 2 days post-vaccination on, the density of these cells varied around 10% area until the end of the experiment. Control animals had a lower amount of infiltrating granulocytes, except on day 2 post-sham-challenge (3 days old) (Figure 4).

Macrophages started infiltrating in the caecal lamina propria of vaccinated animals at about 16 hours post-vaccination, as shown in figure 5. At twenty-four hours after vaccination (time of challenge), 6 to 7 percent of the caecal lamina propria was occupied by this cell type. From about 3 days post-vaccination onwards , the number of macrophages in vaccinated animals decreased to cover almost the same percent of the caecal wall area as non-vaccinated challenged and control animals at 11 days post-(sham-)vaccination, which was 6%.

Figure 6 shows that the infiltration of T-lymphocytes in the caecal lamina propria of vaccinated animals started at about 16 hours post-vaccination, to cover approximately 6% of the lamina propria at 24 hours post-vaccination. A few hours later peak values of about 12% area were detected that remained more or less constant untill 7 days post-vaccination. Afterwards, the percent area covered by T-lymphocytes in vaccinated animals decreased to reach a value of about 6% at the end of the experiment.

Infiltrating B-lymphocytes occupied a much lower percentage area of the caecal wall than all other leukocytes (Figure 7). Differences between vaccinated and control animals were detectable from about 20 hours post-vaccination. From about 2 days post-vaccination, their number increased to reach a percent area of about 6% five days post-vaccination, whereafter their number remained constant. Control animals had a low number of infiltrating T- and B-lymphocytes, compared to the vaccinated animals.

The non-vaccinated challenged animals followed more or less the same course as the vaccinated animals, but starting from the time of infection (Figures 4 to 7).



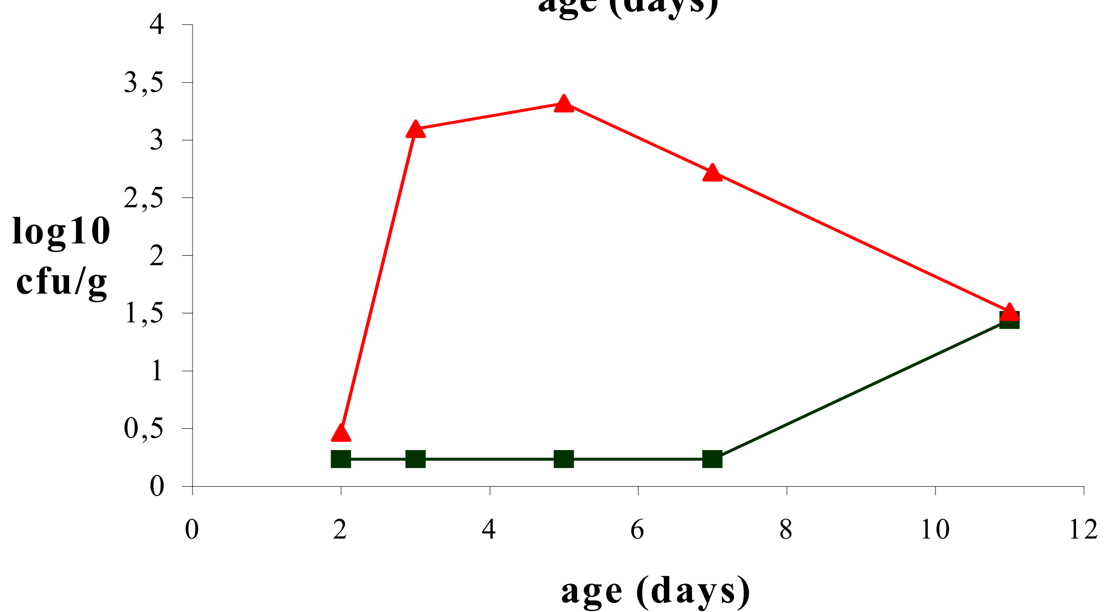
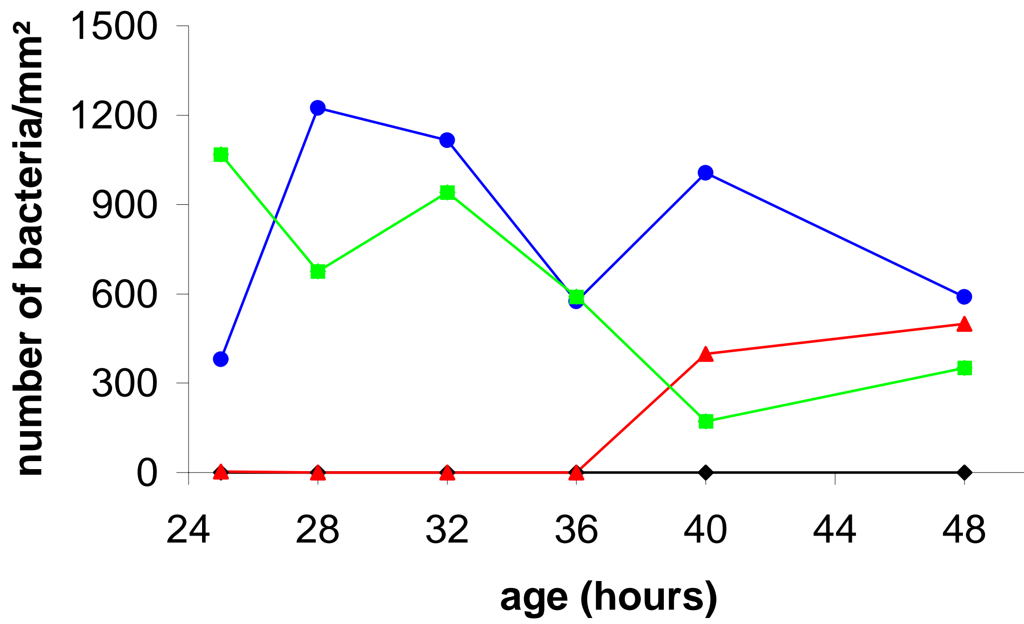


Figure 2. Mean $\log_{10}$cfu *Salmonella* NIDO76Sa88 bacteria present per gram spleen (upper) and liver (lower) in chicks that were (a) vaccinated at their first day of life with 10^8 cfu of *Salmonella* Enteritidis CVL30 and challenged 24 hours later with 5.10^3 cfu *Salmonella* Enteritidis NIDO76Sa88 (■) and (b) sham-vaccinated at their first day of life and challenged 24 hours later with 5.10^3 cfu of *Salmonella* Enteritidis NIDO76Sa88 (▲).



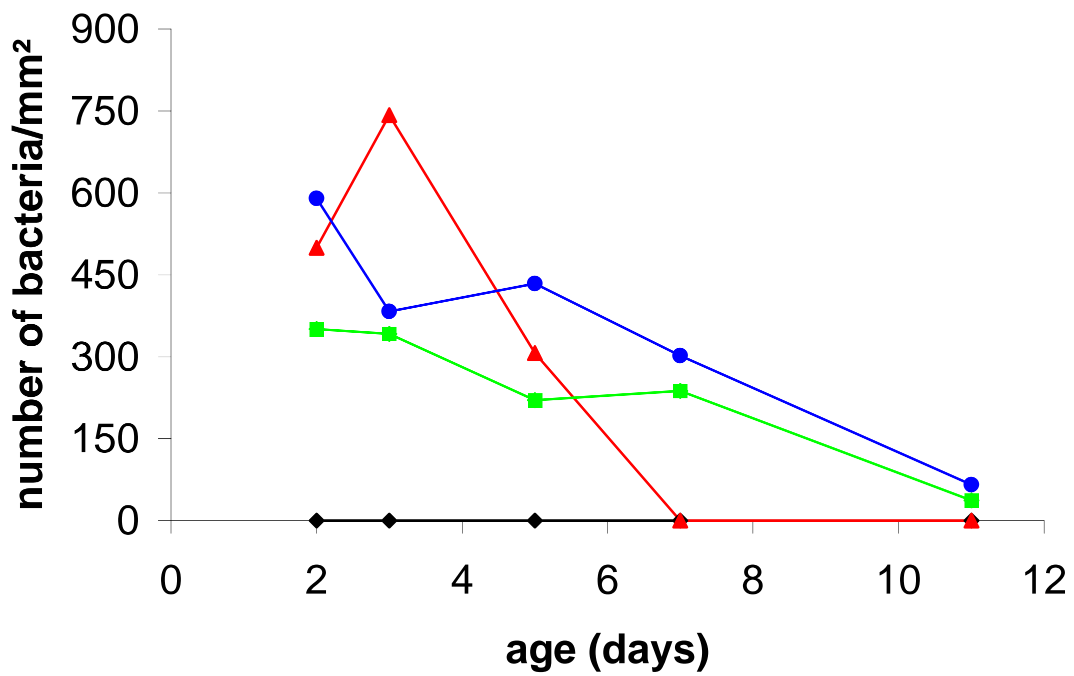


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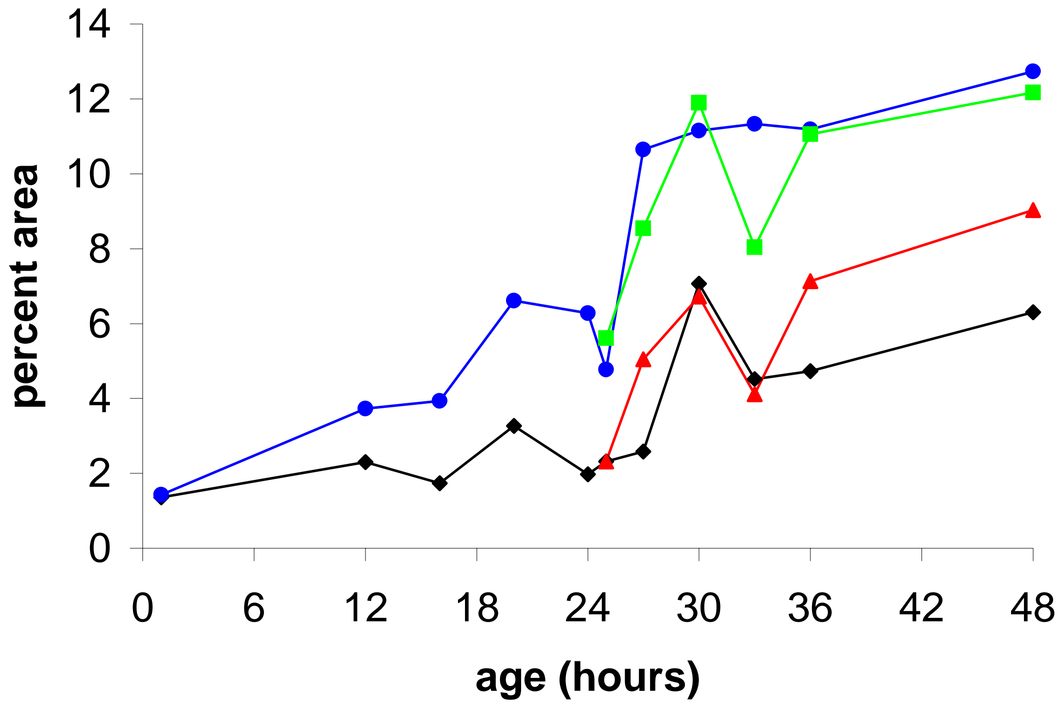
Figure 3. Number of bacteria per mm² in the caecal lamina propria in animals that were (a) sham-vaccinated on their first day of life and sham-challenged one day later (♦), (b) vaccinated with 10^8 cfu *Salmonella* Enteritidis CVL30 on their first day of life and sham-challenged one day later (●), (c) sham-vaccinated at their first day of life and challenged with 5.10^3 cfu *Salmonella* Enteritidis NIDO76Sa88 one day later (▲) and (d) vaccinated with 10^8 cfu *Salmonella* Enteritidis CVL30 on their first day of life and challenged with 5.10^3 cfu *Salmonella* Enteritidis one day later (■). Statistically significant differences ($p < 0.05$) were detected between control and (a) sham-challenged vaccinated animals at all time points (b) challenged vaccinated animals at all time points except 11 days of age (c) sham-vaccinated challenged animals at 36 hours , 2, 3, 5 and 7 days of age. Statistically significant differences ($p < 0.05$) between sham-vaccinated challenged and sham-challenged vaccinated animals were detected at 25, 27, 30, 33, 36 and 7 days of age, while differences between sham-vaccinated challenged and vaccinated challenged animals were observed at 25, 27, 30, 33 and 7 days of age. Only at 36 hours of age statistically different values were observed between vaccinated challenged and sham-challenged vaccinated animals.



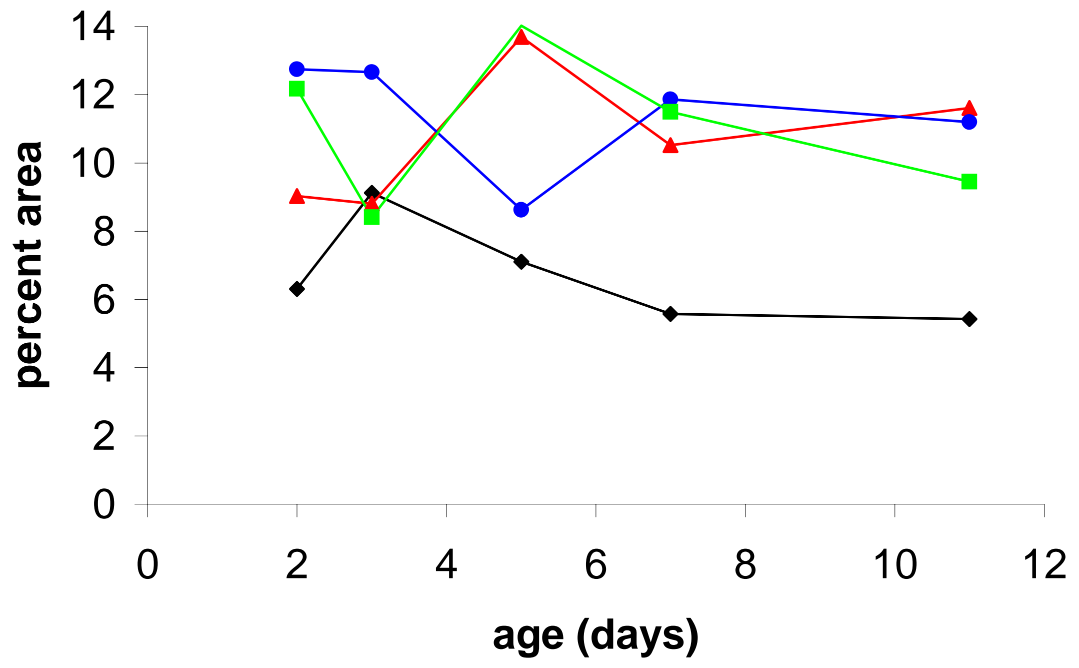


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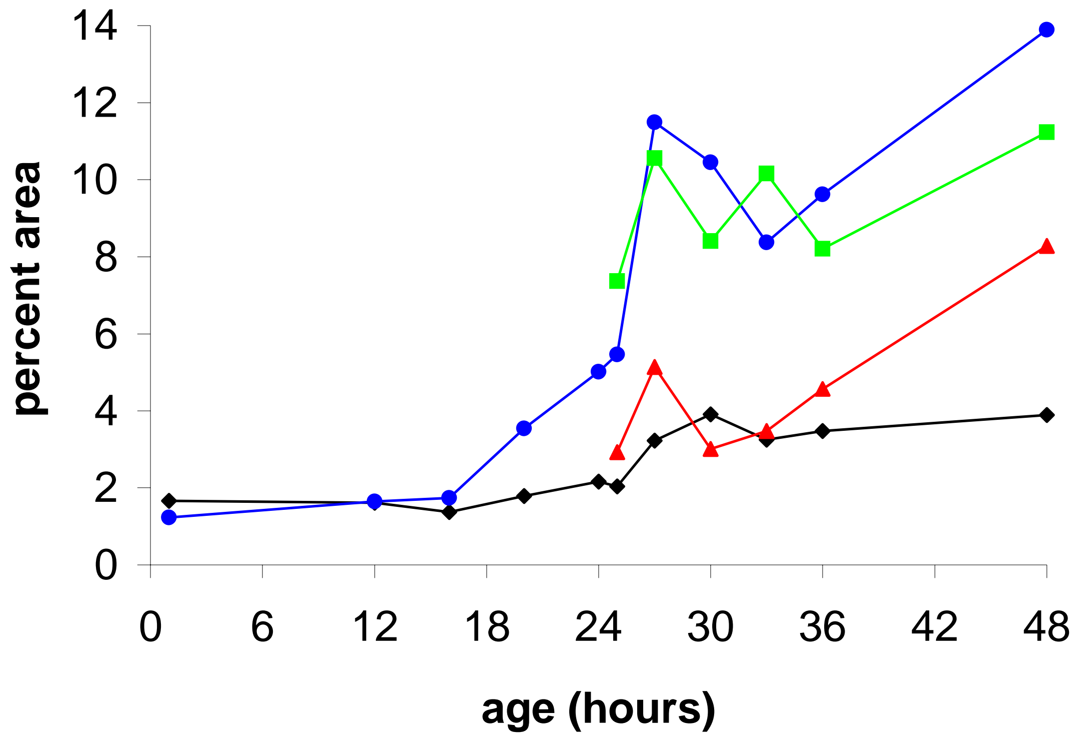
Figure 4. Area percentage of caecal lamina propria occupied by granulocytes in animals that were (a) sham-vaccinated on their first day of life and sham-challenged one day later (♦), (b) vaccinated with 10^8 cfu *Salmonella* Enteritidis CVL30 on their first day of life and sham-challenged one day later (●), (c) sham-vaccinated at their first day of life and challenged with 5.10^3 cfu *Salmonella* Enteritidis NIDO76Sa88 one day later (▲) and (d) vaccinated with 10^8 cfu *Salmonella* Enteritidis CVL30 on their first day of life and challenged with 5.10^3 cfu *Salmonella* Enteritidis one day later (■). Statistically significant differences ($p < 0.05$) were detected between control and (a) sham-challenged vaccinated animals at all time points starting from 12 hours of age except at 30 hours and five days (b) challenged vaccinated animals at all time points except at 3 and 11 days of age (c) sham-vaccinated challenged animals at 5, 7 and 11 days of age. Statistically significant differences ($p < 0.05$) between sham-vaccinated challenged and sham-challenged vaccinated animals were detected at 30, 33, 36 and 48 hours and 5 days af age, while differences between sham-vaccinated challenged and vaccinated challenged animals were observed at 25, 30, 33 and 36 hours of age. Only at 5 days of age statistically different values were observed between vaccinated challenged and sham-challenged vaccinated animals.



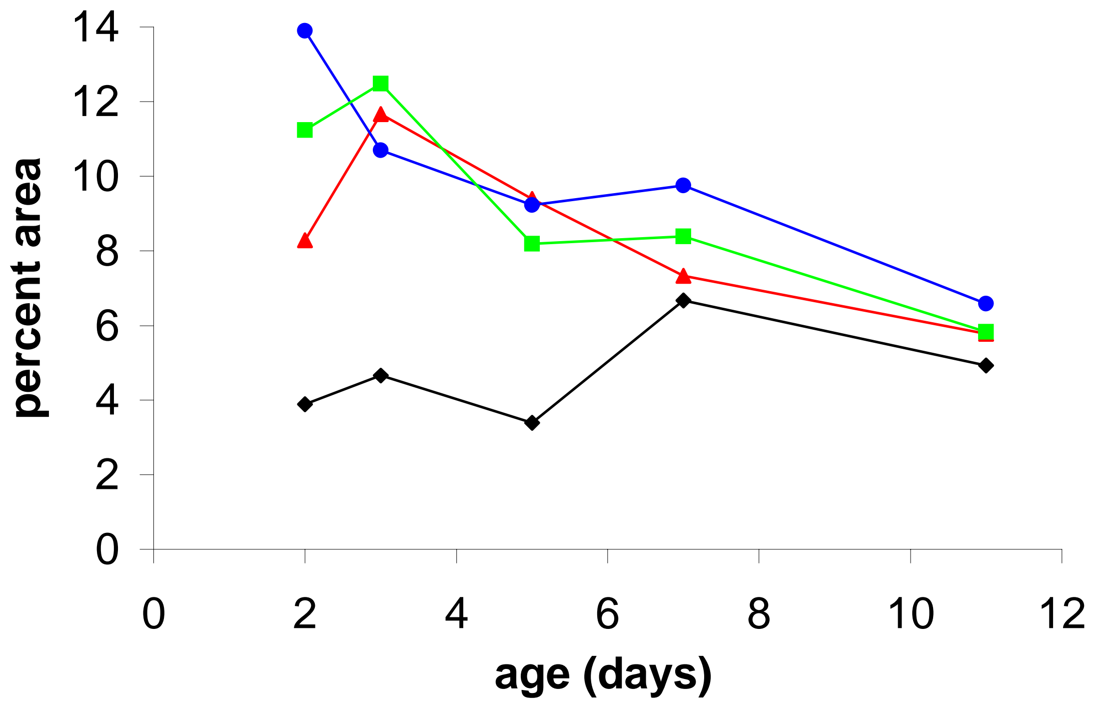


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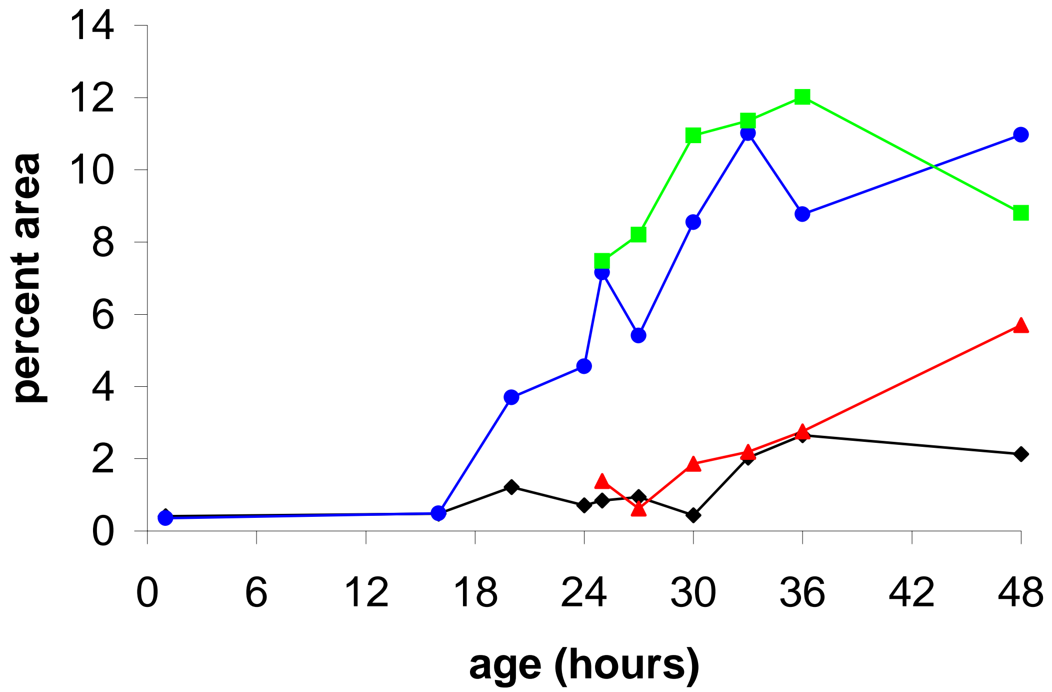
Figure 5. Area percentage of caecal lamina propria occupied by macrophages in animals that were (a) sham-vaccinated on their first day of life and sham-challenged one day later (♦), (b) vaccinated with 10^8 cfu *Salmonella* Enteritidis CVL30 on their first day of life and sham-challenged one day later (●), (c) sham-vaccinated at their first day of life and challenged with 5.10^3 cfu *Salmonella* Enteritidis NIDO76Sa88 one day later (▲) and (d) vaccinated with 10^8 cfu *Salmonella* Enteritidis CVL30 on their first day of life and challenged with 5.10^3 cfu *Salmonella* Enteritidis one day later (■).Statistically significant differences ($p < 0.05$) were detected between control and (a) sham-challenged vaccinated animals at all time points starting from 20 hours of age, except at 5 and 11 days of age (b) challenged vaccinated animals at all time points during except 5 and 11 days of age (c) sham-vaccinated challenged animals at 27 hours, 2, 3 and 5 days of age. Statistically significant differences ($p < 0.05$) between sham-vaccinated challenged and sham-challenged vaccinated animals were detected at 27, 30, 33, 36 and 48 hours age, while differences between sham-vaccinated challenged and vaccinated challenged animals were observed at 25, 27, 30, 33 and 36 hours of age. No statistically significant differences were observed between vaccinated challenged and sham-challenged vaccinated animals.



