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5. Loss of intestinal epithelial barrier function in *Salmonella* Enteritidis infection

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Abstract. Intestinal infection with *Salmonella* enterica serotype Enteritidis, a food-borne infection spread to humans especially through contaminated eggs and egg-products as well as undercooked contaminated fresh meat, is the most common cause of intestinal inflammation in the European Union. Enteritis caused by *Salmonella* Enteritidis is characterized by fever, diarrhoea and abdominal pain. The disruption of the intestinal epithelial barrier function contributes to diarrhoea and is responsible for the perpetuation of the inflammatory process. In this sense, oxidative stress and the proinflammatory cytokines TNF-α, IFN-γ and IL-1β are described to induce the disorganization of the tight junctions (TJ), the most apical epithelial intercellular junctions and responsible for the paracellular permeability. The interest of this chapter relies not only in the investigation dealing with the mechanisms of TJ regulation but also in the contribution to the development of new tools for the prevention of epithelial barrier disruption in enteritis caused by *Salmonella* Enteritidis.

Introduction

Salmonella are gram-negative bacteria consisting of non-spore forming bacilli and are a member of the family *Enterobacteriaceae*. The genus *Salmonella*

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includes two species, *Salmonella enterica* and *Salmonella bongori*. The nomenclature of *Salmonella* is quite complex and is based on both serotype and subspecies names [1]. For the subspecies *enterica* of *Salmonella enterica*, more than 2500 serotypes have been described [2]. Thus, *Salmonella enterica* subspecies *enterica* serotype Enteritidis is shortened to *Salmonella* serotype Enteritidis or *Salmonella* Enteritidis. *Salmonella* Enteritidis is one of the leading causes of food-borne salmonellosis in humans all over the world [3]. The remaining cases of salmonellosis are caused by *Salmonella enterica* serotype Typhimurium, associated with the consumption of contaminated pig and beef meat, and other more minor serotypes. Poultry is considered the single largest reservoir of *Salmonella* Enteritidis and the consumption of chicken meat and egg products is the major source of human infection [4-7]. *Salmonella* causes asymptomatic intestinal infections in adult birds but acute outbreaks exhibiting clinical disease along with high levels of mortality occur in chicks younger than 2 weeks old [8]. Birds that are asymptomatic carriers may facilitate the spread of disease infections among flock, thus constituting the major source of infection [9,10]. Salmonellosis is characterized by fever, diarrhea and abdominal cramps, but if the bacterium invades the bloodstream it can also cause life-threatening infections.

A necessary step in the successful colonization and ultimate production of disease is the ability of bacterial pathogens to adhere to host surfaces, which is an important determinant of virulence. Generally, binding to intestinal host cells is essential for the bacteria to resist the fluid flow of the luminal contents and the peristalsis of intestinal contraction. Once bound to the epithelial surface, bacteria may colonize and establish a permanent residence in the gut. The host cell is often an active participant in the adhesion process and does not function simply as an inert surface for attachment. Indeed, a wide range of mammalian cell surface constituents, including glycoproteins and glycolipids, can serve as receptors for bacterial attachment [11]. *Salmonella* Enteritidis possesses mannose-specific lectins in type-1 fimbriae that adhere to glycoproteins of the intestinal epithelium [12] and allow passage, mainly but not exclusively, through M cells (microfold cells) [13] (Fig. 1). This invasive mechanism is governed by the type III secretion system 1 (T3SS-1), which facilitates epithelial uptake and invasion [14]. The genes that encode the T3SS-1 machinery are associated with Salmonella pathogenicity island 1 (SPI-1) [15]. Pathogenicity islands (PI) are genetic elements that carry genes encoding virulence factors, such as adhesion, invasion, and toxin genes [16,17]. In fact, one of the major clinical features of salmonellosis is diarrhea, which is caused by SPI-1 T3SS