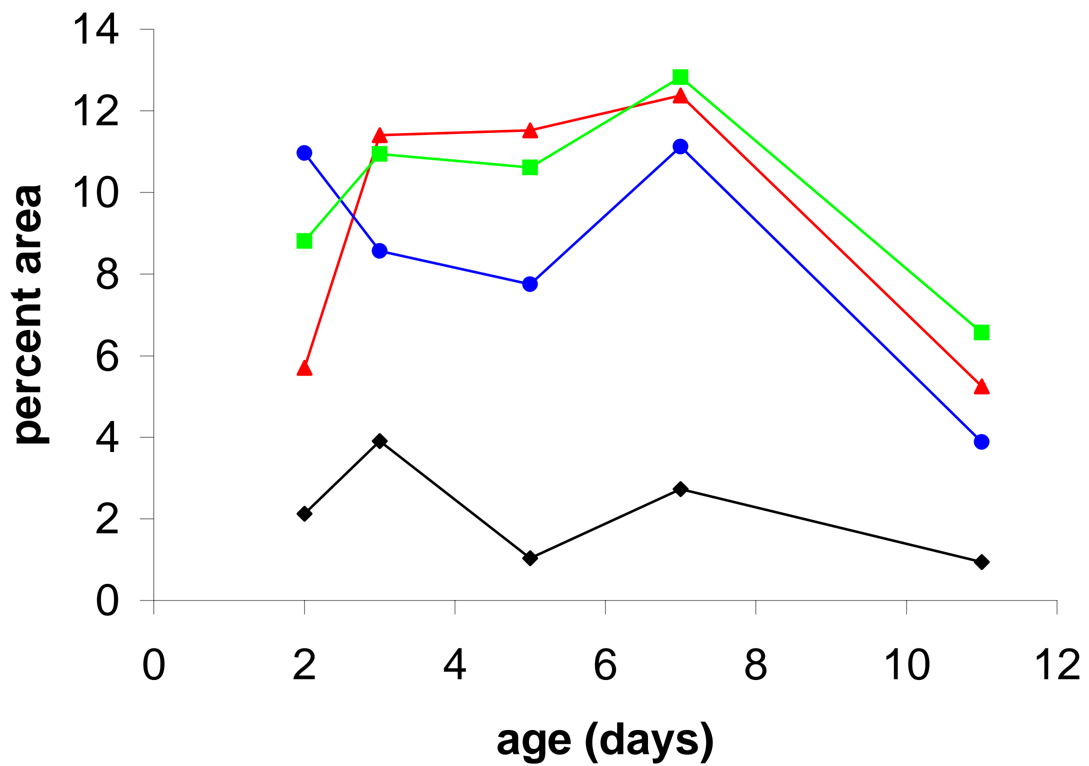


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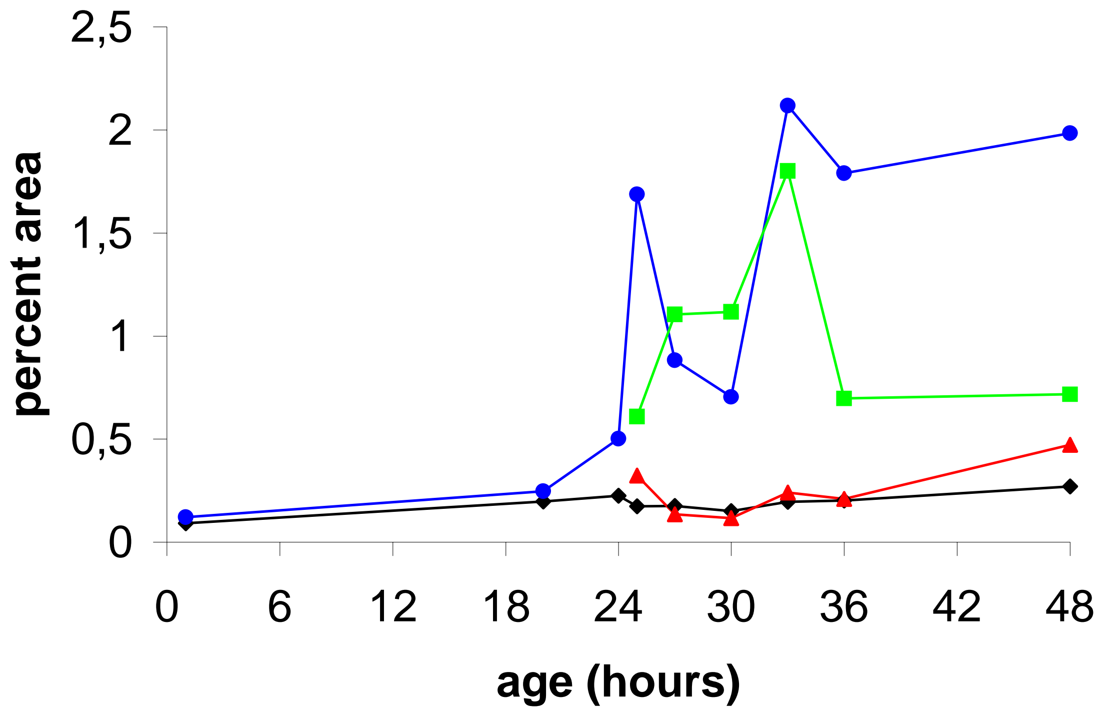
Figure 6. Area percentage of caecal lamina propria occupied by T-lymphocytes in animals that were (a) sham-vaccinated on their first day of life and sham-challenged one day later (♦), (b) vaccinated with 10^8 cfu *Salmonella* Enteritidis CVL30 on their first day of life and sham-challenged one day later (●), (c) sham-vaccinated at their first day of life and challenged with 5.10^3 cfu *Salmonella* Enteritidis NIDO76Sa88 one day later (▲) and (d) vaccinated with 10^8 cfu *Salmonella* Enteritidis CVL30 on their first day of life and challenged with 5.10^3 cfu *Salmonella* Enteritidis one day later (■).Statistically significant differences ($p < 0.05$) were detected between control and (a) sham-challenged vaccinated animals at all time points starting from 20 hours of age (b) challenged vaccinated animals at all time points (c) sham-vaccinated challenged animals at all time points starting from 2 days of age. Statistically significant differences ($p < 0.05$) between sham-vaccinated challenged and sham-challenged vaccinated animals were detected at 25, 27, 30, 33, 36 and 48 hours of age, while differences between sham-vaccinated challenged and vaccinated challenged animals were observed at 25, 27, 30, 33 and 36 hours of age. No statistically significant differences were observed between vaccinated challenged and sham-challenged vaccinated animals.



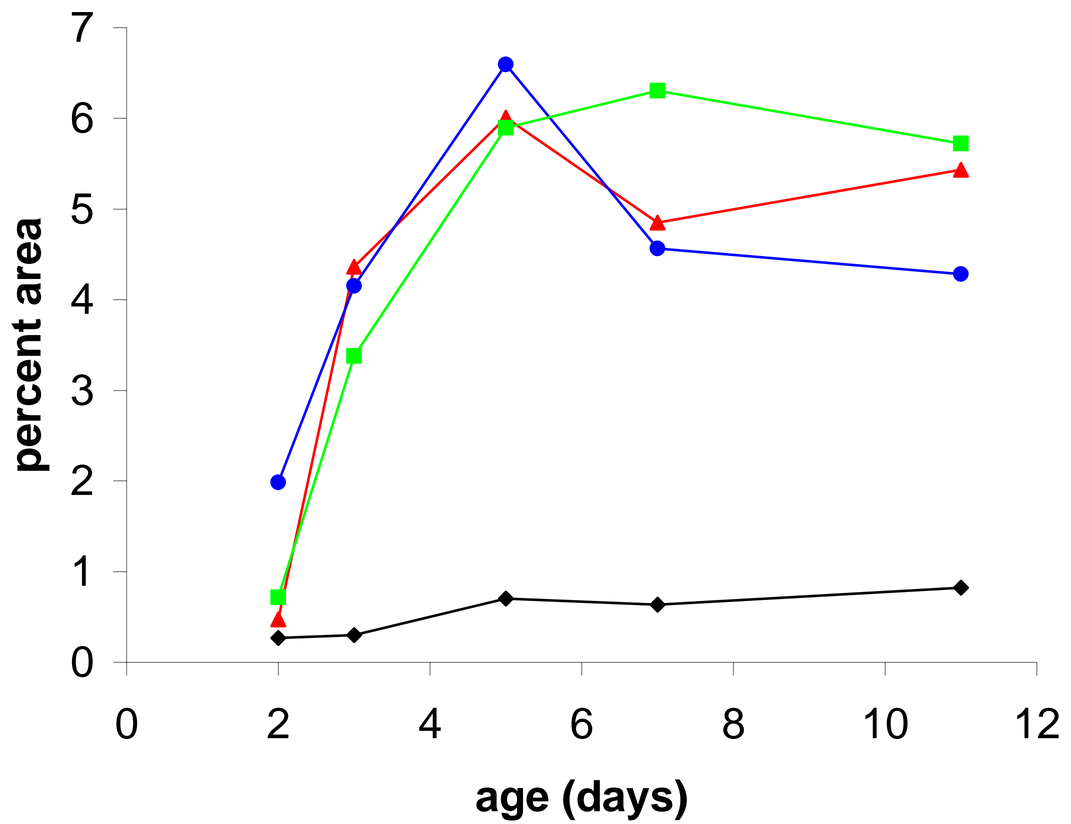


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Figure 7. Area percentage of caecal lamina propria occupied by B-lymphocytes in animals that were (a) sham-vaccinated on their first day of life and sham-challenged one day later (♦), (b) vaccinated with 10^8 cfu *Salmonella* Enteritidis CVL30 on their first day of life and sham-challenged one day later (●), (c) sham-vaccinated at their first day of life and challenged with 5.10^3 cfu *Salmonella* Enteritidis NIDO76Sa88 one day later (▲) and (d) vaccinated with 10^8 cfu *Salmonella* Enteritidis CVL30 on their first day of life and challenged with 5.10^3 cfu *Salmonella* Enteritidis one day later (■).Statistically significant differences ($p < 0.05$) were detected between control and (a) sham-challenged vaccinated animals at all time points starting from 24 hours of age (b) challenged vaccinated animals at all time points except at 27, 30, 36 and 48 hours of age (c) sham-vaccinated challenged animals at 3, 5, 7 and 11 days of age. Statistically significant differences ($p < 0.05$) between sham-vaccinated challenged and sham-challenged vaccinated animals were detected at 25, 33, 36 and 48 hours, while differences between sham-vaccinated challenged and vaccinated challenged animals were observed at 33 hours of age. Only at 33 hours of age statistically different values were observed between vaccinated challenged and sham-challenged vaccinated animals.

Sandwich ELISA for total IgA in caecal contents

During the experiment, no *Salmonella*-specific IgA were detected in the caecal contents (data not shown). For total IgA, the mean absorbance was beneath 0.1 for control animals at all analyzed time points. For all non-control animals, mean absorbances were higher than 0.35 at 3 days and higher than 1.1 at 5 days post-(sham)vaccination (data not shown).

DISCUSSION

Live attenuated *Salmonella* strains can colonize the alimentary tract of neonatal chicks, due to a lack of normal microflora in the intestine of these animals. This prevents colonization by virulent *Salmonella* strains inoculated within the first day afterwards, what is known as colonization-inhibition (Barrow et al., 1987; Berchieri and Barrow, 1990). Currently, research groups are trying to elucidate the mechanisms of this colonization-inhibition phenomenon (Zhang-Barber *et al*., 1999). Analogous inhibitory phenomena were seen when a *Salmonella* strain was inoculated in stationary-phase nutrient broth cultures following pre-inoculation with another *Salmonella* strain (Barrow, 1996). This inhibitory phenomenon however is not observed with all *Salmonella* strains tested. The current hypothesis is that growth of the second strain is suppressed because of the absence of utilizable carbon sources or electron acceptors (Zhang-Barber *et al*., 1997). However, unpublished results of our laboratory indicate that there are large differences in inhibition of a virulent *Salmonella* Enteritidis strain, when strains of different *Salmonella* serovars were pre-inoculated in stationary phase cultures. *AroA* mutant *Salmonella* strains were only partly inhibitory in stationary phase nutrient broth cultures (Barrow, personal communication). Therefore the *in vivo* inhibitory effect of the *Salmonella* Enteritidis *aroA* mutant strain, as seen in our experiments, is not likely to be an exclusively microbiological phenomenon. Host-related mechanisms most probably play also a role in this *in vivo* inhibition.

Our bacteriological data show that inoculation of newly hatched chicks with 10^8 cfu of a *Salmonella* Enteritidis *aroA* mutant, resulted in a decrease in number of challenge bacteria in caecal contents and internal organs, at least during the first 6 days after subsequent experimental infection with a virulent Salmonella Enteritidis strain. These results confirm previous results, in which colonization of the caeca and invasion from the gut was reduced in the vaccinated chicks up to 5 days post-challenge (Cooper *et al*., 1994b). The first days following vaccination and subsequent infection were therefore suitable to study the early colonization-inhibition phenomena, seen when two *Salmonella* strains were subsequently inoculated in young chickens.

Our data suggest that invasion of the caecal wall by *Salmonella* Enteritidis bacteria triggers an immune cell infiltration, with heterophils being the first to react. Macrophages and T-cells infiltrate a few hours after the heterophils. Finally also B-lymphocytes enter the caecal wall. Kinetics of the immune cell infiltration after inoculation of the *Salmonella* Enteritidis *aroA* strain and the virulent strain were similar. This is consistent with the findings of Berndt *et al*. (Berndt *et al*., 2001), who detected similar changes in T-cell populations after inoculation of virulent and attenuated *Salmonella* Typhimurium strains in chickens.

It is unlikely that B-lymphocytes play an important role in the early colonization-inhibition phenomenon. Salmonella-specific IgA were not detected in the caecal content the first days after inoculation of neither of the two *Salmonella* strains, while the total IgA level increased during time after inoculation with the *Salmonella* strains. Serum antibodies against the lipopolysaccharide antigen of *Salmonella* Enteritidis were first detected at 18 days after inoculation of day-old chicks (Desmidt *et al*., 1997). Several studies have shown that B-lymphocytes are immature at hatch. Indeed, the B-cell compartment of the spleen, which is the most important organ for the generation of humoral responses against T-cell dependent antigens, gradually matures during the first week post-hatch (Mast and Goddeeris, 1999). Lymphoid infiltrates of other antibody producing tissues, like the mucosa-associated lymphoid tissues, only start to appear at day 5 post-hatch (Jeurissen *et al*., 1989).

Because of the immature characteristics of T-lymphocytes and macrophages, these cells are also not likely to play an important role in inhibition of colonization during the first days post-hatch. Concerning T-lymphocytes, a period of transient T-cell unresponsiveness to immune stimulation exists the first days post-hatch in chickens (Lehtonen *et al*., 1989; Lillehoj *et al*., 1992). T-cells from day-old chicks fail to proliferate and produce cytokines. Reactivity to immune stimulation gradually develops from the second day of life, reaching normal activities after one week (Lowenthal *et al*., 1994). Also the phagocytic and bactericidal activities of macrophages to *Salmonella* were shown to be age-dependent in chickens, with normal activities being generated a few days post-hatch (Kodama *et al*., 1976, 1977).

There are several indications that heterophils play an important role in the colonization-inhibition phenomenon, when 2 *Salmonella* strains are subsequently administrated. In the present study it was shown that these cells invade the caecal wall within 16 hours after inoculation of the *aroA* mutant. Although age-dependent phagocytic and bactericidal activity have been described in heterophils, these cells efficiently kill *Salmonella* the first day post-hatch (Wells *et al*., 1998). Furthermore, heterophil depletion studies indicate that these cells are decisive in the early protection against *Salmonella* Enteritidis (Kogut *et al*., 1993, 1994).

In conclusion, vaccination of newly hatched with a *Salmonella* Enteritidis *aroA* mutant strain partly protects newly hatched chicks against a subsequent challenge with a virulent strain, during the first days of their life, resulting in reduced numbers of challenge bacteria in the caeca and in liver and spleen. The underlying mechanisms of this protection are possibly both microbiological and host-related. The rapid infiltration of immune cells after inoculation with *aroA* mutant bacteria are indicative for a contribution of the host response in protection. However, the functional activities of these infiltrating cells need to be investigated to get a full understanding of this phenomenon.

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CHAPTER 3

Early protection against *Salmonella* Enteritidis after vaccination with a live strain is abolished by depletion of the heterophilic granulocytes

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ABSTRACT

Vaccination of day-old chicks with a live *aroA* mutant of *Salmonella* Enteritidis results in increased resistance against challenge with a field strain of the same serotype within 24 hours. The vaccination results in massive influx of heterophils in the caecal lamina propria within 24 hours, which in timing matches the early protection against a *S*. Enteritidis field strain. In the present study, the effect of vaccination of day-old chickens with a live *aroA* mutant of *Salmonella* Enteritidis on subsequent challenge with a field strain of the same serotype 24 hours later was studied in heterophil-depleted chickens. Therefore, newly-hatched chickens, heterophil depleted by injection of the cytostatic drug 5-fluorouracil, were divided into 4 groups: O = control, V = vaccinated, C = challenged and VC = vaccinated and challenged. The chickens of groups V and VC were vaccinated orally with 10^8 cfu of *Salmonella* Enteritidis CVL30, an *aroA* mutant, at 1 day of age. The animals of groups C and VC were challenged with 5.10^3 cfu *Salmonella* Enteritidis strain 76Sa88 24 hours later. Administration of wild type *Salmonella* bacteria resulted in extensive colonization of caeca and invasion in liver and spleen in groups C as well as VC. It was concluded that in these heterophil-depleted chickens vaccination with a live *aroA* mutant of *Salmonella* Enteritidis did not afford early protection against challenge with a field strain of the same serotype, thus confirming earlier suggestions that heterophilic granulocytes play an important role in the early protective response induced by live vaccines.

INTRODUCTION

Salmonella enterica serovar Enteritidis is a gram-negative, facultative intracellular pathogen causing disease in men and animals. Humans become infected with *S*. Enteritidis mainly through ingestion of contaminated foods of poultry origin, including eggs and poultry meat (Poppe, 1999). The tremendous increase in isolation of *S*. Enteritidis in poultry during the last 15 years, and the fact that *S*. Enteritidis is one of the major causes of foodborne gastro-enteritis, made monitoring programs indispensable and stimulated research into control strategies. Amongst them, vaccination opens new perspectives in the control of salmonellosis (Zhang-Barber *et al*., 1999).

Several live and killed vaccines have already been tested with variable results (Barrow, 1991). Live vaccines confer better protection than killed vaccines (Griffin, 1991). Amongst them, aromatase deficient strains (*aroA*) offer good perspectives. Vaccination with a *S*. Enteritidis *aroA* mutant reduces faecal shedding of a *S*. Enteritidis challenge strain administered orally, and significantly reduces the colonization of internal organs after intravenous challenge (Cooper *et al*., 1990, 1992). This is not only the result of an acquired specific adaptive immune response which develops only 10 to 14 days after vaccination. Indeed, when newly-hatched chicks are vaccinated with a *S*. Enteritidis *aroA* mutant, and challenged 24 hours later with a wild-type *S*. Enteritidis strain, the number of challenge strain bacteria recovered from liver, spleen and caecal content is considerably reduced (Cooper *et al*., 1994). This phenomenon may be a valuable tool to protect young chicks during the neonatal period, when they are extremely susceptible to *Salmonella* infection (Desmidt *et al*., 1997). A similar phenomenon has been observed using other serotypes in other experimental models (Martin *et al*., 1996; Barrow *et al*., 1997; Foster *et al*., 2003).

Although several authors have confirmed this early protection phenomenon, there is considerable controversy in the literature as to the mechanism involved. Some authors have suggested that this is merely an interaction between two bacterial strains, mediated by bacteriocin-like factors (Martin *et al*., 1996). There have been suggestions that the phenomenon can be reproduced *in vitro* and that it is not the consequence of nutrient depletion (Zhang-Barber *et al*., 1997). The results of these *in vitro* experiments, however, do not always seem to match the results of *in vivo*

experiments. Moreover previous results (Van Immerseel *et al*., 2002a) from our group indicated a very early influx of heterophylic granulocytes in the lamina propria of the caecal wall, which in timing matched the development of the early protection phenomenon, suggesting a role of host non-specific immunity.

The purpose of the present work was to study the very early protective effect of a live *aroA* mutant vaccine of *Salmonella* Enteritidis against challenge 24 hours later with a *Salmonella* Enteritidis field strain in heterophil-depleted chickens.

MATERIALS AND METHODS

Salmonella Strains

An *aroA* mutant of *S*. Enteritidis was used for vaccination: strain LA5, phage type four (PT4), designated CVL30 (Cooper *et al*., 1994). A nalidixic acid resistant strain of *S*. Enteritidis (76Sa88 Nal^R) was used for challenge. For both *Salmonella* strains, a Brain Heart Infusion (BHI; Oxoid, Basingstoke, Hampshire, England) broth culture was made. After inoculation on ISO-sensitest-agar (Oxoid, Basingstoke, Hampshire, England) ten colonies were brought in 2ml BHI-broth and shaked for 20 hours at 37°C. Then, ten-fold dilutions were made with phosphate buffered saline (PBS) and 6 times 20 µl of each dilution was plated out on brilliant green agar (BGA; Oxoid, Basingstoke, Hampshire, England). Colonies were counted to determine the number of colony forming units per ml BHI-broth. The broth was stored at 4°C and the appropriate dilution was made to obtain the desired infection dose.

Assessment of 5-fluorouracil injection route

A preliminary study was carried out to check for the optimal route of 5-fluoruracil (Fluroblastine®, Pharmacia, Puurs, Belgium) injection for depletion of granulocytes in the caecal wall after *Salmonella* infection. Five groups of three chickens were orally inoculated with 5.10^3 cfu *S*. Enteritidis 76Sa88 2 days post-hatch. Of these, four groups were injected with 200 mg/kg 5-fluorouracil. The injection was done by intravenous, intraperitoneal, intramuscular and subcutaneous route at the first day post-hatch, respectively. One group served as a non 5-fluorouracil treated control. Three days post-infection with S. Enteritidis 76Sa88, the animals were euthanised and caecal specimens were frozen. Immunohistochemical stainings were performed for the detection of heterophilic granulocytes as described further.

Animals in the vaccination – challenge experiment

Specific pathogen free (SPF) chicken eggs (Lohmann Tierzucht GmbH, Cuxhaven, Germany) were hatched in isolation. Immediately after hatching, cloacal swabs were

taken from the chicks and tested for *Salmonella*. This was done by direct inoculation on brilliant green agar (BGA), as well as by pre-enrichment/enrichment. Pre-enrichment was done in buffered peptone water (BPW), while enrichment was performed in tetrathionate-brilliant green broth. Also serum samples were taken for the detection of maternal antibodies against *S.* Enteritidis by means of a previously described ELISA (Desmidt *et al*., 1996).
The chicks were divided at random into four groups of 18 chickens each. Each group was housed under the same conditions, but in different isolation units. From the second day after hatching, chicks had access to ad libitum sterile water and feed (Versele-Laga, Deinze, Belgium) treated with 25 kGy of γ-radiation (Griffith-Mediris, Fleurus, Belgium).

Vaccination – challenge experiment

Seventy two newly-hatched chickens each received 200 mg/kg body weight 5-fluorouracil, to induce heterophil depletion, as decribed by Kogut *et al*. (1993). Then the animals were randomly divided into four groups (O = control, V = vaccinated, C = challenged, VC = vaccinated and challenged) of 18 chicks each.
The day after hatching chicks of the groups V and VC all received 10^8 cfu of *S.* Enteritidis CVL30 in 0.5 ml BHI-broth, while groups O and C were sham-inoculated with 0.5 ml sterile BHI-broth. Twentyfour hours later, the chicks of groups C and VC were inoculated with 5.10^3 cfu of *S.* Enteritidis NIDO76Sa88 Nal^R in 0.5 ml BHI-broth while groups O and V were sham-inoculated. Inoculations were performed orally in the crop using a sterile plastic tube.
At 1 and 24 hours and at 3, 4 and 5 days post-challenge, three chicks from each treatment group were euthanised. Samples of liver, spleen and caecal content were collected aseptically for bacteriological analysis. Specimens of the central and distal part of the remaining caecum were submerged in a cryoprotectant gel (Tissue-tek® O.C.T™ Compound, Sakura, Zoeterwoude, The Netherlands), snapfrozen at –70°C and used for immunohistochemical analyses.

Bacteriological examination

Organs were weighed, homogenized and diluted (caecum 1/5, liver 1/10 and spleen 1/20) in buffered peptone water (BPW; Oxoid, Basingstoke, Hampshire, England). Decimal dilutions were made in PBS. From each dilution, 6 x 20 µl was plated out on BGA and incubated overnight at 37°C. Growth of the challenge strain (Nal^R) was evaluated on BGA with 20 mg/l nalidixic acid, and growth of the vaccine strain on BGA without nalidixic acid. When no growth was visible in direct inoculation, additional samples were pre-enriched in BPW (overnight, 37°C), thereafter enriched in tetrathionate-brilliantgreen broth (overnight, 37°C) and plated out on BGA with and without nalidixic acid. For liver, spleen and caecum, viable *Salmonella* bacteria were counted and the number of cfu/g tissue was determined. Samples that were negative after direct inoculation but positive after enrichment, were presumed to have 10^1 (liver), 2.10^1 (spleen) or 5 cfu/g (caecum). Samples that remained negative were presumed to have 0 cfu/ml. The mean number of cfu/g tissue was calculated for 3 chickens of the same treatment group and for each time point.

Immunohistochemistry

From the snapfrozen caecal specimens, 6 µm thick cryostat sections were cut and fixed in acetone. Immunohistochemical labelling of heterophylic granulocytes was done as described by Mast (1998), using IC3, a monoclonal antibody recognising 90% of chicken granulocytes (Mándi *et al*., 1987). Briefly the sections were rinsed with PBS to wash-out the cryoprotectant. The primary antibody (IC3) to chicken heterophils was diluted 1:1600 in PBS pH 7.85 with 2% skimmed milkpowder (PBS+smp) and was applied during 45 minutes at room temperature. After rinsing with PBS, goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Dako, Glostrup, Denmark in a dilution of 1:200 in PBS+smp) was added and tissue sections were incubated at room temperature for 30 min. After rinsing, 3.3'-diaminobenzidine tablets (3.3', 4.4' Tetraaminobiphenyl Tetrahydrochloride 10mg/tablet; Sigma, St.Louis, USA) were dissolved in TRIS-HCl-buffered saline (pH 7.6) with 0.003% fresh hydrogen peroxide. This solution was filtered through a 0.2 µm filter immediately prior to use. Sections were incubated at ambient temperature for 10 min. The sections were counterstained with hematoxylin and mounted. Positive

cells stained brown. The amount of heterophils was scored with a computerised image-analysis system (Optimas 6.5., Media Cybernetics, Silver Spring, USA), measuring the percent area occupied by the labelled cells in the caecal lamina propria. For each time point and for each chicken, 2 locations (proximal and central part of the caecum) and 8 randomly chosen microscopic fields (High Power Field examined at x 400) per location were analysed. For each time point, the mean of the percent area was calculated for the 3 chickens of the same treatment group.

Statistical analysis

All data were analysed by a general analysis of variance method using SPSS 9.0 software. Treatment (O, V, C and VC), location in caecum (proximal or central) and time after challenge were used as crossed factors (Maxwell and Delaney, 1990). An arcsine transformation was applied to the response variable (percent area occupied by the labelled cells), in order to comply to the assumption of normality of the statistic. The Least Significant Difference-test was used to evaluate the effect of the treatment versus the control treatment, while the inter-treatment effects were evaluated with the Scheffé test (Maxwell and Delaney, 1990). The effect of the treatments at a specific time after infection was considered significant if this was effectively so at both caecum levels.

RESULTS

Assessment of the route of injection of 5 fluoro-uracil

Quantitative image analysis of immunohistochemically stained sections revealed a thin caecal wall with a low amount of heterophilic granulocytes 3 days post-infection, in all animals that were treated with 5- fluoro-uracil. The group that was injected subcutaneously with 5-fluoro-uracil gave the lowest average of area percentage of the caecal wall occupied by heterophilic granulocytes : a 4 times reduction in the area percentage of the caecal wall occupied by these cells was observed, as compared to non 5-fluorouracil treated animals.

Bacteriological data of the vaccination – challenge experiment

The vaccine strain efficiently reached internal organs to a density of about 5.10^3 cfu/g of liver and 10^5 cfu/g of spleen at 6 days after vaccination (Fig. 1). More than 10^8 cfu/g caecal contents of the vaccine strain was detected throughout the experiment (data not shown) in vaccinated animals.
One hour after challenge infection, challenge bacteria were already detected in the caecal contents and levels of 10^9 cfu/g were reached at 5 days post-challenge (Fig. 2). In non-vaccinated, challenged animals, the number of challenge bacteria reached maximum levels of about 10^9 cfu/g in liver (Fig. 3) and spleen (Fig. 4) at 4 days post-infection, decreasing afterwards. In the vaccinated animals, the challenge strain started its invasion later compared to unvaccinated chickens, reaching maximum levels of 10^5 cfu/g in liver (Fig. 3) and 10^7 cfu/g in spleen (Fig. 4) six days after challenge.

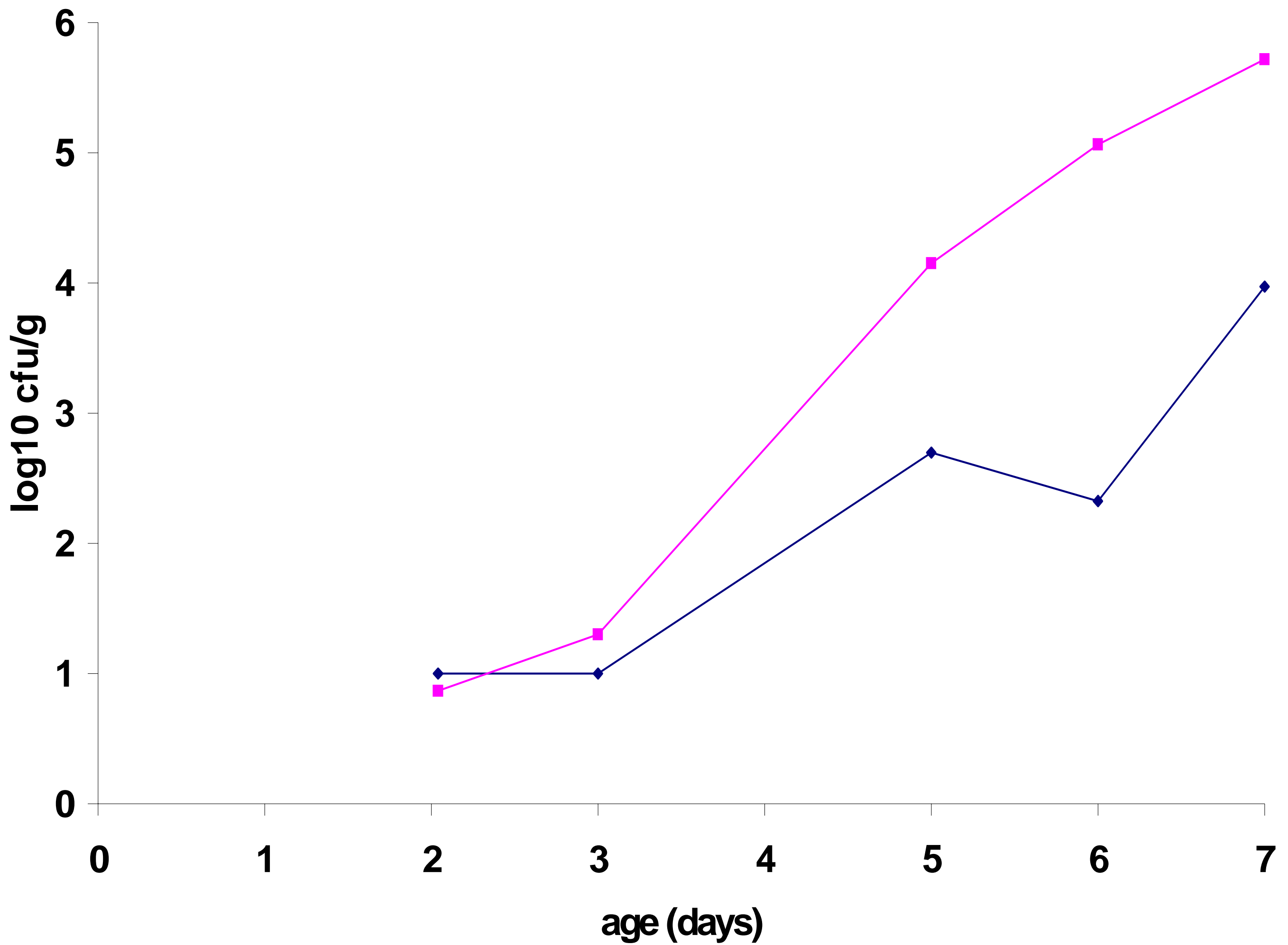

Figure 1. Mean $\log_{10}$ cfu *Salmonella* Enteritidis CVL30 bacteria (vaccine strain) present per gram liver (♦) and spleen (■) in chickens that were injected with 5-fluorouracil (200 mg/kg body weight) at hatch, vaccinated with 10^8 cfu of *Salmonella* Enteritidis CVL30 at their first day of life and sham-challenged 1 day later.

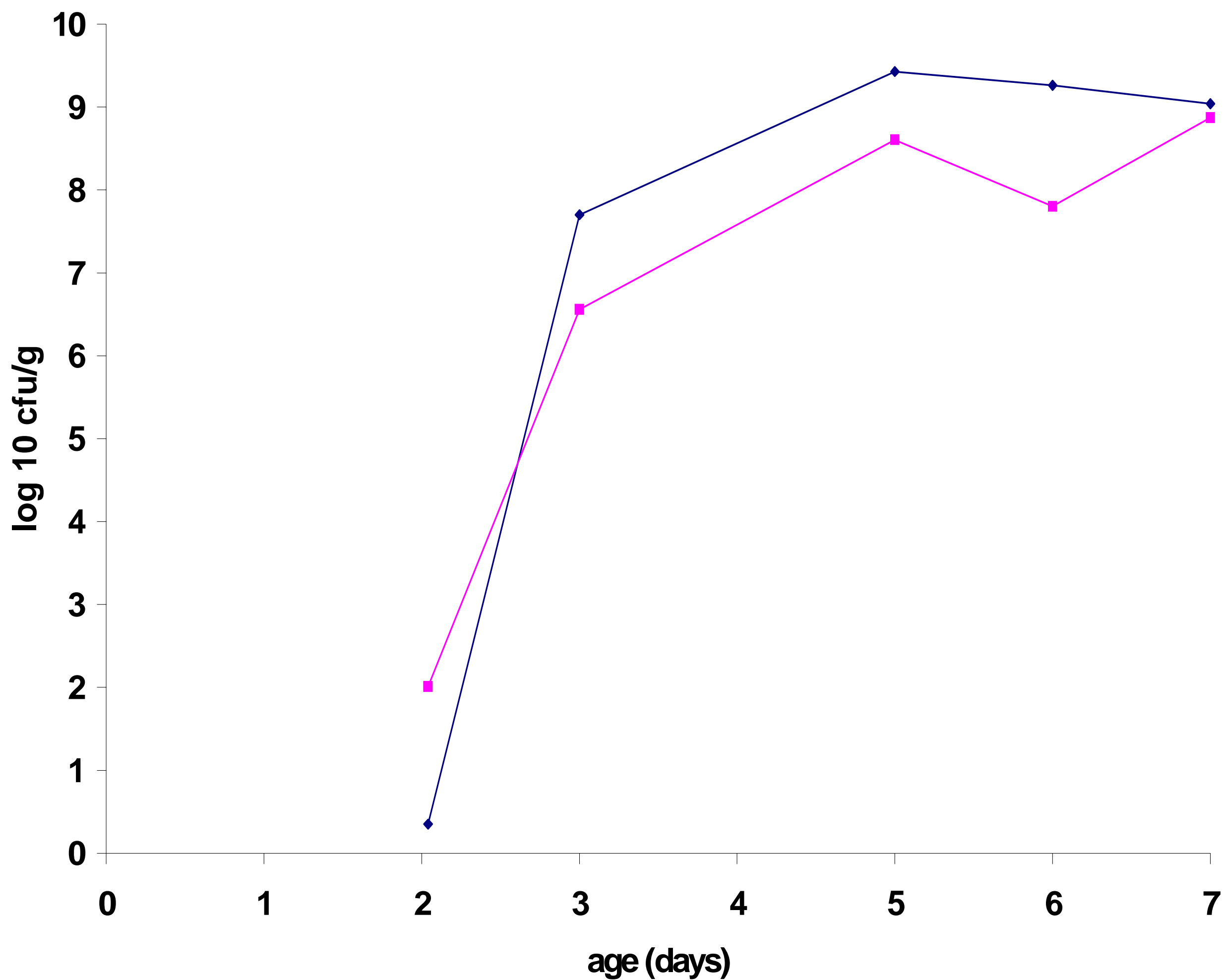

Figure 2. Mean $\log_{10}$ cfu *Salmonella* bacteria (challenge strain 76Sa88) present per gram caecum in chicks that were injected with 5-fluorouracil (200 mg/kg body weight) at hatch. Chickens of group C (♦) were sham-vaccinated after 24 hours and challenged 24 hours later with 5.10^3 cfu wild type *Salmonella* Enteritidis strain 76Sa88. Animals of group VC (■) were vaccinated 24 hours after 5-fluorouracil injection with 10^8 cfu of *Salmonella* Enteritidis CVL30 and challenged 24 hours later with 5.10^3 cfu wild type *Salmonella* Enteritidis strain 76Sa88.

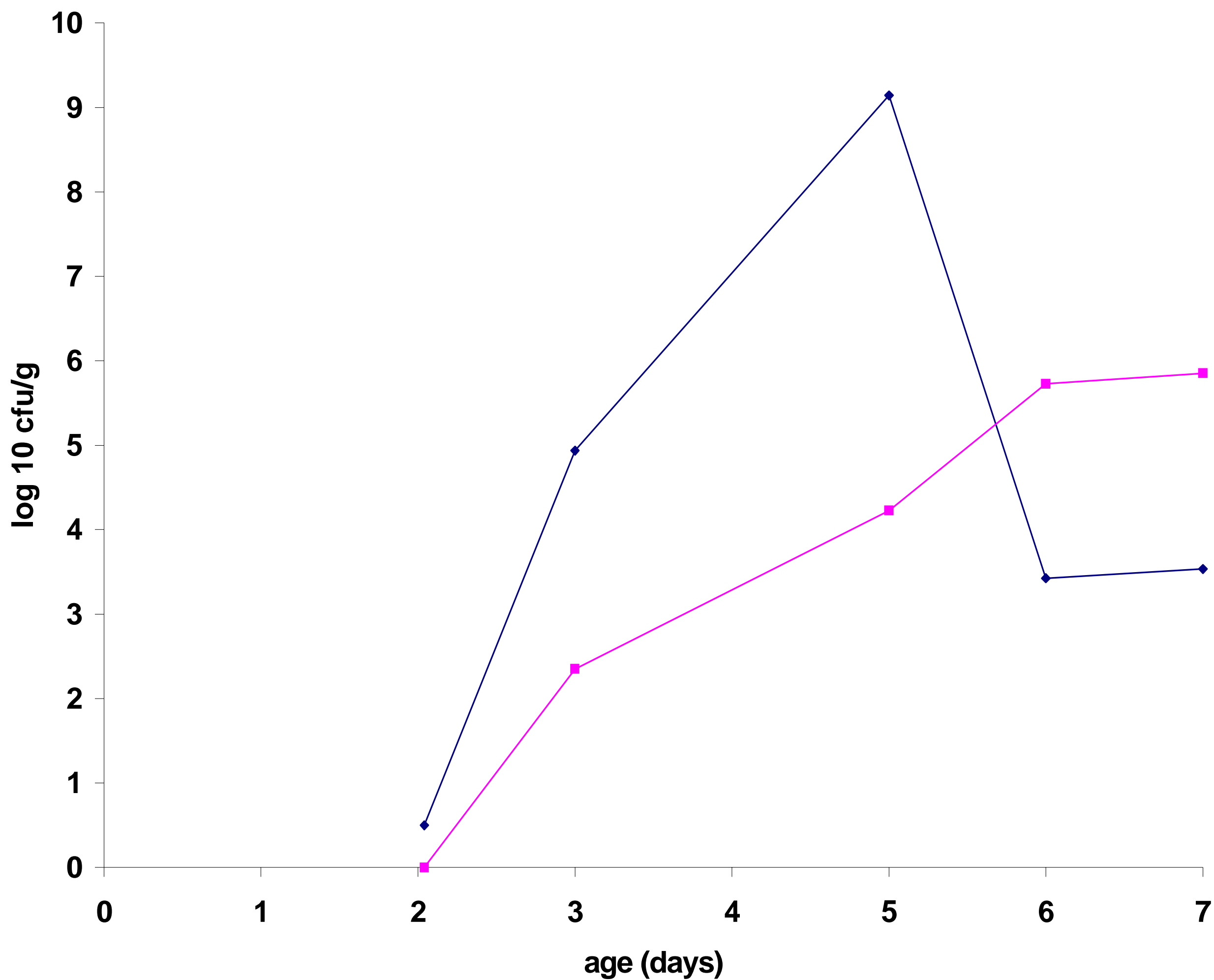

Figure 3. Mean $\log_{10}$ cfu *Salmonella* bacteria (challenge strain 76Sa88) present per gram liver in chicks that were injected with 5-fluorouracil (200 mg/kg body weight) at hatch. Chickens of group C (♦) were sham-vaccinated 24 hours after 5-fluorouracil injection and challenged 24 hours later with 5.10^3 cfu *Salmonella* Enteritidis strain 76Sa88. Animals of group VC (■) were vaccinated 24 hours after 5-fluorouracil injection with 10^8 cfu of *Salmonella* Enteritidis CVL30 and challenged 24 hours later with 5.10^3 cfu *Salmonella* Enteritidis strain 76Sa88.

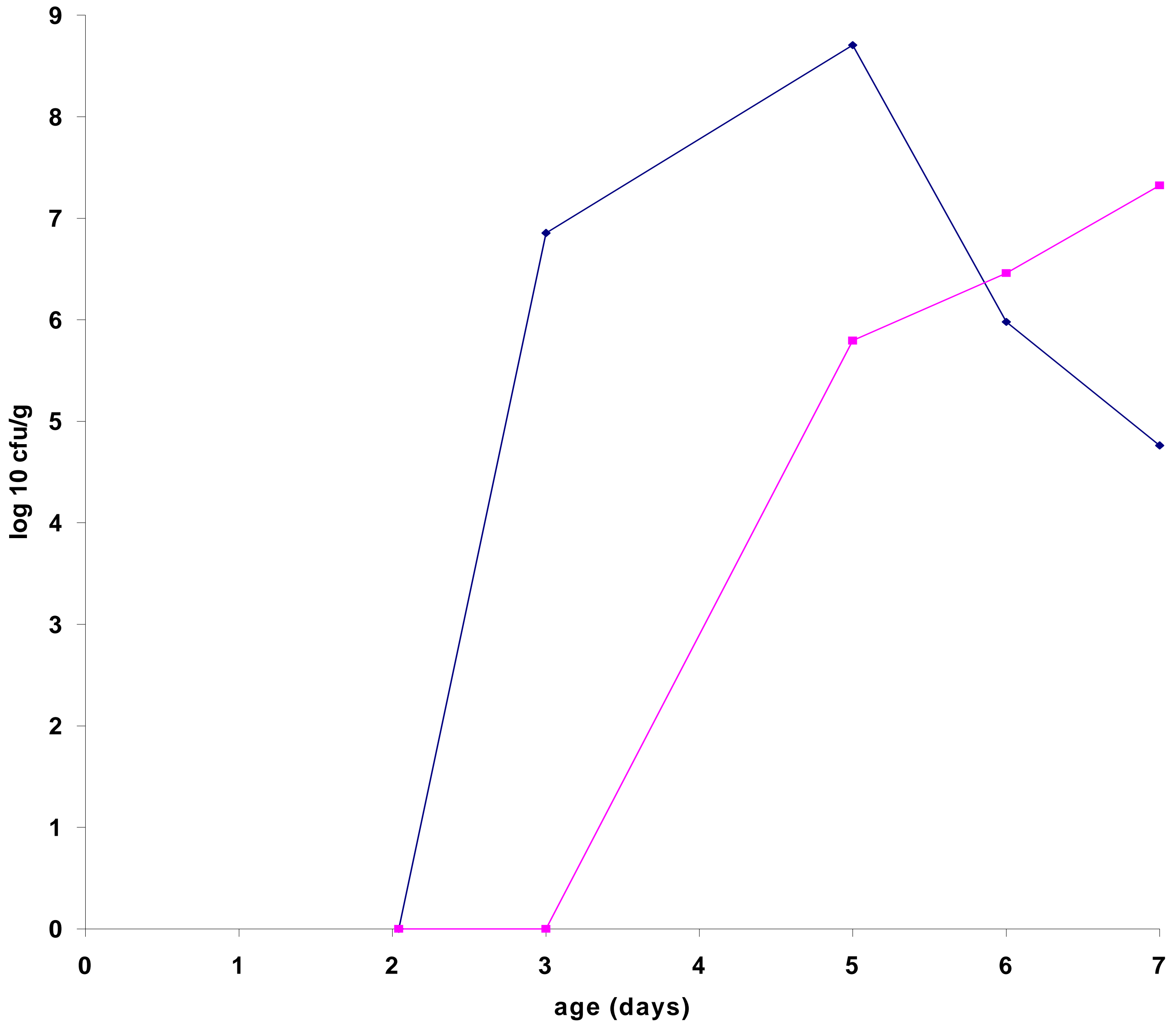

Figure 4. Mean $\log_{10}$ cfu *Salmonella* bacteria (challenge strain 76Sa88) present per gram spleen in chicks that were injected with 5-fluorouracil (200 mg/kg body weight) at hatch. Chickens of group C (♦) were sham-vaccinated after 24 hours and challenged 24 hours later with 5.10^3 cfu wild type *Salmonella* Enteritidis strain 76Sa88. Animals of group VC (■) were vaccinated 24 hours after 5-fluorouracil injection with 10^8 cfu of *Salmonella* Enteritidis CVL30 and challenged 24 hours later with 5.10^3 cfu wild type *Salmonella* Enteritidis strain 76Sa88.

Immunohistochemical data of the vaccination – challenge experiment (Fig. 5)

In unvaccinated and unchallenged control animals, the heterophils never occupied more than 2% of the area of the caecal lamina propria. No statistically significant differences were detected between different time points in this group.
In challenged sham-vaccinated animals, a slight increase of heterophils was noticed from about 2% to 4% of the area in the first 3 days post-challenge, then followed a 3 to 5-fold decrease. No statistically significant differences were found between the different time points in this group.
In the vaccinated sham-challenged chickens (group V), the density of heterophilic granulocytes was about 5% of the caecal lamina propria area 1 hour after sham-challenge (25 hours post-vaccination), followed by a peak 24 hours later and then a fast decrease. The peak was mainly caused by one of the three chickens having a very high percent of the caecal wall occupied by heterophils. Statistically significant differences in this group were detected between 1 hour post-sham-challenge (25 hours post-vaccination) and 4 and 5 days post-sham-challenge.
For chickens of the group VC, the heterophils occupied about 5% of the area when the first samples were taken at 1 hour post-challenge, and decreased to approximately 2% over a period of 5 days. No statistically significant differences were detected between the time points in this group.
Statistically significant differences were detected at the age of 49 hours (1 hour post-(sham-)challenge) between groups O and V, O and VC, C and V and finally C and VC. At 24 hours post-(sham-)challenge, differences were significant between group V and all other groups and between group V and VC. At the age of 5 days, percent area of the caecal wall occupied by heterophils of group O was statistically significant different from the values of group V and C, while one day later group C was significant different from all other groups. Finally, at the last time interval, group V was significant different from group VC.

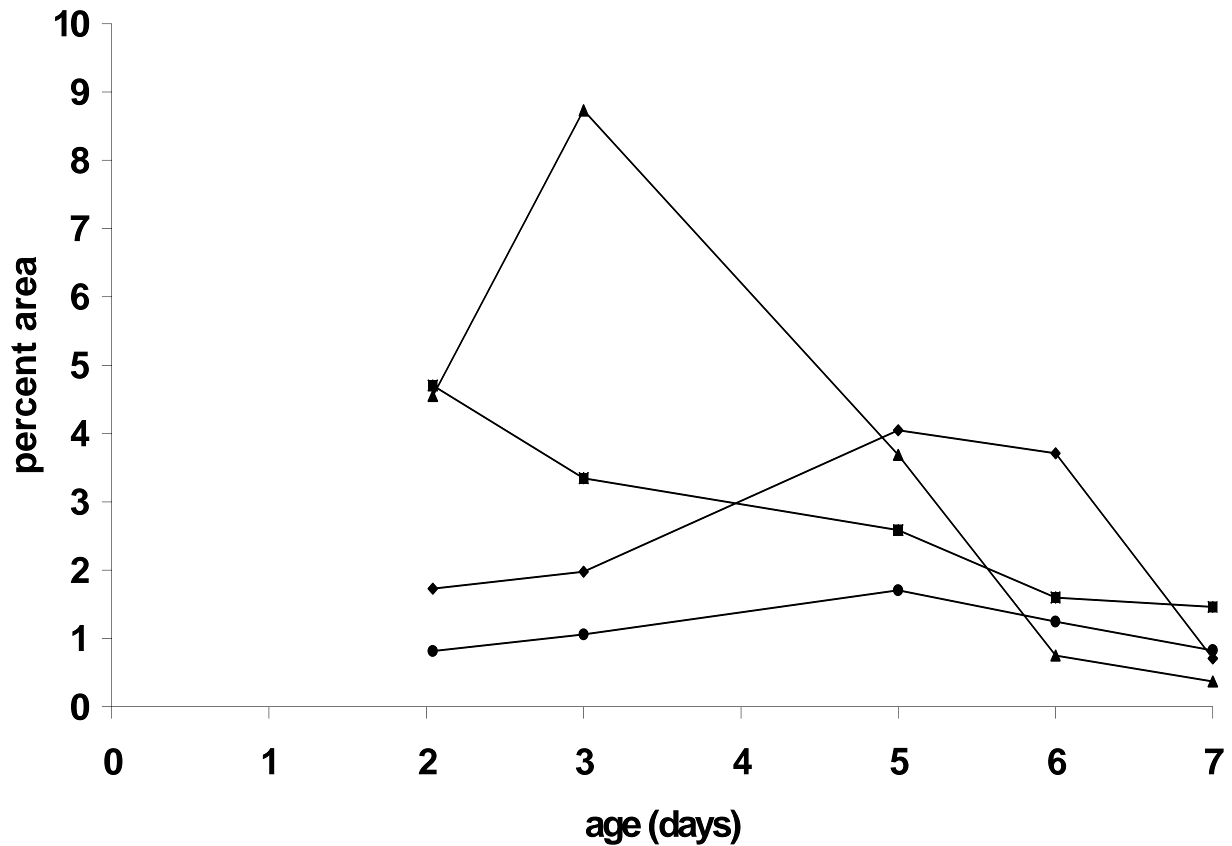

Figure 5. Percent area of the caecal lamina propria occupied by heterophilic granulocytes in chickens injected with 5-fluorouracil (200mg/kg body weight) at hatch. The animals of group O (●) were sham-vaccinated 24 hours after 5-fluorouracil injection and sham-challenged 24 hours later. Chicks of group V (▲) were vaccinated 24 hours after 5-fluorouracil injection with 10^8 cfu of *Salmonella* Enteritidis CVL30 and sham-challenged 24 hours later. Chickens of group C (♦) were sham-vaccinated 24 hours after 5-fluorouracil injection and challenged 24 hours later with 5.10^3 cfu *Salmonella* Enteritidis strain 76Sa88. Animals of group VC (■) were vaccinated 24 hours after 5-fluorouracil injection with 10^8 cfu of *Salmonella* Enteritidis CVL30 and challenged 24 hours later with 5.10^3 cfu *Salmonella* Enteritidis strain 76Sa88.