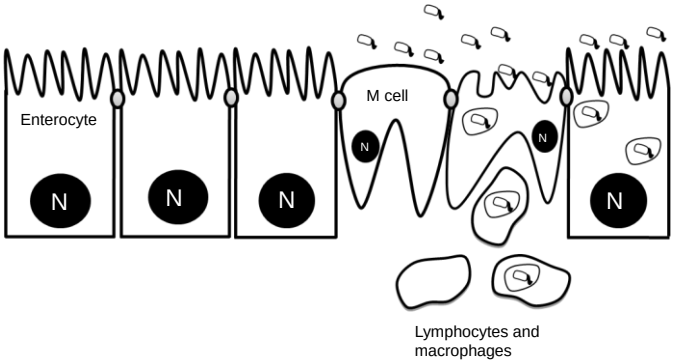

Figure 1. *Salmonella* Enteritidis entry into intestinal epithelial cells, mainly via M cells. After crossing the M cell, the bacteria are engulfed by macrophages and induce their apoptotic death. This process allows the bacteria to invade adjacent epithelial cells and to elicit an inflammatory response.

translocated proteins [18]. A number of additional proteins are translocated via the SPI-1 T3SS including SipA, SipC, and SopB (SigD), which interact with the actin cytoskeleton causing cytoskeletal rearrangements leading to membrane ruffling [19,20]. Membrane ruffling is characterized by a rearrangement of the cell membrane and cytosol such that the *Salmonella* is surrounded by the host cell and internalized within a membrane containing vacuole that serves as a protective niche from lysosomal degradation [21,22].

1. Intestinal epithelial barrier function

The gastrointestinal epithelium is a selective barrier that allows the absorption of nutrients, electrolytes and water, but restricts the passage of larger potentially injurious compounds such as allergens, toxins and pathogens. Thus, this barrier prevents inflammation, mucosal injury, bacterial translocation and systemic infection. The epithelial cells create this selective permeability, constituting the so called epithelial barrier function, by two pathways: the transcellular and the paracellular pathway. The transcellular pathway is involved in the transport of nutrients, including sugars, amino acids, peptides, fatty acids, minerals and vitamins. As the cell membrane is impermeable, this process is predominantly mediated by specific transporters and channels (Fig. 2) located on the apical and basolateral membranes. The paracellular pathway is associated with diffusion though the intercellular space between adjacent epithelial cells. Therefore, the ability of the intestinal

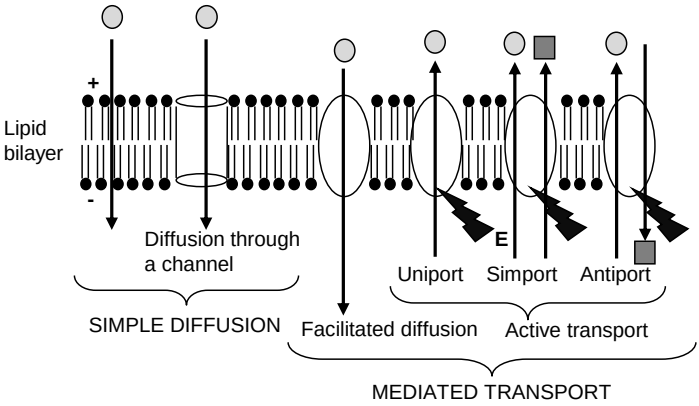

Figure 2. Schematic representation of simple diffusion and mediated transport mechanisms.

epithelium to establish the equilibrium between nutrient absorption and the prevention of harmful element entry constitutes the key backbone of intestinal barrier function. In addition, other elements also contribute to mucosal protection, such as the luminal mucus layer, antibacterial products and microbiota which not only protects against pathogens but also forms a sophisticated intestinal homeostatic colonization system, as well as intraepithelial lymphocytes and subepithelial immune cells with innate and adaptive immune systems [23].

The structural integrity of the epithelium is maintained by three distinct adhesion systems: tight junctions (TJ), adherent junctions, and desmosomes. The adherent junctions, along with desmosomes, provide strong adhesive bonds between the epithelial cells and also aid intercellular communication, but does not determine paracellular permeability (PP) [24]. TJ, the most apical component, are the rate-limiting step for PP and constitute the interface (fence) between apical and basolateral membrane domains [25]. TJ are formed by transmembrane proteins associated with cytosolic proteins and the cytoskeletal perijunctional actomyosin ring (Fig. 3). Five transmembrane proteins of the TJ have been identified until now: occludin, the claudin family, tricellulin, crumbs, and junctional adhesion molecules (JAM). These proteins are associated with a wide spectrum of cytosolic proteins, of which *zonula occludens* (ZO)-1, ZO-2, ZO-3, AF6, and cingulin are described as forming the nexus with cytoskeletal proteins [26]. Although the structure and in some cases the role of these proteins have been well described, the manner they work together to determine selective PP is not already completely well-defined [27].

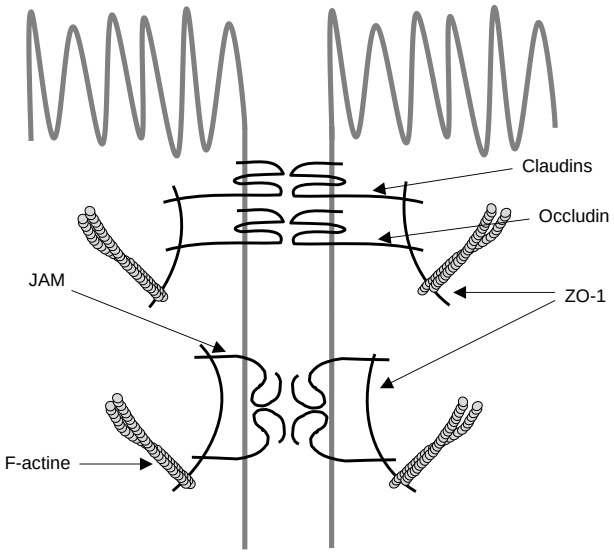

Figure 3. Molecular structure of the tight junction (TJ) of intestinal epithelial cells. The TJ complex consists of transmembrane and intracellular scaffold proteins. The extracellular loops of the transmembrane proteins (occludin, claudins, JAMs, and tricellulin) create a selective barrier in the paracellular pathway. The intracellular domains of the transmembrane proteins interact with the intracellular scaffold proteins such as *zonula occludens* (ZO) proteins and cingulin, which in turn anchor the transmembrane proteins to the actin cytoskeleton.

2. Disruption of the intestinal epithelial barrier function

The modification of TJ structure and therefore PP is dynamically regulated by various extracellular stimuli and is closely associated with health and susceptibility to disease [28]. In this sense, evidence from basic science and clinical studies indicate that the intestinal TJ barrier has a critical role in the pathogenesis of intestinal diseases such as inflammatory bowel disease, celiac disease and irritable bowel syndrome [28]. TJ barrier disruption and increased PP, followed by permeation of luminal proinflammatory molecules, can induce activation of the mucosal immune system, resulting in sustained inflammation and tissue damage. Thus, pro-inflammatory cytokines, antigens, and pathogens contribute to barrier impairment [29]. In contrast, food factors and nutrients also participate in intestinal TJ regulation, and some of these could be developed as preventive and therapeutic tools for defective barrier-associated diseases [30].

Tumour Necrosis Factor-α (TNF-α), mainly produced by activated macrophages and T lymphocytes, has been described to be one of the main cytokines involved in the disruption of epithelial barrier function in intestinal inflammatory processes [31]. TNF-α regulates barrier function indirectly by the generation of oxidative stress and directly via an increase in myosin light chain kinase (MLCK) expression and activity [32,33] and TJ remodelling [34]. In this regard, MLCK activation triggers different cellular contractile events including a) occludin endocytosis to the cytosol leading to the disorganization of the TJ, and the b) contraction of the perijunctional actomyosin which generates mechanical tension at the TJ and induces its opening [35]. In fact, all these effects of TNF-α on TJ structure and permeability are prevented by genetic or pharmacological MLCK inhibition, as is diarrhea [32]. This highlights the close link between changes in TJ, epithelial barrier disruption and water loss.

The production of reactive oxygen and nitrogen species (ROS and RNS) has gained relevance in recent years in the context of the regulation of TJ permeability. In Caco-2 cells, H_2O_2 has been reported to induce the dissociation of the complex formed by occludin and ZO-1 and the separation of this complex from the cytoskeleton via a Tyr kinase-dependent mechanism [36]. This effect is accompanied by the disorganization of actin and the redistribution of occludin and ZO-1 from the TJ to the cytosol [37]. In this sense, the increased production of ROS has also been reported to play an important role in a number of intestinal disorders including inflammatory bowel disease [38]. Moreover, a significant body of evidence suggests that oxidative stress disrupts epithelial barrier function [39]. Indeed, the protective role of many substrates with antioxidant properties (such as taurine, quercetin and epithelial growth factors) has been proven as useful in TJ sealing [40-42].