DISCUSSION

Due to the lack of a protective microflora in the caecum (Nuotio *et al*., 1992), newly-hatched chickens are very susceptible to colonization by *Salmonella* strains. Different studies report the phenomenon of colonization-inhibition where the intestinal colonization by live attenuated *Salmonella* strains very rapidly prevents colonization by virulent *Salmonella* strains (Barrow *et al*., 1987; Berchieri *et al*., 1990). It has been suggested that this phenomenon is due to microbial interactions (Martin *et al*., 1996). However, previous results from our laboratory suggest that host-related mechanisms also may play an important role in this phenomenon. We showed that *Salmonella* Enteritidis field strain infection in newly hatched chicks results in a rapid influx of heterophilic granulocytes in the caecal lamina propria, starting within 12 hours after infection (Van Immerseel *et al*., 2002b). This observation of rapid influx of heterophils in caecal lamina propria after *Salmonella* inoculation was in accordance with previous literature data (Kogut *et al*., 1994). We also confirmed that, when day-old chickens are vaccinated with the *S*. Enteritidis aroA mutant CVL30 and challenged 24 hours later with a field strain of *S*. Enteritidis, organ invasion and fecal shedding is reduced during the first six days after vaccination (Van Immerseel *et al*., 2002a). This is accompanied by an increased infiltration of heterophils, macrophages and lymphocytes in the caecal lamina propria, starting within 24 hours post-vaccination, the heterophil influx starting first. Thus vaccination rapidly protects newly-hatched chickens against a subsequent challenge with *S*. Enteritidis and this is accompanied by a simultaneous influx of heterophils in the caecal lamina propria. A causal relationship between the two observations, however, was not proven.

In the present study we used an established method to deplete heterophils in day-old chicks. Kogut *et al.* (1993) showed that a single 5-fluorouracil injection results in a dose-dependent three- to fivefold reduction in circulating polymorphonuclear leukocytes (PMN) 5 to 10 days later. Our data show that this treatment also induces a marked heterophil-depletion in the caecal lamina propria. Moreover Kogut *et al.* (1994) showed that 5-fluorouracil treatment does not cause intestinal damage. The experiment was of too short duration and the dosage too low to expect possible other undesirable side-effects.

After experimental infection of non-immune SPF chicks, *Salmonella* Enteritidis rapidly colonizes the caeca and invades internal organs, the number of bacteria

reaching values of about 10^3 cfu/g liver and 10^4 cfu/g spleen between 3 and 5 days after infection (Desmidt *et al*., 1997; Van Immerseel *et al*., 2002b). Vaccination with a *S*. Enteritidis *aro*A mutant strain immediately after hatching results in reduced organ invasion by a challenge strain administered 24 hours later (Van Immerseel *et al*., 2002a). Indeed, less than 10 cfu/g challenge bacteria are found in liver and spleen until 6 days post-challenge. The phenomenon of early colonisation-inhibition is already described for different vaccine strains, challenge strains and animal species (Berchieri and Barrow, 1990; Methner *et al*., 1999; Nogrady *et al*., 2003; Foster *et al*., 2003).

In group VC of the present study, the organ invasion of bacteria started almost immediately after infection of the chickens, reaching peaks at 5 days post-challenge (10^5 cfu/g liver and 10^7 cfu/g spleen). In the present study using heterophilic granulocytes depleted chickens, the colonization and invasion pattern in vaccinated and challenged chicks resembled that of the non-vaccinated challenged chicks. This is in contrast with numerous studies reported in the literature using normal chickens (Berchieri and Barrow, 1990; Methner *et al*., 1999; Nogrady *et al*., 2003), indicating that heterophilic granulocytes play an important role in the early protection after vaccination with an *aroA* mutant.

In conclusion, it was shown that the well-known phenomenon of early protection against *Salmonella* colonization by vaccination with a live avirulent *Salmonella* strain is abolished when heterophil-depleted chickens are used. Eliciting a heterophilic granulocyte influx into the caecal lamina propria may protect young chicks during the neonatal period, when they are very susceptible to *Salmonella* infection.

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CHAPTER 4

Invasion of *Salmonella* Enteritidis in avian intestinal epithelial cells *in vitro* is influenced by short-chain fatty acids

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ABSTRACT

Fermentation reactions in the caeca of chickens, the predominant place for *Salmonella* colonization, result in high concentrations of short-chain fatty acids (SCFA). Thus *Salmonella* bacteria are in close contact with SCFA during their life cycle. A study was carried out to analyze the effects of SCFA on invasion of *S*. Enteritidis in an avian intestinal epithelial cell line. Pre-incubation of *S*. Enteritidis for 4 hours in growth media supplemented with various concentrations of propionate or butyrate, resulted in decreased invasion compared to bacteria, preincubated in non-supplemented media, and to bacteria, pre-incubated in media supplemented with formate or acetate. Incubation of the *S*. Enteritidis bacteria in media supplemented with mixtures of SCFA mimicing the *in vivo* caecal concentrations, resulted in increased invasion compared with butyrate exposed bacteria, but equal invasion compared with non-exposed bacteria. Increasing the butyrate concentration in these mixtures did not modify invasion compared with the original mixtures.

INTRODUCTION

Salmonella enterica serovar Enteritidis is the leading cause of human foodborne infections, associated with the consumption of chicken eggs and meat (Guard-Petter, 2001; Olsen *et al*., 2001). The pathogenesis of *Salmonella* Enteritidis in the chicken starts with adhesion to and invasion of intestinal epithelial cells. Thereafter, bacteria are carried within non-activated macrophages to reach other tissues in the animal (Barrow, 2000). Invasion of *Salmonella* into intestinal epithelial cells is regulated by genes of the *Salmonella* pathogenicity island I (SPI-1) (Galán, 1996).

Salmonella bacteria encounter various environmental stress conditions in different niches during their life cycle. Nutrient starvation, pH extremes, osmotic shocks and oxidative stress are only a few examples (Foster *et al*., 1995). The expression of genes of *Salmonella* pathogenicity island I (SPI-1) is regulated by environmental stress conditions (Leclerc *et al*., 1998). *Salmonella* bacteria also have a high chance to encounter short-chain or volatile fatty acids (SCFA or VFA). Fermentation processes in the intestine of animals can yield high concentrations of acetate, propionate and butyrate (Cummings *et al*., 1987; Corrier *et al*., 1994). Short-chain fatty acids induce *hilA* and *invF* expression in *S. typhimurium* (Durant *et al*., 2000a). The HilA and InvF proteins are transcriptional activators that regulate the expression of invasion genes in response to environmental stimuli. Moreover, short-chain fatty acids affect cell-association and invasion of *S.* t*yphimurium* in a human epidermal carcinoma cell line (Durant *et al*., 1999, 2000b). Exposure of the bacteria to acetate leads to a dramatic increase in invasion properties of the bacteria compared to control conditions, whereas propionate and butyrate exposure leads to a decrease in invasion of the human cells (Durant *et al*., 1999). These processes are influenced by pH and the growth state of the bacteria.

Short-chain fatty acids are used in commercial mixtures to control *Salmonella* in poultry, and are end-products of fermentation reactions in the gut, that can be driven by addition of prebiotics to the feed. Thus knowledge of the effects of the different SCFA on the virulence of *Salmonella* in poultry is of practical importance. Therefore a study was carried out to evaluate the effects of SCFA on the invasion properties of *Salmonella* Enteritidis in an avian intestinal epithelial cell line *in vitro*.

MATERIALS AND METHODS

Bacterial growth curves

Salmonella Enteritidis phage type four, strain 76Sa88, a well-characterised strain isolated from a poultry farm, (Desmidt *et al*., 1997, 1998) was used in our experiments.

Bacteria were grown for 20 hours in Luria-Bertoni medium (LB), consisting of 10 g bacto-tryptone (Oxoid, Basingstoke, Hampshire, England), 5 g yeast extract (Oxoid, Basingstoke, Hampshire, England) and 5 g NaCl (Merck, Leuven, Belgium) per l distilled water. Then the suspension was 1:50 diluted in LB-medium supplemented with formate, acetate, propionate or butyrate (Sigma, St. Louis, USA). For each SCFA, three solutions were made with the following concentrations: 25, 50 and 100 mM. LB-medium without supplements was used as control. The pH of all solutions was brought to pH 6 and pH 7 by addition of NaOH and HCl. Osmolarity of all solutions was adjusted to the same value (610 mmol/kg), by addition of NaCl in solutions with lower osmolarity. Osmolarity was measured by a Fiske One-Ten osmometer (Indumed, Dendermonde, Belgium). This was done because differences in osmolarity influence invasion of *Salmonella* (Galan and Curtiss, 1990). After 1:50 dilution of the bacteria in these media, the suspensions were statically incubated at 37°C. The number of cfu in these suspensions was determined each half hour until 5.5 hours of growth, and at 24 hours of growth, by titration. This was done by making 10-fold dilution series of 20 µl of the bacterial suspensions for each time point. Then 6 x 20 µl of each dilution was inoculated on Brilliant Green Agar (Oxoid, Basingstoke, England), that was incubated overnight at 37°C, whereafter colonies were counted.

Gas chromatographical analysis of caecal contents

Eighteen week old and 4-day old ISA Warren Brown chickens (layers) were derived from a local company and 10 chickens per age were euthanased by intravenous embutramid (T61, Intervet, Mechelen, Belgium) injection. The diet of the animals is shown in table 1. From 9 weeks of age, the diet of the animals was changed to diet 2

(see table 1). The caeca were removed and the caecal contents were acidified by addition of 2 % (final concentration) sulfuric acid (10N), centrifuged (15 min at 22000 x g), filtered and the filtrate stored at 4°C until further analyses. Short-chain fatty acid concentrations in the acidified caecal samples were analysed by gas liquid chromatography (GC 14, Shimadzu, ‘s Hertogenbosch, the Netherlands) as described by Van Nevel and Demeyer (1977).

Table 1. Composition of the diet of the animals

Ingredient	Percentage	
	Diet 1	Diet 2
Mais	45 %	50 %
Wheat	20 %	10 %
Soya	25 %	25 %
Luzern	3 %	4 %
Fat (animal origin)	2 %	6 %
Vitamin Premix	1 %	1 %
Mineral Premix	1 %	1.1 %
Lysine	0.9 %	0.85 %
Methionine	0.6 %	0.65 %
Threonine	1.5 %	1.4 %

Cell line

The avian intestinal cell line DIV-1 was used in our experiments (Velge *et al.*, 2002). These cells have the characteristics of moderately differentiated intestinal epithelial cells. Cells were grown in DMEM, supplemented with 10 % fetal calf serum and 1% penicillin/streptomycin (all components of Life Technologies, Paisley, Scotland) in 75 cm² cell culture plates (Greiner, Frickenhausen, Germany) at 37°C and 5% CO_2. Every other day, cells were diluted 1:4 into fresh culture medium.

Cell invasion assay

Cells of the chicken intestinal epithelial cell line DIV-1 were seeded in 24-well cell culture plates (Greiner, Frickenhausen, Germany) at a density of 5.10^5 cells/ml culture medium (without antibiotics) and grown overnight. Since the surface of a well was 1.75 cm² and 1 ml of cell suspension was seeded per well, a cell density of 2.86×10^5 cells per cm² surface was seeded. After growth overnight at 37°C, the cells were confluent. Bacteria were grown for 20 hours in LB-medium, whereafter the suspension was diluted 1:50 in the SCFA solutions, from which the compositions were described above. After 4 hours of incubation at 37°C, suspensions were centrifuged and diluted in DMEM with 10 % FCS. The number of cfu/ml was determined by plating 6 x 20 µl of a dilution series of the suspensions on BGA, whereafter the plates were incubated overnight at 37°C. The suspensions were put at 4°C until they were used in the assay. The bacterial suspensions were diluted to a density of 5.10^6 cfu/ml. The growth medium of the cells was then replaced by 1 ml of the diluted bacterial suspensions. This was centrifuged for 10 min at 1500 rpm, whereafter the plates were incubated for 1 hour at 37°C and 5% CO_2. Then cells were rinsed 3 times with Hanks' Balanced Salt Solution (HBSS, Life Technologies, Paisley, Scotland), cell culture medium with gentamicin (50 µg/ml) was added and plates were incubated for 1 hour at 37°C and 5% CO_2. Hereafter, cells were rinsed 3 times with PBS and lysed with 1% Triton X-100 in distilled water (Sigma, St. Louis, USA). From this lysate, a 10-fold dilution series was made. From each dilution, 6 x 20 µl was inoculated on BGA, to determine the number of cfu of *S*. Enteritidis per ml. The percentage of invaded *S*. Enteritidis bacteria, relative to the number of *S*. Enteritidis bacteria, initially brought on the cells, was then calculated.

In a second series of invasion assays, mixtures of SCFA at concentrations mimicking those found in caecal contents of 18 week old chicks, as measured by gas chromatographical analysis, were used to supplement LB-medium. Medium was therefore supplemented with 33 mM acetate, 12 mM propionate, 6 mM butyrate and 3 mM formate. Next to this, also the butyric acid concentration was varied in the mixtures to mimic the *in vivo* situation of enhanced fermentation to the end-product butyric acid. Therefore the above concentrations of acetate, propionate and formate were used, with 20 or 40 mM butyrate. Also controls were included, i.e. media

supplemented with 20 mM and 40 mM butyrate and unsupplemented medium. Bacteria were grown for 4 hours in these media, whereafter an invasion assay was performed, as described above.

Statistical Analysis

All experiments were carried out in triplicate with 3 repeats per experiment. For the growth curves, the growth rate (doublingd/hour) was calculated for the first 6 hours for all conditions. The percentages of invasion were divided by the mean of the respective experiment and multiplied by the mean of the three experiments, to lower inter-experimental variations. The data were analysed by one-way analysis of variance methods (Neter *et al*., 1996), using the SPSS 10.0 software.

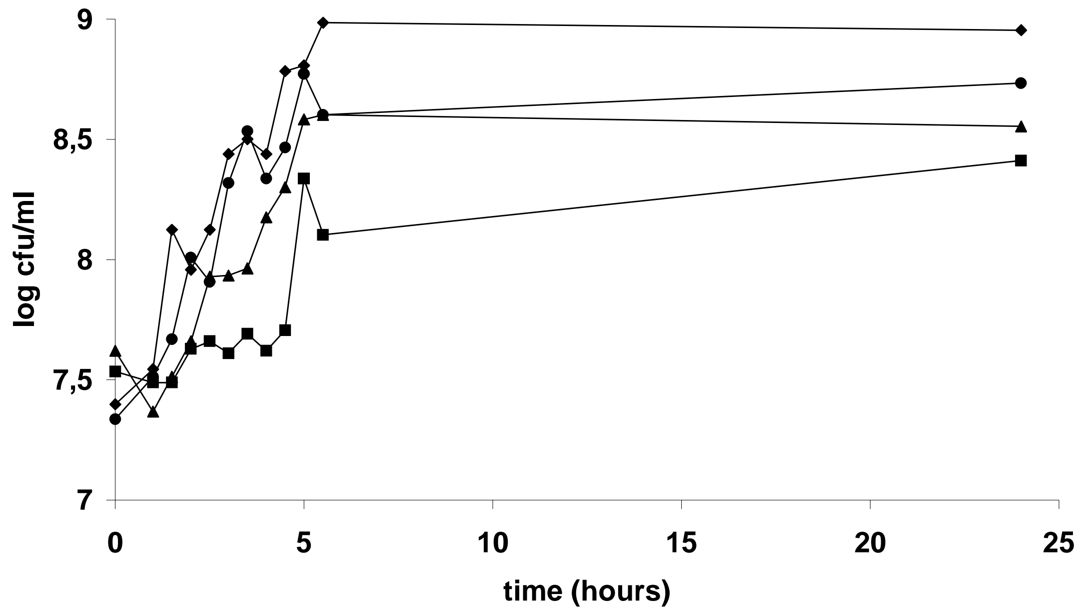
RESULTS

Bacterial growth curves (Figures 1 to 4)

Growth of *S*. Enteritidis was inhibited when the growth medium was supplemented with short-chain fatty acids at pH 6. Concentrations of 25mM propionic acid, 50mM of acetic, propionic and butyric acid, and 100 mM of all tested VFA yielded statistically significant different growth rates ($P < 0.05$) compared with control conditions. At pH 7, no significant differences in the growth curves were observed between *S*. Enteritidis grown in medium supplemented with different concentrations of VFA and the control medium.

Gas chromatographical analysis

The total concentration of VFA in the caeca of 18-week old chicks was 50.98 ± 13.70 µmole/g caecal contents. The mean acetate, propionate and butyrate concentrations were 33.17, 12.03 and 5.77 µmole/g caecal contents respectively. Expressed as relative concentrations, 652.82, 232.68 and 114.48 mmole/mole total VFA of acetate, propionate and butyrate was measured. In 4-day old chickens, relative concentrations were 961.83, 21.05 and 17.11 mmole/mole total VFA for acetate, propionate and butyrate, respectively.



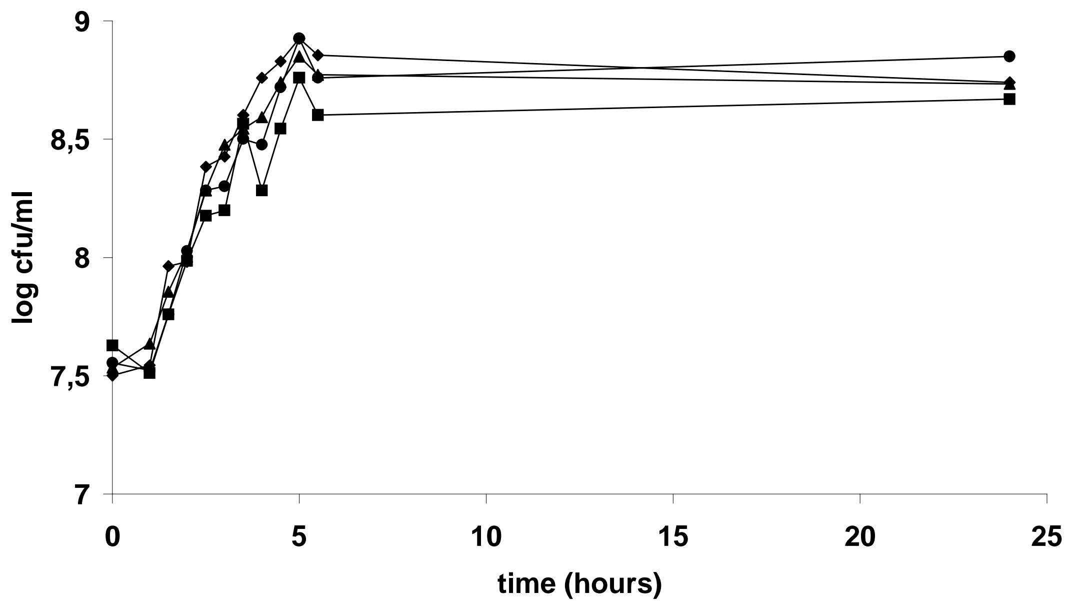


Figure 1. Growth curves of *Salmonella* Enteritidis were determined in media supplemented with 25 mM (♦), 50 mM (▲) and 100 mM (■) formiate. Unsupplemented medium served as control (•). The pH of the media was adjusted to pH = 6 (upper figure) and pH = 7 (lower figure).

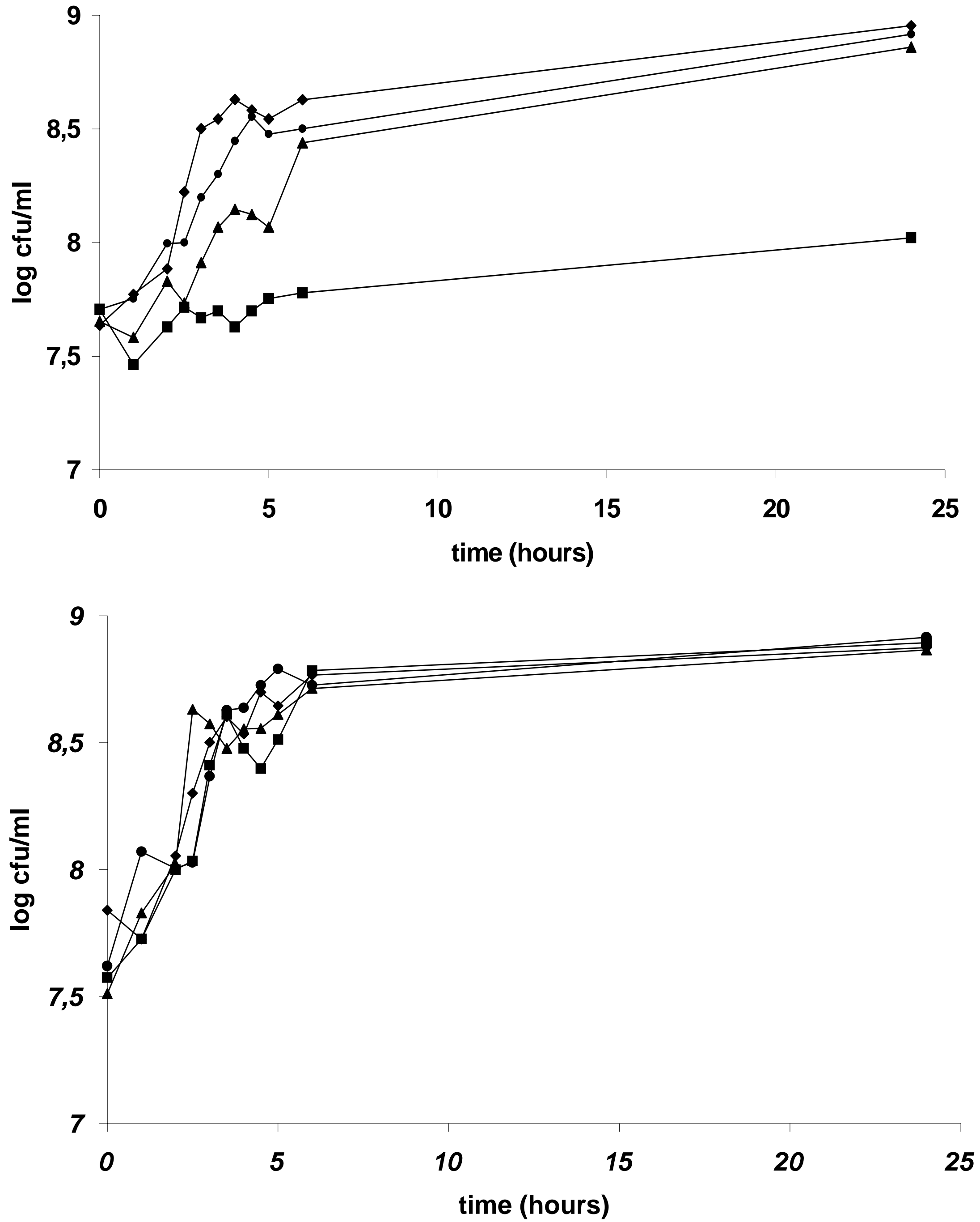
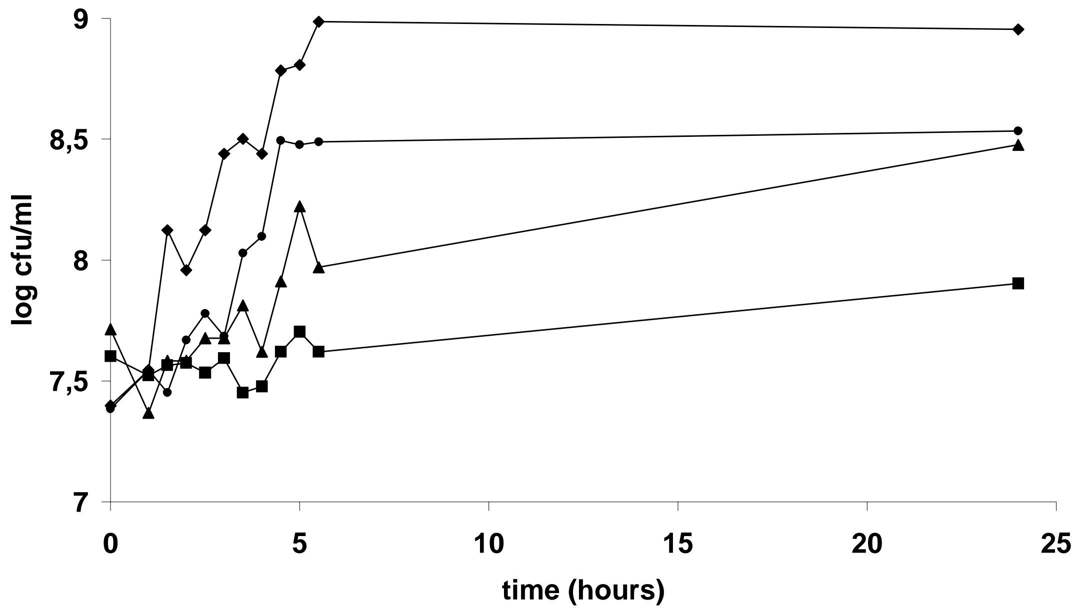


Figure 2. Growth curves of *Salmonella* Enteritidis were determined in media supplemented with 25 mM (♦), 50 mM (▲) and 100 mM (■) acetate. Unsupplemented medium served as control (●). The pH of the media was adjusted to pH = 6 (upper figure) and pH = 7 (lower figure).



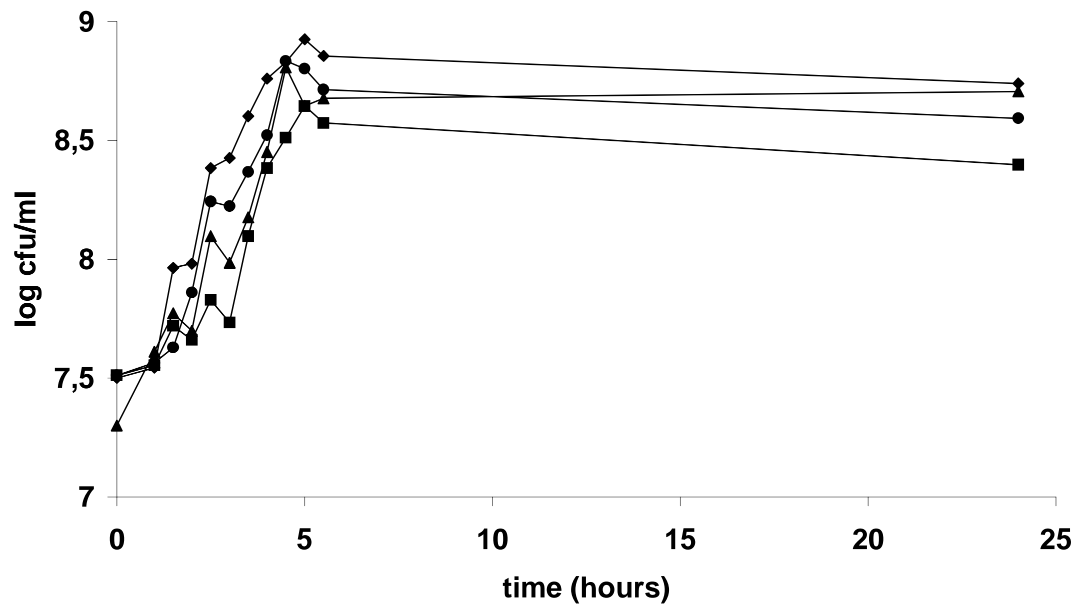
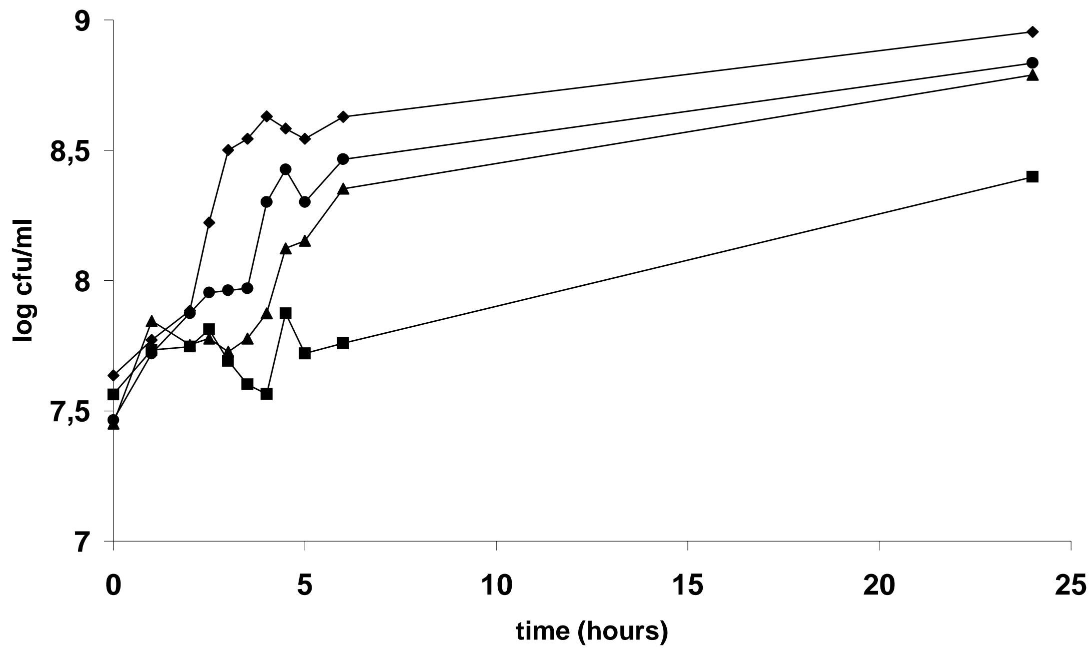


Figure 3. Growth curves of *Salmonella* Enteritidis were determined in media supplemented with 25 mM (♦), 50 mM (▲) and 100 mM (■) propionate. Unsupplemented medium served as control (•). The pH of the media was adjusted to pH = 6 (upper figure) and pH = 7 (lower figure).



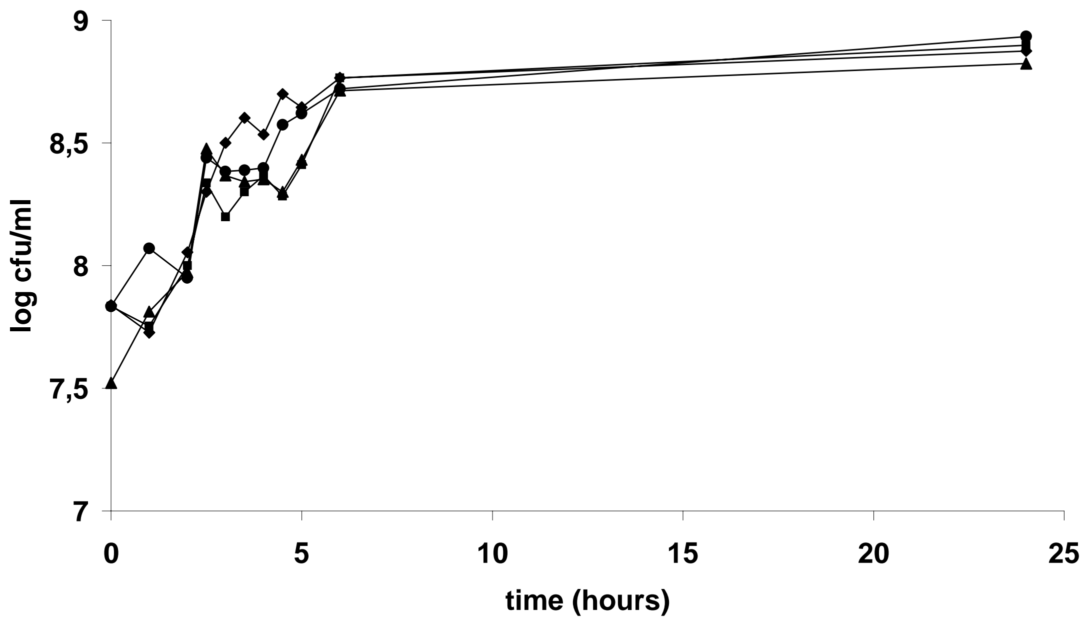


Figure 4. Growth curves of *Salmonella* Enteritidis were determined in media supplemented with 25 mM (♦), 50 mM (▲) and 100 mM (■) butyrate. Unsupplemented medium served as control (•). The pH of the media was adjusted to pH = 6 (upper figure) and pH = 7 (lower figure).

Cell invasion assays

a) single VFA

When single VFA were used to supplement the LB-media at pH 6, a decreased percentage of invaded bacteria was detected when bacteria were pre-incubated in propionate and butyrate supplemented medium, relative to invasion of bacteria pre-incubated in acetate and formate supplemented medium. These differences were significant ($P < 0.05$) in all cases within the same concentration of the VFA (Figure 5). The propionate and butyrate exposed bacteria invaded also significantly less ($P < 0.05$) than control bacteria (Figure 5). When the test was performed at pH 7, an overall trend of lower invasion percentages was detected for bacteria grown in propionate and butyrate supplemented medium, relative to the other conditions. Pre-exposure to acetate and formate resulted in invasion percentages of the bacteria that were significantly higher than the control values (Figures 5 and 6).

b) mixtures of SCFA mimicking the concentrations in adult chicken caeca

Salmonella Enteritidis bacteria grown for 4 hours in LB-medium supplemented with mixtures of SCFA, mimicking the *in vivo* concentrations, invaded to the same extent as bacteria grown in non-SCFA supplemented LB-media. *Salmonella* Enteritidis bacteria grown for 4 hours in LB-medium supplemented with 20 or 40 mM butyrate invaded statistically significant less ($P < 0.05$) than control bacteria and bacteria grown in LB-medium supplemented with *in vivo*-like caecal SCFA mixtures, whether 20 or 40 mM butyrate were supplemented to these mixtures or not (Figure 7). Additional butyrate supplementation in the *in vivo*-like mixtures did not result in changes of invasion percentages of *S*. Enteritidis in the DIV-1 cells, compared with *in vivo*-like mixtures without additional butyrate supplementation.

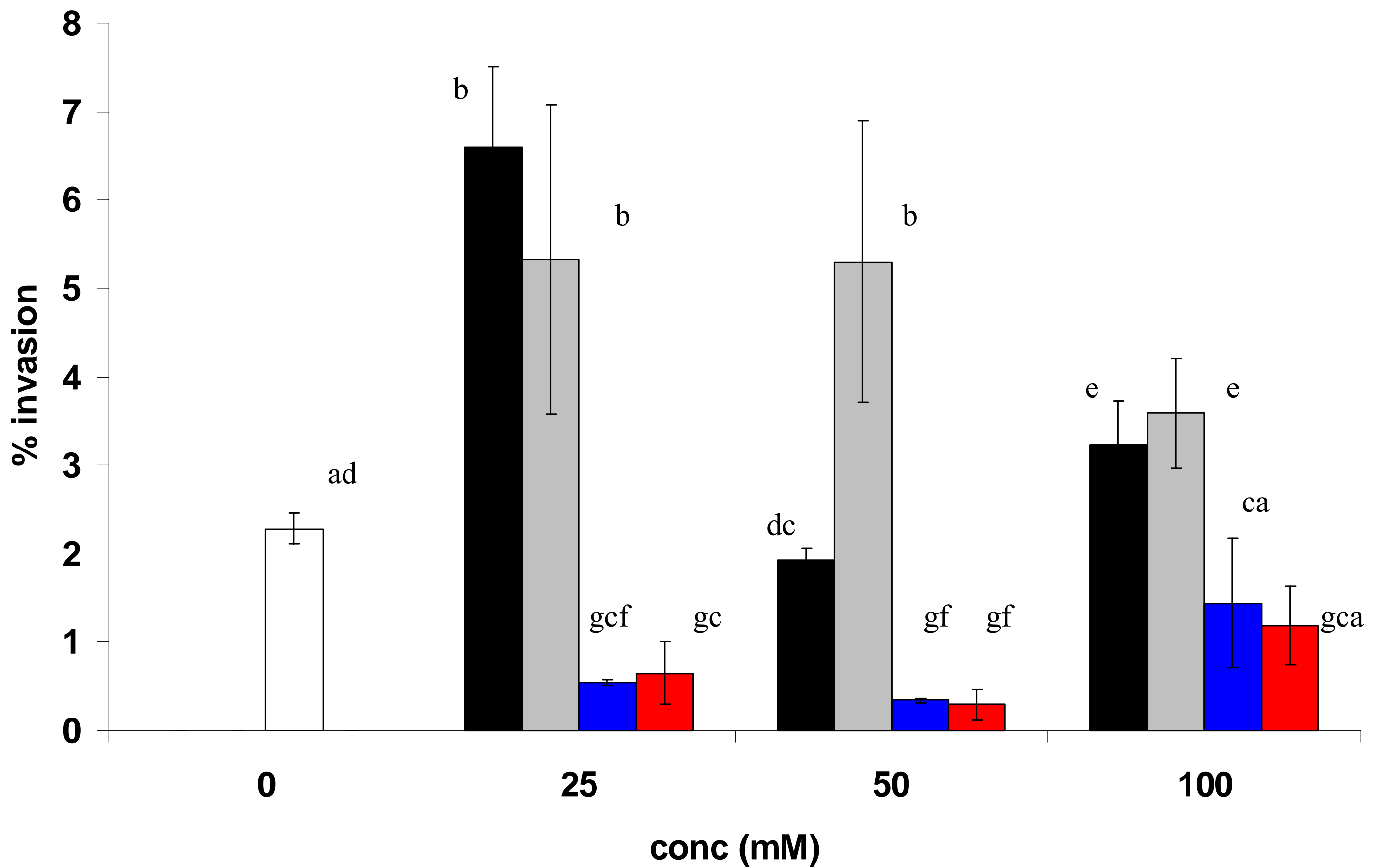


Figure 5. *Salmonella* Enteritidis bacteria were pre-incubated for 4 hours at 37°C in LB-medium with short-chain fatty acids (formic acid : black; acetic acid : grey; propionic acid : blue, butyric acid : red) in different concentrations (pH = 6), whereafter an invasion assay was performed in 24-well cell culture plates. Invasion was calculated as the percent invaded bacteria relative to the initial number of bacteria inoculated in one well of the cell culture plate (y-axis). Different concentrations of short-chain fatty acids are given in the x-axis. Values that do not share the same superscripts are statistically significant from each other ($P < 0.05$).

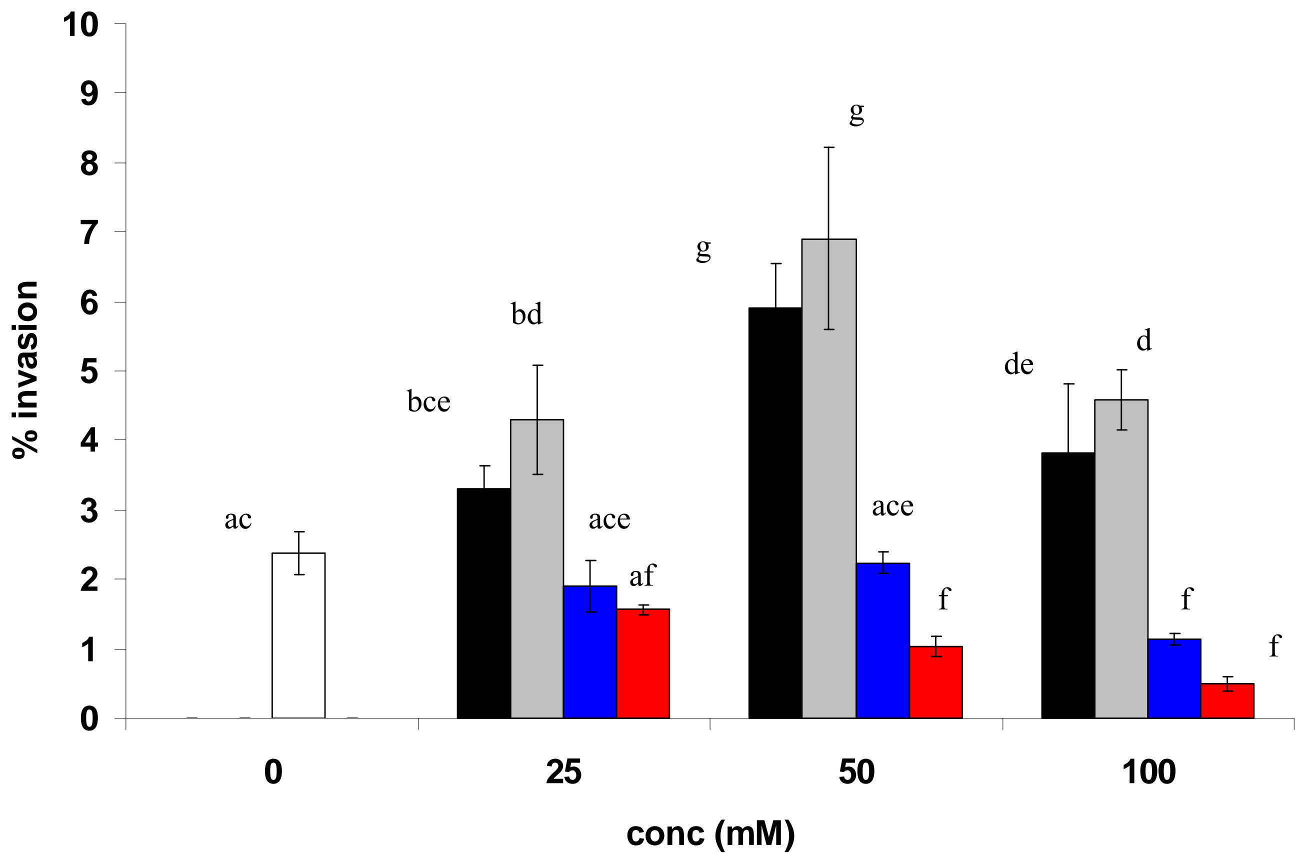


Figure 6. *Salmonella* Enteritidis bacteria were pre-incubated for 4 hours at 37°C in LB-medium with short-chain fatty acids (formic acid : black; acetic acid : grey; propionic acid : blue, butyric acid : red) in different concentrations (pH = 7), whereafter an invasion assay was performed in 24-well cell culture plates. Invasion was calculated as the percent invaded bacteria relative to the initial number of bacteria inoculated in one well of the cell culture plate (y-axis). Different concentrations of short-chain fatty acids are given in the x-axis. Values that do not share the same superscripts are statistically significant different from each other ($P < 0.05$).

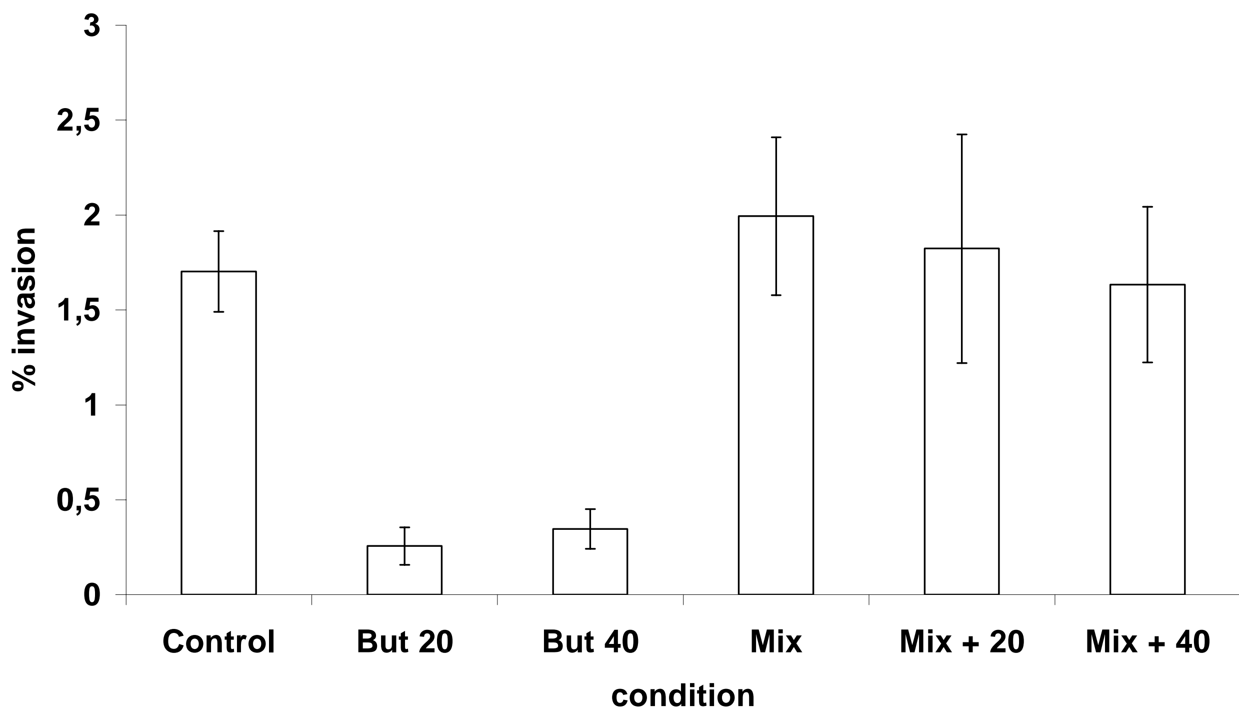


Figure 7. *Salmonella* Enteritidis bacteria were pre-incubated for 4 hours at 37°C in LB-medium, supplemented with various concentrations on different SCFA at pH = 6, whereafter an invasion assay was performed in 96-well cell culture plates. Invasion was calculated as the percent invaded bacteria relative to the initial number of bacteria inoculated in one well of the cell culture plate (x-axis). Different concentrations of short-chain fatty acids are given in the y-axis. Mix is equal to the concentrations of SCFA as detected in adult chickens, i.e. 33 mM acetate, 12 mM propionate, 6 mM butyrate and 3 mM formiate. Mix + 20 and + 40 means that the above described mixture of SCFA was used, supplemented with 20 and 40 mM butyrate. Values of conditions But 20 and But 40 are statistically significant different from all other values ($P < 0.05$).

DISCUSSION

Growth of *S*. Enteritidis was decreased in media supplemented with SCFA at pH 6. This growth inhibition was independent of the SCFA used, and was more obvious with increasing concentrations of the SCFA. At pH 7, no or very little growth inhibition was observed. These growth inhibiting properties of SCFA were similar to those detected for *S*. Tyhimurium (McHan and Shotts, 1993; Durant *et al*., 1999). At pH 6, more undissociated molecules are present. Undissociated SCFA can pass the cell membrane of bacteria and dissociate in the more alkaline cytoplasm, hereby increasing the inward leak of protons so that efflux of protons is not rapid enough to alkalinize the cytoplasm (Kashket, 1987; Cherrington *et al*., 1991). Growth is inhibited because the cell must spend energy in trying to maintain internal pH and this energy cannot be used for other metabolic processes. More recently, pH-gradient mediated anion accumulation was proposed as a mechanistic explanation for the effect of fermentation acids on bacterial growth inhibition (Russell and Diez-Gonzalez, 1998). Short-chain fatty acid resistant bacteria should have a low pH-gradient across the membrane and high potassium concentrations in the cytoplasm, that can act as countercations for the anion of the SCFA.

Acetate is the major SCFA in the caeca of humans (Cummings *et al*., 1987; MacFarlane *et al*., 1992), pigs (Lærke *et al*., 1999,2000) and rats (Le Blay *et al*., 1999). In broiler chickens, acetate can be detected in the ceca of 3-day old animals, and the concentration increases until 15 days of age, after which the acetate concentration stabilizes at 70 µmole/g caecal contents. Propionate and butyrate are detected from 12 days onwards and stabilize at 8 and 24 µmole/g caecal contents, respectively (Van Der Wielen *et al*., 2000). We confirmed that acetate was the predominant SCFA in the caecal contents of both 4-day and 18-week old chickens. The propionate concentration however was higher than the butyrate concentration. This can be due to differences in chicken breed, feed and rearing conditions. In 4-day old chicks, almost exclusively acetate was present in the caecal contents. This indicates that the caecal microflora differs between 4-day and 18-week old chicks. In 0- to 4-day old broilers enterococci, *Enterobacteraceae* and lactobacilli are dominant in the caeca, whereafter total enterococci and *Enterobacteraceae* decrease in numbers (Mead and Adams, 1975; Van Der Wielen *et al*., 2000). Starting from about 10 days

of age, the obligate anaerobic microflora dominates the ceca of broiler chickens (Salanitro *et al*., 1974; Van Der Wielen *et al*., 2000). Since these anaerobic bacteria are responsible for fermentation reactions, it is not surprising that propionate and butyrate are almost absent in 4-day old chickens.