3. Evaluation of epithelial barrier function

Experimentally, TJ barrier integrity and permeability in intestinal tissues and cells are evaluated by measurement of transepithelial electrical resistance (TER) and the paracellular passage of water soluble molecules of different molecular weight, such as mannitol, dextran, and inulin. The use of cultures of intestinal epithelial cells (Caco-2, IEC-6, HT-29, T-84, etc.) is frequently used as a reductionist experimental model to evaluate epithelial barrier function. The cells are allowed to grown on to semipermeable filters to create an apical and a basolateral compartment to measure transepithelial fluxes of paracellular markers and TER [43-45]. The tightness of the monolayer is indicated by high TER values and low permeability to paracellular markers. TER is considered an indicator of ionic fluxes across the epithelium or cell

monolayer (mainly Na^+ and Cl^-). In addition to charge selectivity, TJ differ in size selectivity. In this sense, it is described a pore pathway that is permeable to molecules with radii of 4 Å or less and a second pathway, which is refered to as the leak pathway, for flux of larger noncharged solutes [27]. The small ions do not discriminate between pore and leak pathways, and therefore TER cannot be used to investigate the selectivity of TJ size or charge. Increased permeability of both pathways reduces TER values. The permeability of the leak pathway is then evaluated from transepithelial fluxes of paracellular markers. In cells in culture the more commonly used tracers include D-mannitol, sucrose, inulin, PEG or dextrans of different molecular weights [27].

The Caco-2 cell system is an efficient model to study the changes in epithelial barrier function induced by several infectious microorganisms that are also involved in gastrointestinal disorders, such as rotavirus, *Escherichia coli*, *Vibrio parahaemolyticus* and *Salmonella* [46-49]. Moreover, the role of the toll-like receptor 2 pathway, which plays a key role in microbial recognition and immune modulation in the regulation of TJ permeability, has also been described in this experimental model [50]. Similarly, the protective effect of probiotics, such as *Lactobacillus rhamnosus* and *Lactobacillus casei*, against the effect of pro-inflammatory cytokines has also been demonstrated in Caco-2 cells [51,52]. In this sense, intestinal cells in culture, mainly Caco-2 cells, can also be co-cultured with different immune cells to also consider the mechanisms of cellular interaction in the regulation of TJ permeability [53].

4. Intestinal epithelial barrier function disruption by *Salmonella*: Nutritional strategies to prevent this effect

The invasion of *Salmonella* Enteritidis into the intestinal epithelium triggers diverse transduction signals at the subepithelial compartment which induce the activation of immune cells and therefore the onset of the inflammatory process [14]. The secretion of proinflammatory cytokines disrupts epithelial barrier function which in turn contributes to water loss and bacterial translocation, perpetuating the inflammatory process and initiating systemic invasion [35]. In this sense, the infection of cultures of human intestinal Caco-2 cells with different strains of *Salmonella* confirms the ability of these bacteria to increase PP [48]. This is exemplified through a decrease in TER, increase in tracer permeability and TJ protein alterations when assessed in a variety of cell lines including MDCK, Caco-2 and T84 cells [54-56]. In this regard, it has been concluded that *Salmonella* causes a decrease in both ZO-1 expression and in the amount of phosphorylated

occludin in the TJ by 2 h of T84 cell infections [56]. Studies aimed at elucidating the specific bacterial proteins involved in the documented *Salmonella*-induced TJ alterations have primarily used mutated strains of *Salmonella* to identify those that do not alter TER, ZO-1 and occludin localization. Through these experiments, the SPI1 effectors; SopB, SopE, SopE2 and SipA have all been implicated in TJ alterations [57]. Moreover, since *Salmonella* infection induces an increase in TNF-α production [58,59], the effect of infection on PP can be, almost in part, attributed to this cytokine. This suggests that synergistic and potentially redundant mechanisms are in place to ensure TJ are modified as part of the disease imparted by *Salmonella*. Since 2006, the use of antibiotics as chicken growth promoters has been baned (January, 1, 2006; Regulation 1831/2003 of the European Parliament and of the Council). This prohibition arises from the increase of antibiotic resistant bacteria in animal farms and represents a health hazard after resistance transfer to human pathogenic bacteria [3]. For this reason, extensive research has been conducted on the development of naturally occurring antimicrobials as alternatives to antibiotics. In this sense, probiotic and prebiotic feed additives are promising alternatives because they control intestinal microbiota, reducing pathogenic bacteria colonization and enhancing mucosal immune system [60].