Pre-exposure of *S*. Enteritidis bacteria to propionate or butyrate for 4 hours at 37°C results in a lower invasion of the avian intestinal epithelial cell line, compared with pre-incubation of the bacteria with formate or acetate. Similar phenomena were already observed when *S*. *typhimurium* bacteria were pre-incubated for 4 hours in media supplemented with SCFA and tested for invasion into Hep-2 cells, human larynx carcinoma cells (Durant *et al*., 1999). *Salmonella* t*yphimurium* bacteria showed increased invasion when grown in acetate supplemented medium, while pre-incubation with propionate and butyrate lead to decreased invasion, relative to non-preexposed bacteria (Durant *et al*., 1999). Our findings confirm these observations in chicken intestinal epithelial cells. Moreover, unpublished results of our laboratory show an increased invasion of acetate-exposed bacteria relative to control conditions, in the case of *S. hadar*, *S. typhimurium* F98 and *S. blockley*. Also a butyrate and propionate induced decrease in invasion was detected for these serovars. *S*. Enteritidis bacteria grown in formate supplemented media showed similar characteristics as bacteria grown in acetate supplemented media, i.e. an increased invasion of DIV-1 cells compared with propionate and butyrate exposed bacteria. A full explanation for the above described differences in invasion can not be given. All SCFA induce *hilA* and *invF* expression in *S*. *typhimurium* at pH 6, while at pH 7 only acetate, but not propionate or butyrate, induce *hilA* and *invF* expression (Durant *et al*., 2000). HilA and invF are transcriptional activators that regulate the expression of genes, involved in entry into epithelial cells, in response to environmental stimuli (Bajaj *et al*., 1996). As a consequence, all SCFA should therefore stimulate invasion at pH 6. For butyrate and propionate however, decreases in invasion were detected. It is suggested that entry of SCFA into the bacterial cell is necessary for induction of the invasive phenotype (Durant *et al*., 2000). Acetate can traverse the bacterial membranes in the undissociated as well as the dissociated form while this is not the case for propionate and butyrate (Axe and Bailey, 1995; Russell and Diez-Gonzalez, 1998). It is not known whether formate can cross the membrane in both forms. Acetate can therefore

generate other intracellular signals and affect transcription of invasion genes differently (Durant *et al*., 1999).

The growth inhibition of bacteria by the SCFA can not be responsible for the decreases in invasion properties. Indeed, at pH 7, also decreases in invasion of DIV-1 cells were detected while there was no growth inhibition. Furthermore, the same amount of viable bacteria were brought on the cells, since the number of cfu/ml of the bacterial suspensions, that were brought onto the cells, was determined in each trial. These data indicate that the individual SCFA play a role in the invasion process.

Short-chain fatty acid mixtures mimicing *in vivo* concentrations found in the caeca of 18 week-old chickens, supplemented with additional butyrate, showed no effects on bacterial invasion, compared with control bacteria or mixtures without additional butyrate. It is clear that SCFA in the mixture, different from butyrate, or at least one of them, are reponsible for the fact that adding more butyrate did not decrease invasion, since LB-media with only butyrate decreased invasion in DIV-1 significantly compared with the mixtures. Acetate is probably responsible for antagonizing the butyrate effect, because of the high invasion properties of acetate exposed bacteria and the high concentration in the *in-vivo* like mixtures. It can therefore be questioned whether in chicks, increasing butyrate caecal concentrations, as can be reached by addition of fructo-oligosaccharides or butyrate-coated microcarriers, will affect bacterial invasion into epithelial cells. Moreover, young chicks seem to have acetate as the only SCFA in their caecal contents (Van der Wielen et al., 2000). Based on our results, this possibly increases the invasion of ingested bacteria. Newly hatched chicks are highly susceptible to *Salmonella* Enteritidis infection (Desmidt *et al*., 1997). Possibly the acetate content in the caeca of young chickens and the lack of other SCFA adds to the susceptibility of these young animals.

In summary, our results show a decreased invasion of *S*. Enteritidis in an avian intestinal epithelial cell line, when the bacteria are pre-incubated with propionate and butyrate supplemented medium, relative to bacteria, pre-incubated in acetate supplemented medium. This can have important practical consequences when adding prebiotics to chicken feed, in order to drive fermentation reactions to a certain end-

product, such as butyrate. Unfortunately, the acetate load in caecal contents of chickens is high and therefore, beneficial effects of butyrate production in the caecum on *S*. Enteritidis invasion of epithelial cells *in vivo* can be questioned.

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CHAPTER 5

Protection against *Salmonella* colonization in chickens by feed supplementation with short-chain fatty acids

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ABSTRACT

Short-chain fatty acids (SCFA) are widely used as feed additives in poultry for the control of pathogenic bacteria, such as *Salmonella* Enteritidis. Recently, a new range of products was developed in which short-chain fatty acids are encapsulated in mineral carriers, resulting in a slow release during the transport of these carriers through the intestinal tract. To test the efficacy of this type of products against *Salmonella* in poultry, a challenge experiment with *S*. Enteritidis was performed. Five groups of 20 chickens were given feed with no supplement, or feed supplemented with acetic acid (0.24%), formic acid (0.22%) or propionic acid (0.27%) as film coated microbeads, or butyric acid (0.15%) as spray cooled microcapsules. The five groups were challenged with 5.10^3 cfu *S*. Enteritidis at day 5 and 6 post hatch and samples of caeca, liver and spleen were taken at day 8 and analysed for the number of cfu *Salmonella* per g tissue. Feed supplementation with acetic acid, and to a lesser extent formic acid, resulted in an increase of colonisation of caeca and internal organs. Birds receiving propionic acid coated microbeads as feed supplement were colonized with *Salmonella* to the same extent as control animals. Butyric acid impregnated microbeads in the feed, however, resulted in a strong decrease of colonization by *S*. Enteritidis in the caeca, but not in liver and spleen.

INTRODUCTION

Salmonella is still one of the leading causes of foodborne infections in the world, mainly due to the consumption of poultry meat and eggs (Mead *et al*., 1999 ; Poppe, 1999). Control measures for reducing the risk of introduction of *Salmonella* on the farm and to control *Salmonella* in chicken flocks are widely applied in Western countries (Van Immerseel et al., 2002b).

Short-chain or volatile fatty acids (SCFA) have been used as feed additives in poultry since many years. SCFA are bacteriostatic or bactericidal *in vitro* for gram-negative bacteria, provided that there are sufficient undissociated acid molecules present and that they are in contact with the bacteria for sufficiently long time (Thompson and Hinton, 1997). It is commonly accepted that the SCFA diffuse into the bacterial cell in undissociated form, which is favoured by low pH. Inside the bacterial cell, the acid is expected to dissociate, resulting in reduction of intracellular pH and anion accumulation (Russell and Diez-Gonzalez, 1998). It is proposed that the SCFA in the feed will have antibacterial effects in the crop, but will have no effects further down in the gastrointestinal tract (Thompson and Hinton, 1997). This would implicate that *Salmonella* bacteria, that have reached the small intestine or caeca, would be protected against the antibacterial activity of these agents. SCFA however can be impregnated in micropearls, from which they are released slowly during transport in the gastrointestinal tract. In this way SCFA could also reach the small intestine and the caeca, the latter being the predominant site for *Salmonella* colonization (Anonymous, 1997). It has been shown that feed supplementation with mixtures of propionic and formic acid, coated on a carrier, resulted in decrease of intestinal colonization, mortality and morbidity after *Salmonella* infection (Hinton and Linton, 1988; Berchieri and Barrow, 1996; Iba and Berchieri, 1995). Data about effects of acetic and butyric acid on colonization of *Salmonella* in chickens *in vivo* are not reported yet.

In the present study the efficacy of microencapsulated SCFA in reducing the colonization of caeca and internal organs of chickens by *Salmonella* Enteritidis was assessed. For this purpose, microbeads carrying either formate, acetate, butyrate or propionate are supplemented to the feed.

MATERIALS AND METHODS

Salmonella strain

Salmonella Enteritidis phage type four, strain 76Sa88, a well-characterised strain isolated from a poultry farm, (Desmidt *et al*., 1997, 1998; Van Immerseel *et al*., 2002a) was used in the experiments. The strain was grown for 6 hours in Luria-Bertoni medium (LB), whereafter the number of cfu/ml was determined by plating ten-fold dilutions of the bacterial suspension on brilliant green agar (BGA, Oxoid, Basingstoke, England). Then the bacteria were diluted in phosphate buffered saline (PBS) to reach the inoculation titer.

Chickens

Specific pathogen free (SPF) chickens (Iffa-Credo, Brussels, Belgium) were hatched and housed in isolation. Before the start of the experiment, 20 chickens were euthanised and serum samples were taken for the detection of maternal antibodies against *S*. Enteritidis by means of a previously described anti *S*. Enteritidis ELISA (Desmidt *et al*., 1996). All birds were seronegative. Chickens received autoclaved drinking water and irradiated feed (25 kGy of γ-irradiation), supplemented with the feed additives described below, ad libitum.

Feed additives

Different short-chain fatty acids were used as feed additives. Formic, acetic and propionic acid were film coated on microbeads, while microbeads containing butyric acid were made by spray cooling (all products manufactured by SODA Feed Ingredients, Dublin, Ireland). Concentrations of different components of the microbeads are shown in table 1. For all products, 0.5 % of micropearls was included in the feed, so that final in-feed concentrations of SCFA were 0.217 % for formic acid, 0.242 % for acetic acid, 0.275 % for propionic acid and 0.156 % for butyric acid.

In vivo trial

Chickens were randomly divided in five groups of 20 chickens. From the day of hatch, four groups received feed supplemented with the above described feed additives, while one group received unsupplemented feed. The animals were orally inoculated with 2.10^3 cfu *S*. Enteritidis 76Sa88 at both day 5 and 6. At day 7, cloacal swabs were taken from 5 animals per group to detect *Salmonella* bacteria. At day 8, chickens were euthanised and samples of caecum, liver and spleen were taken for bacteriological analysis.

Table 1. Composition of the microbeads, used as feed additives. (FOR = formic acid impregnated microbeads; ACE = acetic acid impregnated microbeads; PROP = propionic acid impregnated microbeads; BUT = butyric acid impregnated microbeads)

	FOR	ACE	PROP	BUT
Calcium	190 g/kg	165 g/kg	150 g/kg	0 g/kg
Respective short-chain fatty acid	435 g/kg	485 g/kg	550 g/kg	313 g/kg
Citric acid	0 g/kg	200 g/kg	200 g/kg	0 g/kg
Phosphoric acid	60 g/kg	0 g/kg	0 g/kg	0 g/kg
Silica	54 g/kg	0 g/kg	0 g/kg	195 g/kg
Carrier	147 g/kg	0 g/kg	0 g/kg	40 g/kg
Hydrogenated vegetable oil	114 g/kg	150 g/kg	100 g/kg	452 g/kg

Bacteriological analysis

Cloacal swabs were directly inoculated on BGA-plates, which were incubated overnight at 37°C. When negative after direct inoculation, samples were pre-enriched in buffered peptone water (BPW, Oxoid, Basingstoke, England) overnight at 37°C, whereafter samples were enriched by addition of 1 ml of this suspension to 9 ml brilliant green tetrathionate broth (Oxoid, Basingstoke, England). After incubation overnight, a drop of this suspension was plated on BGA.
Samples of caeca, liver and spleen were homogenised and 10-fold dilutions were made in buffered peptone water starting from 5-, 10- and 20-fold dilutions for caeca, liver and spleen, respectively. For each dilution 6 x 20 µl were inoculated on BGA. After incubation overnight (37°C) the number of cfu/g tissue was determined by counting the bacterial colonies. For samples which were negative after titration, pre-enrichment and enrichment was performed, as described above. Samples that were negative after titration but positive after *Salmonella* enrichment, were presumed to contain 0.5×10^1 (caeca), 10^1 (liver) or 2×10^1 cfu/g (spleen). Samples that were negative after enrichment were presumed to have 0 cfu/g. The mean cfu/g tissue was calculated for each group. The SPSS 9.0 software was used for statistical analysis. The non-parametric Kruskal-Wallis test was used to check for inter treatment effects. Since for each organ, inter treatment effects were detected, the non-parametric Mann-Whitney test was used to determine significant differences between the treatment groups.

RESULTS

One day after the second infection 4/5 animals of the group that received acetic acid as feed supplement had positive cloacal swabs, compared with none of the animals of the other groups.

Table 2 shows the number of animals classified according to caecal bacterial count of *Salmonella* (log scaled intervals). In the control group, caeca of 6/20 animals were only positive after enrichment, while 9/20 animals had more than 10^6 cfu/g *Salmonella* in their caeca. In the group, that had received formic acid as feed supplement, 10/20 animals had more than 10^6 cfu/g *Salmonella* in the caeca, and only 1 animal was positive after enrichment. Thus, a higher number of animals had intermediate *Salmonella* titers, compared with the control animals. For the group receiving acetic acid as feed supplement, 16/20 animals had a *Salmonella* count of more than 10^6 cfu/g, from which 13 had even more than 10^7 cfu/g caecum. In the propionic acid supplemented group, 7 animals had more than 10^6 cfu of *Salmonella* per g caecum, while 8/20 animals were only positive after enrichment. Finally, in the group receiving butyric acid impregnated microbeads, no animals had a caecal *Salmonella* count of more than 10^6 cfu/g, while 11/20 animals were only positive after enrichment.

Table 3 shows the number of animals classified according to bacterial counts of *Salmonella* in liver and spleen. While for the control group and the butyric acid group only a few animals had a bacterial count of more than 10^2 cfu/g of spleen, 5 animals in the propionic acid group had *Salmonella* numbers between 10^2 and 10^3 cfu/g spleen. In the formic acid group, 11/20 animals had a *Salmonella* count of more than 10^2 cfu/g, from which 6 had a count of more than 10^3 cfu/g and one of more than 10^4 cfu/g. In the acetic acid group, 17/20 animals had more than 10^2 cfu/g spleen, from which 6 more than 10^3 cfu/g, 5 more than 10^4 cfu/g and even one animal having more than 10^5 cfu/g spleen. For liver, comparable results were obtained (Table 3).

Table 2. Colonization of the caeca at day 8 of life (inoculation with 10^3 cfu S. Enteritidis 76Sa88 on day 5 and 6) in chickens fed a diet supplemented with formic, acetic, propionic and butyric acid or no feed additives.

	CTRL^{A} (n=20)	FOR^{A} (n=20)	ACE^{B} (n=20)	PROP^{AC} (n=20)	BUT^{C} (n=20)
Negative	0*	0	0	0	0
Positive after enrichment	6	1	1	8	11
$10^2 < x < 10^3$ cfu/g	0	1	1	1	2
$10^3 < x < 10^4$ cfu/g	0	4	0	1	1
$10^4 < x < 10^5$ cfu/g	3	2	0	2	3
$10^5 < x < 10^6$ cfu/g	2	2	2	1	3
$10^6 < x < 10^7$ cfu/g	8	7	3	3	0
More than 10^7 cfu/g	1	3	13	4	0

* Number of chickens in a group of 20 that has a given amount of *Salmonella* bacteria in the caeca.

^{A,B,C} Groups not having equal superscripts are statistically significant different.

Mean log cfu/g of caeca, liver and spleen for the different animal groups are shown in figure 1. The mean log cfu *S*. Enteritidis per g caecum was significantly higher in acetic acid treated groups than in all other groups. The butyric acid treated group had a significantly lower mean log cfu/g caecum than all other groups, except the propionic acid treated group. For liver and spleen, the acetic and formic acid group had significantly higher values of mean log cfu/g compared with all other groups.

Table 3. Colonization of the liver (L) and spleen (S) at day 8 of life (inoculation with 10^3 cfu S. Enteritidis 76Sa88 on day 5 and 6) in chickens fed a diet supplemented with formic, acetic, propionic and butyric acid or no feed additives.

	CTRL		FOR		ACE		PROP		BUT	
	L^{A}	S^{A}	L^{BD}	S^{B}	L^{B}	S^{C}	L^{AD}	S^{A}	L^{A}	S^{A}
Negative	0*	8	0	0	0	0	1	5	0	7
Positive after enrichment	19	10	11	9	8	3	14	10	20	12
$10^2 < x < 10^3$ cfu/g	0	0	6	4	6	4	5	5	0	1
$10^3 < x < 10^4$ cfu/g	1	1	3	6	3	6	0	0	0	0
$10^4 < x < 10^5$ cfu/g	0	1	0	1	2	5	0	0	0	0
More than 10^5 cfu/g	0	0	0	0	1	2	0	0	0	0

* Number of chickens in a group of 20 that has a given amount of *Salmonella* bacteria in the caeca.

[A,B,C] Within each organ, groups not having equal superscripts are statistically significant different.

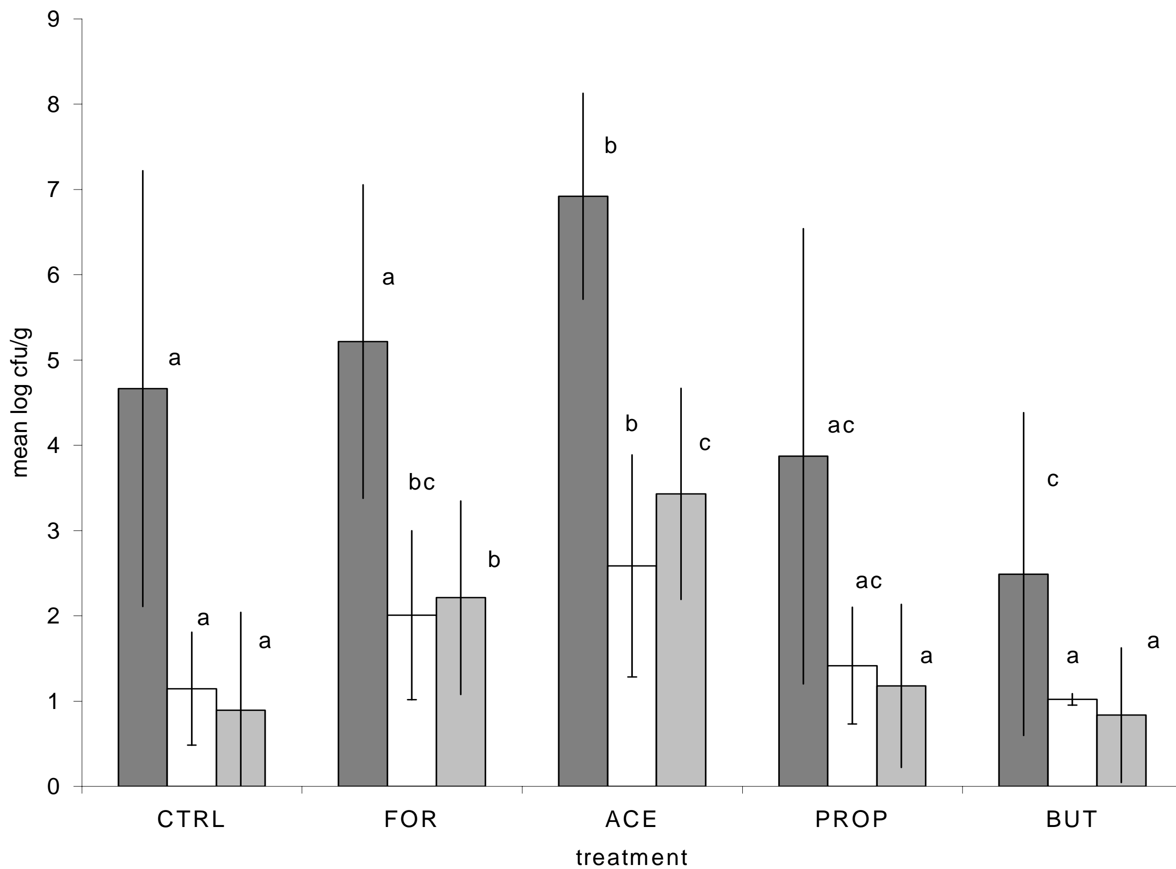

Figure 1. The mean log cfu/g in caeca (dark grey); liver (white) and spleen (light grey) at day 8 of life of chickens, that were orally inoculated with 10^3 cfu *S*. Enteritidis 76Sa88 at day 5 and 6. Chickens received diets supplemented with formic acid (FOR), acetic acid (ACE), propionic acid (PROP), butyric acid (BUT) and non-supplemented feed (ctrol). Between conditions; for each organ, values not having the same superscripts are statistically significant different.

DISCUSSION

Short-chain fatty acids (SCFA) are being used for years in poultry to control *Salmonella* (Van Immerseel et al., 2002b). Experimental use of single propionic and formic acid as feed or water additives yielded variable results in protection experiments against *Salmonella*, depending on the experimental setup (Izat *et al*., 1990a, 1990b ; McHan and Shotts, 1992 ; Patten and Waldroup, 1988). The choice of SCFA in commercial products, however, is empirical. Information on the effects of butyric or acetic acid supplementation in the feed on *Salmonella* infections is lacking. In this study, the single SCFA (formic, acetic, propionic and butyric acid) were used to supplement the feed of the animals so that comparison between effects of the single SCFA on *Salmonella* colonization is possible.

It is believed that the direct (non-encapsulated) incorporation of SCFA in the feed or in the drinking water only results in activity in the crop and not further in the gastro-intestinal tract (Thompson and Hinton, 1997). Recently, a delivery system for feed ingredients has been developed to improve protection, bio-availability and sustained slow selective release of feed ingredients, such as polyunsaturated fatty acids (Anonymous, 1997). This is done by microencapsulation and coating of these feed ingredients in micropearls. Microencapsulation of SCFA would result in a slow release of the SCFA after uptake by the animal, so that the SCFA also reach the intestinal tract and the caeca of chickens.

The choice of the SCFA, to be encapsulated in the micropearls, seems to be important for control of *Salmonella*. In the present study we found that different SCFA, encapsulated in micropearls, in the feed, resulted in different levels of colonisation of caeca and internal organs shortly after *S*. Enteritidis inoculation in young chickens.
The effects of the different SCFA on colonization with *Salmonella* can be explained by the alteration of *Salmonella* virulence gene expression and invasion of epithelial cells after contact with the respective short-chain fatty acids. In a previous study, we observed that exposure of *S*. Enteritidis 76SA88 to acetate or formate for 4 hours resulted in an increase in invasion of the chicken intestinal epithelial cell line DIV-1. Contact of the bacteria with propionic and butyric acid resulted in a decrease in invasion (Van Immerseel *et al*., 2002c). It is known that the expression of two

transcriptional activators of genes of *Salmonella* pathogenicity island I, involved in invasion of intestinal epithelial cells, are induced by SCFA in *S*. Typhimurium. At pH 6, expression of these genes increased with time after exposure of the bacteria to acetate, while this was not the case after exposure with propionate or butyrate (Durant *et al*., 2000). More recently, it was shown that expression of hilA and invF, regulators of gene expression in pathogenicity island I of *Salmonella,* and expression of sipC, a protein of the type three secretion system, are upregulated after contact of *S*. Typhimurium with acetate at pH 6.7 (Lawhon *et al*., 2002). The underlying mechanism was proved to be the activation of the BarA/SirA system by acetylphosphate through phosphorylation. The BarA/SirA system in turn is involved in activation of hilA and invF (Lawhon *et al*., 2002). Propionic and butyric acid led to a decrease in hilA, invF and sipC expression, due to a hitherto unknown mechanism. The caeca are the predominant site for *Salmonella* colonization (Desmidt *et al*., 1997, 1998). The pH of the caecal lumen in chickens is about 6. Changes in expression of genes of pathogencity island I due to the contact with SCFA probably play a role in colonization of caeca and internal organs, as seen in our study.

The butyric acid containing microbeads were developed by spray cooling, while the other SCFA products were developed by film coating. The formulation of the beads differed between the four different preparations. The amount of hydrogenated vegetable oil in the butyric acid impregnated microbeads is, for example, higher than in all other SCFA products, used in this study. The relevance of these differences for anti-*Salmonella* activity is not clear.

In conclusion, the choice of which SCFA is encapsulated in micropearls, for feed supplementation of chickens, seems to affect the anti-*Salmonella* activity. Butyric acid impregnated microbeads resulted in a decrease of caecal and organ colonization shortly after inoculation of young chickens with *S*. Enteritidis. Formic and propionic acid, which are most commonly used in coated commercial preparations, however seem to have some antagonistic effects on colonization and invasion. Therefore butyric acid, encapsulated in micropearls, seems to be a good candidate for use as a feed additive to control *Salmonella*.

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GENERAL DISCUSSION

Despite the introduction of monitoring programs and control schemes, the prevalence of *Salmonella* in poultry in Western countries is still high (Wilson, 2002; Van Pelt *et al.*, 2003). Following the introduction of the EU-directive EU-92/117 in 1992, strict control measures have been applied in breeder flocks. It was hoped that this would result in a serious decrease in *Salmonella* prevalence in broiler chickens and laying hens. Strict control measures in breeder farms, however, are insufficient to control *Salmonella* downstream the production chain. Indeed, vertical transmission occurs, but horizontal transmission is at least equally important (Gast and Holt, 1999). Especially broiler chickens are housed in circumstances that permit horizontal transmission and rapid spread within the flock. The range of serotypes isolated from broilers in Belgium in 2002 (more than 30 serotypes) and the number of isolates per serotype, compared to broiler parents, underline the importance of horizontal spread. Indeed, vertical transmission is frequently documented for the serotype Enteritidis, and only sporadically for other serotypes (St. Louis *et al.*, 1988; Okamura *et al.*, 2001a,b).
Numerous preventive and curative measures have been proposed for the control of horizontal spread of the infection in the poultry population at the level of the production flocks. Laying hens are currently vaccinated against *Salmonella*, in contrast to broiler chickens. A variety of products for *Salmonella* control is on the market at the moment, for use in broiler and laying hens (Van Immerseel *et al.*, 2002). These include disinfectants, feed additives, water sanitizers, and many more. Many products only claim a decrease in shedding of the pathogen in treated birds. *Salmonella* can however reside intracellularly in epithelial cells and macrophages and can induce carrier animals, shedding intermittently (Nakamura *et al.*, 1993; Wigley *et al.*, 2001; Aabo *et al.*, 2002). Own unpublished results show that infection of day old chicks with 10^2 cfu *Salmonella* Enteritidis results in 100 % of the animals shedding the bacteria, the percentage decreasing to about 20 % after 10 weeks. After that, 20 % of the animals keep shedding the bacteria, but at each monitoring time point, different animals are shedding *Salmonella*. When only reduction of shedding is achieved, there is little doubt that *Salmonella* will never disappear from the food chain. Indeed, the carrier animals that do not shed bacteria but are colonised in intestinal tract or internal organs can still restart shedding and thus contaminate their environment, when subjected to stress. Moreover, these carrier animals can contaminate the slaughter

line. *Salmonella* contaminated broilers thus continue to constitute an important threat to public health.

Control of *Salmonella* contamination in broilers is complicated by the wide variety of serotypes infecting the birds, the variation of contamination sources and the extreme susceptibility of the birds in the early post-hatch period. The extreme susceptibility is due to the fact that a protective gut flora is not yet established and also because in the first days post-hatch, immunity is not fully developed to combat an infection. The goal of our studies was to investigate tools that could be used to protect these vulnerable newly hatched chicks from *Salmonella* infection.

The newly hatched chicken is indeed immunologically immature. The gut of the hatchling is poorly populated by both innate immune leukocytes and lymphocytes (Friedman *et al.*, 2003). Priming of the immune response of the gut-associated immune system is thought to be an important factor when combating *Salmonella* infections. *Salmonella* preferentially colonizes the caeca of chickens, so attraction of immune cells to this site at young age and priming of the infiltrating cells so that their activity is enhanced is to our opinion one of the possibilities for *Salmonella* control early after hatching. In chapter one, it was found that macrophages, granulocytes and T-lymphocytes infiltrated the caecal lamina propria starting within less than 24 hours after infection with a virulent *Salmonella* strain, followed by an increase in infiltrating B-cells. In chapter two, administration of an avirulent strain, a *Salmonella* Enteritidis *aroA* mutant, to newly hatched chickens, was shown to induce more or less the same dynamics of influx of all types of leukocytes in the caecal wall. Moreover, administration of the aroA mutant strain to one day-old chicks protected against a virulent *Salmonella* strain administered one day later. The protection lasted for a few days, whereafter the virulent bacteria increased in number again. This phenomenon has been reported repeatedly in the literature.

There is controversy on the exact mechanism of this early colonization-inhibition phenomenon. *In vitro* data suggested that the growth suppression of a challenge strain operates by depletion of utilizable carbon and electron acceptors (Zhang-Barber *et al.*, 1997). This hypothesis can be questioned, since in the gut lumen, there should be plenty of nutrients available for the challenge strain. Production of antibacterial substances by *Salmonella* strains inhibiting other *Salmonella* strains has not been described. Moreover, the *aroA* mutant strain, used in this study, did not suppress

growth of the *Salmonella* Enteritidis 76Sa88 challenge strain *in vitro* (own unpublished data). Therefore the theory put forward by Zhang-Barber *et al*. (1997) is probably not explaining the *in vivo* colonization-inhibition in this particular case. Another possible explanation is blocking of receptor sites on intestinal epithelial cells resulting in inhibition of colonization. Own unpublished *in vitro* data showed that adhesion to, and invasion of intestinal epithelial cells by the *S*. Enteritidis 76Sa88 strain was about 10 to 20% decreased when bacteria of another *Salmonella* strain were inoculated on the cells before contact of the challenge strain with these cells. Since invasion is required to reach internal organs, the decrease in colonization of internal organs by the challenge strain in our *in vivo* experiments can partly be explained by such a mechanism. There is however a third possible explanation : administration of the *aroA* mutant induced massive infiltration of immune cells in the caecal wall very shortly after inoculation. The caeca are the predominant site of colonization by *Salmonella.* Thus it can be hypothesized that the host response will have a role in the colonization-inhibition phenomena. This host response may be either a specific or a non-specific immune response. The activity of immune cells in the early post-hatch period is poorly studied. At hatch, the gut associated lymphoid tissue contains immature leukocytes. In unprimed chickens, maturation of T-lymphocytes occurs in two stages or waves (Bar-Shira *et al.*, 2003). The first wave of maturation occurs at about 4 days post-hatch, and is demonstrated by increases in IL-2 and IFN-γ production. Antibody responses can not explain the early colonization-inhibition phenomena after administration of the aroA mutant. Antibodies to LPS of *Salmonella* are only formed at about two weeks after infection of newly hatched chickens with *Salmonella* (Desmidt *et al.*, 1998). Moreover, no *Salmonella* specific antibodies were detected in the first days post-infection of hatchlings. Priming of the B-cells by the *aroA* mutant strain was detected by the functional arrangement of leukocytes into follicles, starting from about 6 days post-challenge.

The chicken homologue of IL-8 might play a role in chemotaxis of inflammatory cells towards the caecal lamina propria. Heterophilic granulocytes probably are the first cells that reach the site of bacterial entrance, being the caecal epithelium. It has been shown that heterophilic granulocytes from one day old chicks efficiently kill *Salmonella* after phagocytosis, although the phagocytic index of these cells is much lower compared to granulocytes of one-week old chickens (Wells *et al.*, 1998).

Whether heterophilic granulocytes, attracted by signals derived from epithelial cells, have the same phagocytic index and killing ability as peripheral blood granulocytes is not known.

If the host response plays an important role in the colonization-inhibition phenomenon, granulocytes seem to be the most likely candidate effector cells due to their rapid influx in the caecal wall and their known *Salmonella* killing activity. Indeed, as shown in chapter 3, depletion of the heterophilic granulocytes by 5 fluoro-uracil resulted in a lack of protection against the virulent S. Enteritidis 76Sa88 in newly hatched chickens, when the aroA mutant vaccine strain was administered 24 hours before challenge.

Since 5 fluoro-uracil is a cytostatic drug, the possible interference with proliferation of other cell types than granulocytes had to be analysed with care. Neither a lympho- or a monocytopenia was detected when the same dose of 5 fluoro-uracil was given to adult chickens (Kogut *et al.*, 1994). Comparisons of blood smears of the animals of the 5 fluoro-uracil treated group and the control group of our experiment showed no differences in number of monocytes and lymphocytes. It is of utmost importance that 5 fluoro-uracil does not influence the intestinal epithelial cells. In humans, epithelial cell damage is reported in persons receiving the drug as anti-cancer agent, leading to diarrhoea, in some cases. For anti-cancer treatment however, multiple intravenous administrations are given over a long period of time, while the animals in our experiments received a single subcutaneous dose. Moreover, on cryostat sections, no epithelial cell damage and villus length alterations were observed in the 5 fluoro-uracil treated animals. We can therefore conclude that the 5 fluoro-uracil treatment does not have major side effects that could account for the altered response to the Salmonella inoculation. The data presented in this work show that heterophilic granulocytes most likely have a major contribution to the early anti-*Salmonella* protection phenomenon. Overall, both microbiological phenomena, such as blocking of receptor sites on the epithelium of the gut, and the innate host response, will play a role in the early colonization-inhibition.

When the aroA mutant vaccine strain was administered to day-old chicks, and these birds were challenge infected at day 2, there was a protective effect the first days after infection, whereafter there was an increase in colonization with the challenge strain again. Probably the real gap in protection is not the first days of age, since this can be

overcome by the colonization-inhibition phenomenon or maternal immunity, but the time between this and the rise of the humoral or specific cellular responses. A possibility to overcome this is the combination of *Salmonella* vaccine strains and competitive exclusion flora (Methner *et al.*, 1999). Also multiple administrations of the vaccine strain in the first days post-hatch could possibly solve this problem.

The effects of administration of attenuated *Salmonella* strains immediately post-hatch on performance of broiler chickens need to be further investigated. Indeed, vaccination should not affect daily weight gain or feed conversion. There is an antagonism between the physiological processes of immunity and growth, yet the severity of this interaction may depend upon the component of the immune system responding (Humphrey and Klasing, 2003). An innate immune response results in tissue specific changes in protein metabolism that may be explained by an organ's role during infection (Barnes *et al.*, 2002). In general, tissues engaged in host defense require greater amounts of amino acids to synthetize protective factors and to support leukocyte proliferation. Those tissues not directly involved in immune defense are either unaffected or increase their rates of protein degradation. Skeletal muscle is the largest pool of amino acids, and infection increases protein degradation in muscle and release of amino acids into plasma (Humphrey and Klasing, 2003). The release of amino acids from skeletal muscle plays an important role in supplying amino acids for the synthesis of hepatic acute phase proteins involved in host defense. In chickens, macrophage derived cytokines, such as IL-1, have been shown to decrease growth (Klasing, 1988). Based on these data, it can be speculated that growth of broilers would be impaired after administration of the live vaccine strains. However, when the strains are only administered in the first days of life of the animals, a possibility exists that the animal will undergo compensatory growth. If this occurs, it should be beneficial since it decreases disorders and mortality caused by excessive weight gain at a premature age (Lippens *et al.*, 2002). Furthermore, it is known that broilers, due to their genetic characteristics, have a blunted inflammatory response compared to laying hens after *S*. Typhimurium LPS injection. This is probably caused by an increased production of the immunosuppressive cytokine TGFβ_2, decreasing levels of IL-1 and IFN-γ (Leshchinsky and Klasing, 2001).

Protection against colonization can be induced by increasing the infection threshold, i.e. the dose to infect an animal, or by reducing the number of bacteria the birds come in contact with. This can be achieved by making the birds less susceptible to the infection through a non-specific or a specific mucosal and systemic immune response. It can also be achieved by reducing the numbers or the colonization capacity of the bacteria. It is again important that anti-*Salmonella* products that reduce the colonization capacity should work immediately after hatching of the chicken, because of the ability of *Salmonella* to induce long lasting carriage after infection at early age and because of the high chance of early infection in broiler houses. Inclusion in the feed or drinking water of products with a direct effect on *Salmonella* would aid in early protection. Short-chain fatty acids (SCFA) are bactericidal against *Salmonella*, dependent on the concentration and the pH. In chapter 4 we reported that incubation of the bacteria in LB-medium, supplemented with butyric or propionic acid, decreased invasion of the bacteria in an intestinal epithelial cell line, while incubation in acetic acid supplemented medium increased invasiveness. Since it is believed that invasion into intestinal epithelial cells is necessary for colonization of the animals, decreasing the invasion will help in controlling colonization (Wallis and Galyov, 2000).

Currently, SCFA are commonly used to supplement poultry feed. The basis of their use is the fact that these acids have an anti-*Salmonella* activity. All SCFA have more or less the same antibacterial effects on *Salmonella in vitro*, with propionic acid being slightly more potent in inhibiting the bacterial growth. Therefore formic, acetic and propionic acid are all used to supplement poultry feed. Recently, also butyric acid has been introduced as poultry feed supplement. Various combinations of the different SCFA in different concentrations are currently used. Also the formulation of SCFA products can differ considerably. SCFA can be used in liquid form to supplement drinking water or in powder form to supplement poultry feed (Hinton and Linton, 1988; Patten and Waldroup, 1988; Izat *et al.*, 1990a,b; Thompson and Hinton, 1997). In these forms, the acids will have their effects limited to the crop and the proximal part of the intestine, due to resorption (Thompson and Hinton, 1997). Preparations in which the acids are coated also are on the market. The purpose of coating is to ensure that the acids will be released further down the intestinal tract (Iba and Berchieri, 1995; Berchieri and Barrow, 1996). There are coated SCFA products, for which is claimed that the acids are slowly released, ensuring that the acids would have their

effects throughout the full length of the gastro-intestinal tract (Anonymous, 1997). The formulation will affect the activity of the SCFA feed additive. Uncoated powder or liquid products will increase the threshold for infection due to killing of the bacteria in the first part of the gastro-intestinal tract (Thompson and Hinton, 1997). However, when the *Salmonella* bacteria pass the stomach, they will no longer be influenced by the acids further down the gastro-intestinal tract. Thus effects on invasion will probably not be very important, due to the short contact of the bacteria with the acids and the change in environment when the bacteria reach the intestine. Moreover, we showed that contact of *S*. Enteritidis with short-chain fatty acids for 30 minutes did not influence invasion to the same degree as incubation for 4 hours (unpublished results). Therefore it is probably not important which acid is used in the case of liquid or powder preparations. In numerous publications, powder or liquid preparations were tested as feed additives to control *Salmonella* shedding with variable results (Hinton and Linton, 1988; Patten and Waldroup, 1988; Izat *et al.*, 1990a,b; Thompson and Hinton, 1997). When coated or micro-encapsulated SCFA are used, then the bacteria that reside in the lumen of the lower intestinal tract are also in contact with these acidic compounds. It is even possible that the acids only exert their action in the neighbourhood of the micropearls, due to local acidification. In this case, the effects of the acidic compounds on invasion of the *Salmonella* bacteria would also play an important role. Therefore it will be very important to choose the appropriate SCFA to be used in this type of preparations. Indeed, our results reported in chapter 5, show that supplementing acetic acid, and to a lesser extent formic acid, in micro-encapsulated form, to poultry feed, resulted in a serious increase in both organ and caecal colonization. When propionic and butyric acid impregnated microbeads were supplemented to the feed of the animals, colonization of the caeca was decreased relative to the control, early after infection with S. Enteritidis. It is proposed that butyric or propionic acid should be used for the coated or micro-encapsulated preparations, or even combinations of these two SCFA. Research should be done on the additive effect of powder or liquid products and coated products. In this case the SCFA should be available throughout the gastro-intestinal tract, from crop to lower intestine. Attempts should be made to obtain release of the acids in the caeca, since this is the predominant site of colonization for *Salmonella*. Elevated butyric acid concentrations in the caeca of animals have been associated with

decreased Salmonella colonization indeed (Bailey *et al.*, 1991; Oyarzabal and Conner, 1996; Fukata *et al.*, 1999).

Elevated butyric acid concentrations in the intestinal tract of chickens can also be obtained by the use of probiotic bacterial strains and prebiotic products. These prebiotics will probably not result in increases in butyric acid concentrations very early after hatch. Most prebiotic products have to be fermented before they are active and therefore these products will only be active after a stable gut flora has formed (Gibson and Roberfroid, 1995; Grizard and Barthomeuf, 1999). The onset of the effect will require some time (several days) after hatching. It is known that addition of fructo-oligosaccharides (FOS) in the feed of animals results in high butyrate concentrations starting a few days after administration of the FOS (Le Blay *et al.*, 1999). FOS addition to the feed results in increases in bifidobacteria, that are known to produce butyrate from FOS (Olano-Martin *et al.*, 2000). These bacteria ferment sugars to the end product butyric acid (Kaplan and Hutkins, 2000). It is hypothetized that the use of combinations of FOS and bifidobacteria may result in increased butyric acid concentrations in caeca and intestine of chickens early after administration of these preparations to the chickens. This would be due to the availability of a substrate for fermentation by the bifidobacteria. The concept of combining pro- and prebiotics, termed synbiotics, is proposed to increase the efficacy and speed of the activity of both products. It is already known that FOS, as well as bifidobacteria, have protective effects against *Salmonella* in chickens through the production of butyric acid (Fukata *et al.*, 1999; Asahara *et al.*, 2001; Henriksson and Conway, 2001). It is less clear how propionic acid concentrations can be increased in the intestine.

Simultaneously to our report, more authors reported other beneficial effects of butyric acid on different parameters of gut health. While butyric acid decreases mitoses and induces differentiation in tumor cell lines and in tumours, this acid also increases cell division in primary intestinal epithelial cells (Lallemand *et al.*, 1999; Hodin, 2000; Greenberg *et al.*, 2001). Indeed, it is already described that villus length and mitotic index of intestinal epithelium increase when butyric acid concentration is increased in the intestine. Butyric acid also has an anti-inflammatory effect in the gut, by decreasing IL-8 production by intestinal epithelial cells, through inhibition of the action of the transcription factor NF-κB (Segain *et al.*, 2000; Hodin, 2000). In human

medicine, butyric acid is already used in patients with Crohn's disease and the anti-tumor activity is receiving great attention (Hodin, 2000; Gassull and Cabre, 2001, Goh and O'Marain, 2003). Moreover, the acid will repair intestinal damage due to the mitotic activity (Butzner *et al.*, 1996). The relevance for anti-*Salmonella* activity is not clear, but it could possibly also control infections that damage the epithelium or require preceding epithelial damage in order to set off the disease process, as is the case in *Clostridium perfringens* infection.

The antagonistic effects of different SCFA such as acetic and butyric acid on invasiveness of *Salmonella* in intestinal epithelial cells is partly explained by a sensor mechanism regulating virulence gene expression in *Salmonella*. It is already described that virulence gene expression of *Salmonella* is regulated by osmolarity, pH and oxygen tension (Foster *et al.*, 1995; Leclerc *et al.*, 1998). It is interesting to speculate how the different SCFA change the invasion pattern. Recently, the mechanism of acetic acid induced increase in invasion was explained by the actions of acetyl-phosphate (Lawhon *et al.*, 2002). Acetic acid, once in the bacterial cell, can be converted to acetylCoA and acetyl-phosphate. Acetyl-phosphate was proven to activate the *Salmonella* sensor/kinase system BarA/SirA by transphosphorylation. This sensor kinase system then activates HilA and InvF, which are the key regulators of *Salmonella* pathogenicity island I. Further in the cascade, expression of effector proteins of the Salmonella Pathogenicity Island I, such as SipC, will increase, directly resulting in increases in invasion. It is not known how propionic acid and butyric acid exert their invasion reducing activity. By the use of transcriptional fusions of the promoters of *Salmonella* virulence genes with lacZ or luxCDABE genes, we were able to measure virulence gene expression by measuring beta-galactosidase activity or light production, respectively. It was shown that propionic acid and butyric acid decreased expression of BarA, SirA, HilA, InvF, SipA and SipC (Van Immerseel and Rychlik, unpublished data). Measuring the expression of SipA and SipC is proposed to be a test system for predictions on how bacteria are going to be influenced by feed additives, which are thought to influence virulence gene expression, since these genes code for the downstream effector proteins having direct effects on invasion (Van Immerseel *et al.*, 2003). An interesting finding is the upregulation of the SdiA protein by butyric acid and propionic acid, and the downregulation of SdiA by acetic acid (Rychlik and Van Immerseel, unpublished data). A S. Typhimurium mutant in sdiA was more virulent in mice than the wild type parent strain (Volf *et al.*, 2002).

Apparently butyric acid not only downregulates virulence genes but also actively upregulates proteins that are involved in reducing virulence. The exact function of the SdiA protein however is not yet known.
Propionic and butyric acid probably exert their effects through the same mechanism. In biochemical pathways of *E. coli*, both propionic and butyric acid metabolisation result in production of the same intermediates, such as succinate and 4-hydroxy butyric acid (EcoCyc Pathway database, http://biocyc.org). We are currently trying to elucidate the mechanism of action of propionic and butyric acid by making mutants in enzymes involved in their metabolic pathways.
Medium-chain fatty acids also decrease invasion and virulence gene expression to the same extent as butyric acid (Van Immerseel *et al.*, unpublished data). These are catabolised to butyric and propionic acid as intermediates. When caproic acid was supplemented to poultry feed, colonisation of caeca and internal organs were decreased (Van Immerseel *et al.*, unpublished data). Probably a common mechanism is involved.

In a *Salmonella* control program, both vertical and horizontal transmission of *Salmonella* should be blocked to control *Salmonella* in chickens. Vaccination of laying hens and parent flocks is an important tool to control vertical transmission. Controlling horizontal spread of *Salmonella* is probably more difficult, since environmental contamination with *Salmonella* is high. Once animals are infected with *Salmonella*, the bacteria can persist in the animal for many weeks, even when the animals are infected at their first day of life. It is moreover likely that horizontal transmission occurs at young age when the environmental pressure is high. It is therefore of utmost importance that, especially for broiler chickens, that are slaughtered already at 6 weeks of age, protection measures start their action immediately post-hatch and preferably continue their action throughout the rearing period. This will require a combination of different measures. Reduction of the infection pressure to prevent *Salmonella* from infecting the animals is the first barrier that should be maintained. Hygienic measures and decontamination of broiler houses are important in this regard. Secondly, the susceptibility of the animals should be decreased by stimulating immune responses. Vaccination of parent flocks should be performed, since maternal antibodies are known to confer protection. Secondly, oral vaccination of broiler chickens with attenuated Salmonella strains immediately post-

hatch can stimulate aspecific immune responses, that combat early *Salmonella* challenges. Thirdly, next to the use of hygienic measures and the stimulation of the host response, it should be tried to maintain a high antibacterial pressure inside the animals by direct action of antimicrobials. This is of extreme importance in case *Salmonella* infects the animals, despite of the hygienic measures and the host immunity. The use of competitive exclusion products could be considered to be used immediately post-hatch to create a protective gut flora for preventing *Salmonella* to establish an infection. Also short-chain fatty acids can be used for this purpose. Butyric acid seems to be the candidate for this purpose due to the antibacterial and virulence-decreasing effects. These measures can also be useful in laying hens, especially during episode of high susceptibility, i.e. at young age at point of lay and at molting. The work in chapter 4 and 5 moreover shows that protection products should not be empirically chosen or given to animals without clear scientific basis.

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SUMMARY

Since the nineteen eighties, *Salmonella* Enteritidis has emerged as a major chicken and egg associated human pathogen. In Belgium, more than 10,000 *Salmonella* Enteritidis human isolates are recorded annually, mostly linked to consumption of poultry products. Chickens are usually infected by oral uptake from the environment. The pathogenesis of *Salmonella* in chickens can be divided in two distinct phases, i.e. the intestinal and the systemic phase of the infection, both regulated by genes of a different *Salmonella* pathogenicity island. *Salmonella* Enteritidis is able to survive gastric acidity and can therefore pass the stomach and reach the intestine of the chicken. During the intestinal phase, the bacteria adhere to the intestinal mucosa and invade the intestinal epithelial cells. This process is regulated by the *Salmonella* pathogenicity island I type three secretion system, injecting bacterial proteins in the eukaryotic cell to reorganize the actin cytoskeleton, leading to uptake of the bacteria in the intestinal epithelial cell. Due to the pro-inflammatory cytokine production by epithelial cells, macrophages are attracted to the intestinal wall. During the systemic phase of infection, *Salmonella* bacteria are internalized by phagocytosis by macrophages. *Salmonella* bacteria can survive within and replicate in the macrophage, and can thereby spread to the internal organs, such as liver and spleen, where the bacteria can be found in large numbers. The replication in the macrophage is regulated by the *Salmonella* pathogenicity island 2 type three secretion system, which translocates bacterial proteins across the membrane of the vacuole, where the bacteria reside.

Monitoring programs and control schemes to reduce *Salmonella* contamination in poultry in Western Europe, mandatory since the introduction of the EU directive 92/117 in 1992, have been introduced with relatively limited success. While *Salmonella* Enteritidis is still a serious problem in layer flocks, many *Salmonella* serotypes still contaminate broiler flocks, posing serious threats for public health. Conventional vaccination, as used in laying hens, seems insufficient for broilers. Vaccines only claim to induce a reduction of shedding of the bacteria in the faeces and not protection against challenge infection. When only reduced shedding is achieved, there is little doubt that *Salmonella* will never leave the food chain. Indeed, the carrier animals, that do not shed bacteria but are colonised in intestinal tract or internal organs, can still restart shedding and thus contaminate their environment, when subjected to stress. Moreover, these carrier animals can contaminate the

slaughter line. Broiler chickens have only a limited life span. Since immunity is not fully developed in the first days post-hatch and since also no protective gut flora is present at this early age, protection measures should start their action immediately post-hatch and should prevent colonization of the animal. The aim of this work was to explore two tools that could be applied for early protection, being the colonization-inhibition phenomenon and the short-chain or volatile fatty acids.