Probiotics are generally referred to as any live microbial feed supplements that benefit the host animals by largely improving intestinal microbial balance [61]. Intestinal microorganisms that are recognized as possessing probiotic properties include mainly *Lactobacilli* and *Bifidobacteria spp*. They exhibit identifiable beneficial effects for the respective host via promotion of gut maturation and integrity, antagonism against pathogens such as *Salmonella* and immune modulation [62]. The effects of probiotics in poultry also include maintaining normal intestinal microflora by competitive exclusion, increasing metabolism, decreasing enzymatic activity and ammonia production, as well as an increase in feed intake and the neutralization of digestive enterotoxins [63]. Therefore, the overall goal of probiotics intervention is to promote the general growth of healthy microorganisms that are competitive with or antagonistic to enteropathogens [61].

Prebiotics can be defined as non-digestible carbohydrate fractions fed in diets that are beneficial to the host by stimulating the growth of one or more bacteria in the gastrointestinal tract [64]. Prebiotics are predominantly a constituent of plant cell walls and also consist of non-starch polysaccharides along with non-carbohydrate compounds including lignin, protein, fatty acid, and wax [65]. Dietary β-galactomannans are non digestible complexes used as prebiotics due to their ability to confer

favourable conditions to intestinal beneficial *Lactobacillus*. Taking into account that *Salmonella* express mannose-specific lectins involved in the adherence of these bacteria to the intestinal epithelium [66], β-galactomannans show competition thus preventing *Samonella* colonization. The main source of β-galactomannans is the cell walls of *Saccharomyces cerevisiae* which contain 45% of mannose residues [67]. However, new sources are being investigated. In this sense, IRTA, the Research and Technology Food and Agriculture Institute from the *Generalitat de Catalunya*, has developed a food additive extract from the carob bean of the *Ceratonia silliqua* tree (Salmosan®) which approximately contains 88% of β-galactomannans [68]. We have reported, in cultures of intestinal pig cells (IPI-2I), the capacity of Salmosan® to inhibit *Salmonella* Typhimurium epithelial attachment and to reduce up to 70% *Salmonella*-induced mRNA expression of proinflammatory cytokines such as TNF-α, GM-CSF (Granulocyte/Macrophage Colony-Stimulating Factor) and chemokine CCL20 [59]. These results are very similar to the obtained for *Sacharomyces cerevisiae* used as a positive control. Moreover, scanning electron microscopy images obtained in our laboratory confirm that the capacity of Salmosan® to reduce *Salmonella* adhesion in these experimental conditions is due to bacterial attachment to this prebiotic [59] (Fig. 4).

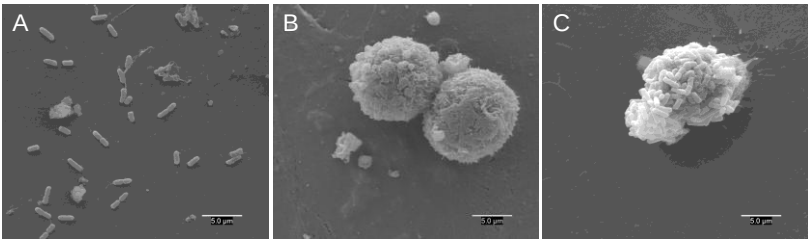

Figure 4. Interaction of *Salmonella* Typhimurium with Salmosan® on the surface of IPI-2I cells assessed by scanning electron microscopy. Images show *Salmonella* attachment on epithelial cells (A), Salmosan® over the control cells (B), and *Salmonella* interaction with Salmosan® (C).

5. Conclusion

Foodborne pathogens such as *Salmonella* possess the capability to survive in external environments during transmission from one host to the next. The determination of microbial genetics and physiology associated with

these mechanisms could have great potential for better control of pathogen colonization. In this sense, although advances have led to an overall greater understanding, the detailed mechanisms that these microorganisms employ to modify TJ remain elusive. For this reason, a better understanding of these indicators could assist in designing more novel approaches to minimize the spread of *Salmonella* in the food animal industry and decrease the consequences to human health.

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