In the first three chapters, the colonization-inhibition phenomenon was investigated. Colonization-inhibition is a phenomenon in which inoculation of a live attenuated *Salmonella* strain to one-day old chickens induces protection against a virulent strain administered one day later. The mechanism that governs this effect *in vivo* is not fully understood. Competition between the strains for receptors on epithelial cells and competition for nutrients and electron acceptors have been proposed as an explanation. When the present studies were started, no information was available on the possible involvement of the host reponse. The first specific purpose of the present studies was to investigate whether the host response could play a role in this colonization-inhibition phenomenon.

In chapter one, data were collected concerning the dynamics of immune cells infiltrating in the caecal wall after neonatal *Salmonella* infection and concerning the course of a *Salmonella* infection in hatchlings. Bacteria invaded the lamina propria of the caecal wall from 12 hours post-challenge onwards. Bacteriological examination of internal organs (liver, spleen) showed a peak in *Salmonella* bacteria at 3 days post-infection, whereafter the number of bacteria decreased. Immunohistochemistry revealed macrophages and T-lymphocytes invading the caecal propria mucosae from 24 hours after challenge onwards, while B-lymphocytes came somewhat later, subsequently organising into follicular aggregates. An early increase in granulocytes was partly masked by the infiltration observed in the control population, which was explained as a response to natural flora. While the B-lymphocyte and granulocyte populations were maintained, T-lymphocyte and macrophage populations were already reducing by 10 days post-challenge.

In chapter two, administration of an avirulent *Salmonella* strain (*S*. Enteritidis *aroA* mutant) inhibited subsequent colonization of the intestine of neonatal chickens by a

virulent *Salmonella* Enteritidis strain. The caecal immune cell infiltration, attracted by the avirulent strain, was analyzed during the first ten days after vaccination of newly hatched chickens with a *Salmonella enterica* ser. Enteritidis *aroA* mutant, and infection one day later with a virulent *Salmonella enterica* ser. Enteritidis strain. These data were correlated with bacterial colonization and clearance of the *Salmonella* Enteritidis challenge strain. Bacteriological data showed that vaccinated animals had a much lower number of challenge bacteria in their organs and caecal contents the first days post-challenge, relative to unvaccinated animals. Immunohistochemical analysis of the caecal lamina propria revealed that heterophils started infiltrating the caecal lamina propria from 12 hours post-vaccination. Macrophages and T-lymphocytes started infiltrating from 20 hours and B-lymphocytes from 24 hours post-vaccination. These data imply that immune cells already colonized the caecal wall at the time of challenge in vaccinated animals. The dynamics of inflammatory cell infiltration led us to the suggestion that the heterophilic granulocyte could be the most important effector cell involved.

In chapter three, the importance of heterophilic granulocytes in the colonization-inhibition phenomenon was further investigated. The effect of vaccination of day-old chickens with a live *aroA* mutant of *Salmonella* Enteritidis on subsequent challenge with a field strain of the same serotype 24 hours later was studied in heterophil-depleted chickens. This heterophil depletion was induced by the administration of 5 fluoro-uracil. Administration of wild type *Salmonella* bacteria resulted in extensive colonization of caeca and invasion in liver and spleen in the challenged groups, whether they were vaccinated or not. The cytostatic drug 5 fluoro-uracil did not induce a monocytopenia or lymphopenia in the blood of the animals. Also no epithelial cell damage or villus shortening was observed in the 5 fluoro-uracil treated animals. Thus heterophilic granulocytes seem to be the only cell type that was seriously affected by the cytostatic drug. Since in the heterophil-depleted chickens, vaccination with a live *aroA* mutant of *Salmonella* Enteritidis did not afford early protection against challenge with a field strain of the same serotype, this study allowed to confirm our earlier suggestion that heterophilic granulocytes play an important role in the early protective response induced by live vaccines. In conclusion, not only microbiological phenomena, such as blocking of receptor sites

on the epithelium of the gut, but also the innate host response, will play a role in the mechanisms of early colonization-inhibition.

Early protection against *Salmonella* infection can also be achieved by reducing the number or the colonization capacity of the bacteria, next to increasing the (immunological) resistance of the chicken. It is again important that anti-*Salmonella* products that reduce the colonization capacity should exert their effect immediately after hatching of the chicken, due to the ability of *Salmonella* to induce long lasting carriage after infection at early age and due to the high chance of early infection in broiler houses. As an example of non-rapid acting anti-*Salmonella* products, most prebiotic products have to be fermented before they are active and therefore these products will only be active after a stable gut flora has formed. Competitive exclusion flora can be administered at hatch and will be active immediately. Also inclusion in the feed or drinking water of products with a direct effect on *Salmonella* would aid in early protection. Short-chain fatty acids (SCFA) are bactericidal against *Salmonella*, depending on the concentration and the pH. Short-chain fatty acids are used as feed additives to reduce *Salmonella* in poultry. The choice of the acids is empirical. Data derived from studies with mammalian cell lines suggest differences in virulence of *Salmonella* after contact with the different short chain fatty acids.
The second specific purpose of this thesis was to examine the effects of different short-chain fatty acids on the early interactions between *Salmonella* and the host, i.e. the chicken.

Therefore in chapter 4 the effects of the short-chain fatty acids formic, acetic, propionic and butyric acid on invasion of *Salmonella* Enteritidis in intestinal epithelial cells was analyzed *in vitro*. Pre-incubation of *S*. Enteritidis for 4 hours in growth media supplemented with various concentrations of propionate or butyrate, resulted in decreased invasion compared to bacteria, preincubated in non-supplemented media, and to bacteria, pre-incubated in media supplemented with formate or acetate. The underlying molecular mechanism is an increase in virulence gene expression after contact of the bacteria with acetic acid, and a decrease in virulence gene expression in *Salmonella* bacteria, after contact with propionic or butyric acid. This was shown by the use of DNA fusion constructs of promoters of the *Salmonella* Pathogenicity Island

I virulence genes and luxCDABE genes, and subsequent measurement of light production after contact of the *Salmonella* bacteria with the respective acids.

In vivo trials described in the literature with uncoated short-chain fatty acids as feed or water additives yield variable results concerning protection against *Salmonella*. No comparisons are made in the literature between the four short-chain fatty acids. Moreover, literature data on the use of impregnated short-chain fatty acids in control of *Salmonella in vivo* are lacking. The use of impregnated short-chain fatty acids is thought to be beneficial since the acids are released further down in the intestinal tract, so that *Salmonella* bacteria that reside in the intestinal tract are also affected. Therefore, in chapter 5, *in vivo* colonization of caeca and internal organs was studied in young chickens in the early phase after *Salmonella* infection, when the animals were given feed supplemented or not with the respective short-chain fatty acids formic, acetic, propionic and butyric acid in microencapsulated form, ensuring the release of the acids in the lower intestinal tract. Five groups of 20 animals each were challenged with 5.10^3 cfu *S*. Enteritidis at day 5 and 6 post hatch and samples of caeca, liver and spleen were taken at day 8 and analysed for the number of cfu *Salmonella* per g tissue. Feed supplementation with acetic acid, and to a lesser extent formic acid, resulted in an increase of colonisation of caeca and internal organs as compared to birds receiving non-supplemented feed. Birds receiving propionic acid coated microbeads as feed supplement were colonized with *Salmonella* to the same extent as animals receiving non-supplemented feed. Butyric acid impregnated microbeads in the feed, however, resulted in a strong decrease of colonization by *S*. Enteritidis in the caeca, but not in liver and spleen.
Simultaneously to our findings, many authors reported positive effects of butyric acid on different parameters of gut health. Especially the increase in villus length and crypt depth in the intestine and the repair of intestinal damage due to the epithelial cell mitosis inducing activity of the acid may have additional beneficial effects.

To our opinion, *Salmonella* control should start early post-hatch due to the susceptibility of the chickens for *Salmonella* at that age. Both stimulation of the host immune system and reduction of virulence of the bacteria could be combined to decrease shedding and colonisation of the animal. The first can be achieved by vaccination with live mutant strains resulting in protective non-specific immune

reponses. The latter can be achieved by the use of short-chain fatty acids propionic and especially butyric acid. These acids are not only bactericidal, but also decrease virulence of *Salmonella* resulting in decreased invasion into intestinal epithelial cells.

SAMENVATTING

Sinds halfweg de tachtiger jaren is *Salmonella* Enteritidis doorgebroken als een belangrijke voedselinfectie bij de mens. In België worden jaarlijks meer dan 10.000 humane *Salmonella* Enteritidis isolaten gerapporteerd, meestal verbonden met de consumptie van pluimveeproducten. Infectie van pluimvee gebeurt gewoonlijk door orale opname van de bacterie vanuit de omgeving. De pathogenese van *Salmonella* in de kip kan worden ingedeeld in 2 fasen : de intestinale en de systemische fase van infectie, die beide gereguleerd worden door genen van verschillende pathogeniciteitseilanden. *Salmonella* Enteritidis kan de zuurtegraad van de maag overleven en kan hierdoor na orale opname de maag passeren en in de darm terecht komen. In de intestinale fase hechten de bacteriën vast aan de intestinale mucosa en dringen binnen in de darmepitheelcellen. Dit proces wordt geregeld door het *Salmonella* Pathogeniciteitseiland I dat codeert voor een type 3 secretiesysteem, dat bacteriële proteïnen injecteert in de eukaryote cel. Dit leidt tot reorganisatie van het actine cytoskelet van de epitheelcel, en tot opname van de bacterie in de epitheelcel. De epitheelcellen produceren pro-inflammatoire cytokines waardoor macrofagen worden aangetrokken naar de darmwand. Gedurende de systemische fase van de infectie worden *Salmonella* bacteriën opgenomen door de macrofaag via fagocytose. *Salmonella* kan overleven en verdubbelen in deze cellen en op die manier verspreiden naar interne organen, zoals lever en milt, waar de bacteriën in hoge aantallen kunnen worden aangetroffen. Verdubbeling in de macrofaag wordt gereguleerd door het *Salmonella* Pathogeniciteitseiland II dat codeert voor een type 3 secretiesysteem, dat ervoor zorgt dat bacteriële eiwitten vanuit de cytoplasmatische vacuole waarin Salmonella zich bevindt, verplaatst worden door de membraan van de vacuole naar het cytoplasma van de gestheercel. Hiermee wordt de fusie van deze vacuole met de lysosoom van de gastheercel geremd.

Monitoring en bestrijdingsprogramma's voor *Salmonella* bij pluimvee werden verplicht in West-Europa vanaf de introductie van de EU-richtlijn 92/117 in 1992. Deze programma's hadden totnogtoe relatief weinig succes. *Salmonella* Enteritidis is nog steeds het belangrijkste probleem bij leghennen. Bij de vleeskippen zijn er veel verschillende *Salmonella* serotypes. Deze besmettingen vormen een groot probleem voor de volksgezondheid. Conventionele vaccinatie, zoals gebeurt bij leghennen, blijkt niet succesvol bij vleeskippen. De beschikbare vaccins verminderen enkel de uitscheiding van *Salmonella* in de faeces. Ze voorkomen niet het aanslaan van een

challenge infectie. Wanneer er alleen een reductie van uitscheiding wordt beoogd, is er weinig twijfel dat *Salmonella* nooit uit de voedselketen zal verwijderd worden. Er bestaan namelijk dragerdieren die geen *Salmonella* uitscheiden maar wel gekoloniseerd zijn in caeca en inwendige organen. Deze dieren kunnen terug beginnen uitscheiden en hun omgeving besmetten, wanneer ze blootgesteld worden aan stress. Deze dragerdieren kunnen eveneens de besmetting overdragen naar de slachtlijn. Vleeskippen hebben slechts een korte levensduur. In de eerste dagen na uitkipping is het immuunsysteem van de kip nog niet volledig ontwikkeld en is er geen beschermende darmflora aanwezig. Hierdoor is het essentieel dat bestrijdingsproducten hun werking reeds direct na uitkipping uitoefenen op zodanige manier dat niet alleen de uitscheiding maar eveneens de kolonisatie van *Salmonella* in het dier zoveel mogelijk voorkomen wordt. Het doel van dit werk was om twee bestrijdingsstrategieën, die kunnen gebruikt worden voor vroege bescherming, nader te onderzoeken, namelijk het kolonisatie-inhibitie principe en het gebruik van korte keten of vluchtige vetzuren.

In de eerste 3 hoofdstukken werd het kolonisatie-inhibitie principe onderzocht. Kolonisatie-inhibitie is een fenomeen waarbij orale inoculatie van een levende verzwakte *Salmonella* stam aan ééndagskuikens bescherming biedt tegen een virulente *Salmonella* stam die 1 dag later oraal wordt toegediend. Deze bescherming duurt slechts enkele dagen. Het mechanisme dat dit *in vivo* effect bewerkstelligt was nog niet gekend bij de start van dit onderzoek. Competitie tussen de *Salmonella* stammen voor receptoren op epitheelcellen en competitie voor nutriënten en electronacceptoren werden voorgesteld als een verklaring. Bij de start van dit werk was er nog geen informatie voorhanden omtrent de rol van de gastheer. Het eerste concrete doel van dit onderzoek was om na te gaan of de gastheer respons een rol speelde in het kolonisatie-inhibitie principe.

In hoofdstuk 1 werden algemene gegevens verzameld omtrent de dynamiek van infiltrerende immuuncellen in de caecale wand na neonatale infectie met een virulente *Salmonella* Enteritidis stam en omtrent het verloop van een *Salmonella* infectie in kuikens. *Salmonella* bacteriën invadeerden de lamina propria van de caecale wand vanaf 12 uur post-infectie. Bacteriologische analyse van interne organen (lever, milt) toonde een piek in aantal *Salmonella* bacteriën 3 dagen post-infectie, waarna het

aantal bacteriën in deze organen daalde. Immunohistochemische analyse toonde infiltrerende macrofagen en T-lympfocyten in de caecale wand aan vanaf 24 uur post-infectie, gevolgd door B-lymfocyten. De B-cellen organiseerden zich vervolgens in folliculaire aggregaten. Een vroege granulocyten respons was sterker bij de geïnfecteerde dieren maar in mindere mate ook aanwezig bij de controle dieren. Dit laatste werd verklaard als een respons op de normale darmflora. Terwijl de B-lymfocyt- en granulocytpopulaties werden behouden tot 10 dagen na infectie, waren de macrofaag en T-lymfocytpopulaties reeds gedaald.

In hoofdstuk 2 werd de kolonisatie van de darm en inwendige organen van jonge kuikens door een virulente *Salmonella* stam geïnhibeerd door voorafgaande orale toediening van een *Salmonella* Enteritidis aroA mutant. De infiltratie van immuuncellen in de caecale wand, aangetrokken door de avirulente aroA mutant, werd geanalyseerd gedurende de eerste 10 dagen na vaccinatie van neonatale kuikens met de *Salmonella* Enteritidis aroA mutant, en infectie 1 dag later met de virulente *Salmonella* Enteritidis stam 76Sa88. Deze gegevens werden gecorreleerd met bacteriële kolonisatie van de virulente *Salmonella* Enteritidis stam. Bacteriologische analyse van orgaanstalen en caecale inhoud toonde aan dat gevaccineerde dieren door een veel lager aantal *Salmonella* bacteriën gekoloniseerd waren gedurende de eerste dagen post-infectie dan niet gevaccineerde dieren. Immunohistochemische analyse van de caecale lamina propria toonde aan dat heterofiele granulocyten begonnen te infiltreren in de caecal wand vanaf 12 uur post-vaccinatie. Macrofagen en T-lymfocyten infiltreerden in de caecale wand vanaf 20 uur post-vaccinatie, gevolgd door B-lymfocyten 24 uur post-vaccinatie. Dit betekent dat de immuuncellen reeds aanwezig waren in de caecale wand op het tijdstip van infectie in gevaccineerde dieren. Het patroon van immuuncelinfiltratie leidde tot de gedachte dat de heterofiele granulocyten de belangrijkste effectorcellen zouden kunnen zijn, betrokken in de bescherming, uitgelokt door de vaccinatie.

In hoofdstuk 3 werd de rol van heterofiele granulocyten in de inhibitie van de kolonisatie verder onderzocht. Daarom werd een analoog experiment uitgevoerd als in hoofdstuk 2, maar dit keer werd de studie uitgevoerd met kuikens die zeer weinig tot geen heterofiele granulocyten meer konden mobiliseren. Deze heterofielendepletie werd bekomen door injectie van 5 fluoro-uracil. Orale inoculatie van virulente

Salmonella Enteritidis bacteriën resulteerde in uitgebreide kolonisatie van caeca en inwendige organen, ongeacht het feit of ze vooral waren gevaccineerd of niet. Het cytostaticum 5 fluoro-uracil induceerde geen monocytopenie of lymfopenie in het bloed van de kuikens. Er werd ook geen epitheelcelschade en villusverkorting waargenomen bij de dieren, die behandeld waren met 5 fluoro-uracil. De heterofiele granulocyt blijkt dus het voornaamste celtype te zijn dat beïnvloed werd door het cytostaticum. Aangezien vaccinatie met een *Salmonella* Enteritidis aroA mutant dus geen vroege bescherming bood tegen een challenge infectie van hetzelfde serotype, in heterofiel gedepleteerde kuikens, werd hiermee dus het oorspronkelijke vermoeden bevestigd dat heterofiele granulocyten een belangrijke rol spelen in de vroege beschermende respons, geïnduceerd door levende vaccins. Samenvattend kan er gesteld worden dat niet alleen microbiologische fenomenen, zoals het blokkeren van bindingsplaatsen op darmepitheel, maar ook de vroege gastheerrespons onder vorm van heterofielen mobilisatie, een rol spelen in het vroege kolonisatie-inhibitie fenomeen.

Vroege bescherming tegen *Salmonella* infectie kan ook worden bereikt door de bacteriële kolonisatiecapaciteit te verlagen, naast de verhoging van de (immunologische) resistentie van de kip. Het is belangrijk dat de anti-*Salmonella* producten, die de kolonisatiecapaciteit verlagen, direct na het uitkippen hun werking starten, vermits infectie van ééndagskuikens aanleiding kan geven tot de inductie van de dragertoestand in de kip en vermits kuikens een hoge kans hebben om geïnfecteerd te raken op deze jonge leeftijd. Een voorbeeld van traagwerkende anti-*Salmonella* producten zijn de prebiotica, die in het algemeen eerst gefermenteerd moeten worden alvorens actief te zijn en dus hierdoor pas actief zijn na vorming van een stabiele darmflora. Toediening van competitieve exclusieflora is een voorbeeld van een product dat direct na uitkipping kan worden toegediend en dat een snelle werking heeft. Ook de toevoeging van producten aan water en voeder van kippen, met een directe antimicrobiële werking op *Salmonella*, kan resulteren in vroege bescherming. Korte keten vetzuren of vluchtige vetzuren (mierenzuur, azijnzuur, propionzuur en boterzuur) werken antibacterieel tegen *Salmonella*, afhankelijk van de concentratie van het zuur en de pH. Korte keten vetzuren worden reeds gebruikt als voederadditief om de besmettingsgraad met *Salmonella* bij pluimvee te verlagen. De keuze van de zuren in deze producten gebeurt empirisch. Studies met zoogdiercellijnen suggereren

echter verschillen in virulentie van *Salmonella* na contact met de verschillende korte keten vetzuren.
De tweede specifieke doelstelling van dit werk was de studie van de effecten van de verschillende vetzuren op vroege interacties tussen *Salmonella* en de gastheer.

Daarom werden in hoofdstuk 4 de effecten van de korte keten vetzuren mierenzuur, azijnzuur, propionzuur en boterzuur op invasie van *Salmonella* in intestinale epitheelcellen onderzocht *in vitro*. Pre-incubatie van S. Enteritidis gedurende 4 uren in groeimedium gesupplementeerd met verschillende concentraties propionzuur of boterzuur resulteerde in een verlaging van invasie vergeleken met *Salmonella* bacteriën, die gedurende 4 uren waren gepreïncubeerd in groeimedium, gesupplementeerd met mierenzuur of azijnzuur, of in ongesupplementeerd medium. Azijnzuur leidde tot een verhoging in invasie ten opzichte van controlemedium. Het onderliggende moleculaire mechanisme is een stijging in expressie van virulentiegenen na contact van *Salmonella* met azijnzuur en een daling na contact met propionzuur en boterzuur. Dit werd aangetoond met behulp van DNA fusieconstructen van promoters van *Salmonella* Pathogeniciteitseiland I virulentiegenen en luxCDABE genen, en daaropvolgende meting van lichtproductie na contact van de *Salmonella* bacteriën met de respectievelijke zuren (deze laatste resultaten zijn niet opgenomen in het proefschrift).

Resultaten uit de literatuur betreffende *in vivo* experimenten met niet-gecoate korte keten vetzuren als voederadditief tonen grote variabiliteit in de bescherming tegen *Salmonella*. In de literatuur worden geen vergelijkingen gemaakt tussen de verschillende vetzuren. Er zijn eveneens geen gegevens over het gebruik van korte keten vetzuren die geïmpregneerd zijn in microbeads. Het gebruik van gecoate of geïmpregneerde vetzuren wordt als voordelig beschouwd vermits de zuren de distale delen van het darmkanaal bereiken, zodat Salmonella bacteriën, die zich hier bevinden, eveneens beïnvloed worden door deze zuren. In hoofdstuk 5 werd de kolonisatie van caeca en inwendige organen bestudeerd in de vroege fase na infectie van jonge kuikens *in vivo*, wanneer de dieren voeder kregen, al dan niet gesupplementeerd met de respectievelijke korte keten vetzuren mierenzuur, azijnzuur, propionzuur en boterzuur. De zuren waren ingekapseld in microbeads, resulterend in geleidelijke vrijgave van de zuren tijdens de passage door het darmkanaal, zodat de

zuren ook de distale delen van het darmkanaal konden bereiken. Vijf groepen van elk 20 kuikens werden geïnfecteerd met 5.10^3 cfu S. Enteritidis op dag 5 en 6 en stalen van caeca, lever en milt werden genomen op dag 8 en bacteriologisch geanalyseerd. Opname van voer, gesupplementeerd met microbeads, geïmpregneerd met azijnzuur, en in mindere mate mierenzuur, leidde tot een verhoging van kolonisatie van de caeca en inwendige organen in vergelijking met niet gesupplementeerd voer. Dieren die als voederadditief propionzuur geïmpregneerde microbeads hadden gekregen werden in dezelfde mate gekoloniseerd door de bacterie als de groep dieren die niet gesupplementeerd voer kreeg, terwijl boterzuur resulteerde in een sterke verlaging van caecale kolonisatie door S. Enteritidis.
Tegelijkertijd met onze bevindingen rapporteerden verschillende auteurs gunstige effecten van boterzuur op verschillende andere parameters van darmgezondheid. Vooral de stijging in lengte van de darmvilli en diepte van de crypten en het herstel van darmschade door verhoging van delingsactiviteit van epitheelcellen, bewerkstelligd door boterzuur, zijn interessante bevindingen.

Bestrijdingsstrategieën en –producten tegenover *Salmonella* moeten dus vroeg na uitkippen beginnen werken, vermits het kuiken op die leeftijd zeer gevoelig is voor de infectie. Stimulatie van het immuunsysteem van de gastheer en het verlagen van de virulentie van de bacterie kunnen gecombineerd worden om uitscheiding en kolonisatie van caeca en inwendige organen te verlagen. Het eerste kan worden bereikt door vaccinatie met levend verzwakte stammen, leidend tot een beschermende aspecifieke immuunrespons. Het tweede kan worden bereikt door het gebruik van de korte keten vetzuren propionzuur, en vooral boterzuur. Deze zuren werken niet alleen antibacterieel, maar verlagen de virulentie van *Salmonella* door invasie in intestinale epitheelcellen tegen te gaan.

CURRICULUM VITAE

CURRICULUM VITAE

Filip Van Immerseel werd geboren op 18 december 1975 te Bonheiden. Hij beëindigde secundaire school in het Koninklijk Technisch Atheneum te Heist-op-den-Berg in 1993, richting Wiskunde-Wetenschappen. Dan startte hij de studies Bio-ingenieur aan de Katholieke Universiteit te Leuven (KUL), waar hij in 1996 het diploma van kandidaat bio-ingenieur behaalde. In 1999 studeerde hij met onderscheiding af als Bio-ingenieur in de cel- en genbiotechnologie. Zijn eerste onderzoekservaring deed hij op in het Laboratorium voor Endocrinologie van het Universitaire Ziekenhuis van de Eberhardt-Karls Universiteit te Tübingen (Duitsland). In 2000 startte hij een onderzoek betreffende mechanismen van vroege bescherming tegen Salmonella infecties, geïnduceerd door levende vaccins, gefinancierd door de Europese Gemeenschap. In 2001 starte hij een onderzoek omtrent de efficiëntie van bestrijdingsproducten tegen Salmonella en hun invloed op de dragertoestand bij de kip, gefinancierd door het Ministerie van Volksgezondheid. Beide Salmonella-projecten werden uitgevoerd aan de Dienst Pathologie, Bacteriologie en Pluimveeziekten van de Faculteit Diergeneeskunde van de Universiteit Gent, met als promotoren Prof. R. Ducatelle en Prof. F. Haesebrouck. In 2003 voltooide hij zijn doctoraatsopleiding en werd hij houder van een 'categorie C' diploma proefdierkunde uitgereikt door FELASA (Federation of European Laboratory Animal Science Associations).

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