Article 1: *Review on the effects of potential prebiotics on controlling intestinal enteropathogens Salmonella and E. coli in pig production*

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Running head: prebiotic in *Salmonella* and *E. coli* in pigs

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1. Summary

Salmonella enterica serotypes (*Salmonella* sp.) are the second cause of bacterial foodborne zoonoses in humans after campylobacteriosis. Pork is the third most important cause for outbreak-associated salmonellosis, and colibacillosis is the most important disease in piglets and swine. Attachment to host cells, translocation of effector proteins into host cells, invasion and replication in tissues are the vital virulence steps of these pathogens that help them to thrive in the intestinal environment and invade tissues. Feed contamination is an important source for *Salmonella* infection in pig production. Many on-farm feeding strategies intervene to avoid the introduction of pathogens onto the farm by contaminated feeds or to reduce infection pressure when pathogens are present. Among the latter, prebiotics could be effective at protecting against these enteric bacterial pathogens. Nowadays, a wide range of molecules can potentially serve as prebiotics. Here, we summarize the prevalence of *Salmonella* sp. and *Escherichia coli in pigs*, understanding of the mechanisms by which pathogens can cause disease, the feed related to pathogen contamination in pigs and detail the mechanisms on which prebiotics are likely to act in order to fulfil their protective action against these pathogens in pig production. Many different mechanisms involve the inhibition of *Salmonella* and *E. coli* by prebiotics such as coating the host surface, modulation of intestinal ecology, downregulating the expression of adhesin factors or virulence genes, reinforcing the host immune system.

Keywords: *Salmonella enterica*, *Escherichia coli* (*E. coli*), prebiotics, pigs

Abbreviations: S, Salmonella; *E. coli*, *Escherichia coli*; ESBL, Extended-Spectrum Beta-Lactamase; GIT, gastrointestinal tract; ETEC, enterotoxigenic *E. coli*; STEC, Shiga toxin-producing E. coli; VTEC, verotoxigenic *E. coli*; EHEC, enterohemorrhagic E. coli; EPEC, enteropathogenic E. coli; EIEC, enteroinvasive E. coli; EAEC, enteroaggregative E. coli; DAEC, diffusely adherent E. coli; AIEC, adherent invasive E. coli; T3SS, syringe-like type-III secretion system

2. Introduction

Zoonotic diseases can be naturally transmitted directly or indirectly between animals and humans, for example through the consumption of contaminated food or through contact with infected animals. The main pathogenic bacteria causing zoonoses in the European Union (EU) are the Gram-negative *Campylobacter* spp., *Salmonella* spp., some strain of *Escherichia coli*

(*E. coli*), *Listeria* spp., and the Gram-positive *Mycobacterium tuberculosis*. *Salmonella enterica* and *E. coli* are Gram-negative rod-shaped non-spore-forming bacteria belonging to the *Enterobacteriaceae* family. *Salmonella enterica* are hosted in the gut of most homoeothermic animals and include various serovars whose pathogenicity can differ widely. *Escherichia coli* is a common intestinal bacterium in humans and animals. Most *E. coli* strains are harmless commensals of the intestinal microbiome, but some serotypes are pathogenic, causing severe intestinal infections (Bhunia, 2008; Kalita et al., 2014).

With 25.8% (88 715 cases in 2013) of all recorded outbreaks, salmonellosis is the second most common zoonosis in humans after campylobacteriosis. Epidemiological studies in the EU in 2013 confirmed that after poultry, sweets and chocolate, pork, with 8.9%, is the third most important cause for outbreak-associated salmonellosis in humans (EFSA, 2015a). the *Salmonella* prevalence in fresh pig meat ranges from 0.7% to 26% (Lin et al., 2014; Ashraf et al., 2015; EFSA, 2015a). While outbreaks caused by pathogenic *E. coli* strains are less frequent although significant (1.7% by verotoxigenic *E. coli*, VTEC) (EFSA, 2015b) with 0–74% of contaminated pig meat (Nørrung and Buncic, 2008; Ashraf et al., 2015; EFSA, 2015a). Nonetheless, post-weaning diarrhoea (PWD) caused by enterotoxigenic *E. coli* (ETEC) is an important cause of economic losses in pigsties due to high morbidity, mortality and reduced growth rates (Luppi et al., 2016).

Control of these pathogens can be implemented at the pre-harvest level (on farm), at harvest level (during transport and slaughter) and at post-harvest level (processing and retailing). Control programmes at farm level are most essential to limit the risks of pathogenic infections into the food chain. Preventing pathogens from entering through the feed is of major significance for the reduction in pathogens in pigs. Different intervention strategies such as using pathogen-free or vaccinated incoming pigs (Andres and Davies, 2015), preventing infection from environmental contamination (Barco et al., 2014; Petruzzelli et al., 2015), antimicrobial medication (Nesterenko et al., 2016), and nutritional supplements have been assessed to reduce pathogenic prevalence in pigsties. Among the latter, non-digestible carbohydrates (NDCs), also known as prebiotics, can be effective by restoring or improving the resistance to colonization, reinforcing the intestinal barrier function against invading pathogens (Bindels et al., 2015a).

This study reviews two major intestinal pathogens in swine: *Salmonella* and *E. coli* infections. After a description of the prevalence of these pathogens, their pathogenicity and the influence of characteristics of feed on contamination in pigs, the mechanisms and effects of some potential prebiotics against these pathogens in pigs are described and analysed.

3. *Salmonella enterica* and *Escherichia coli* prevalence in pigs and pigsties

Salmonella enterica and *E. coli* both pose several human and health concerns worldwide, but also in the EU (Table 2.1). *Salmonella* Typhimurium was the predominant *S. enterica* serotype found in pigs (54.7% of all *Salmonella* isolates recovered), pig meat (27.8%) and compound pig feed (14.3%) in the EU for the last five years, followed by *Salmonella* Derby (17.5%, 24.4% and 0% respectively) (EFSA, 2015b). There are seven major diarrhoeagenic *E. coli* pathotypes in human and mammalian intestines: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC) such as enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), adherent invasive *E. coli* (AIEC) (Croxen et al., 2013). Among them, STEC strains, especially serotype O157, are most prevalent in healthy pigs (Table 2.1). In addition, a high number of Extended-Spectrum Beta-Lactamase (ESBL) bacteria that are resistant to beta-lactam antimicrobials have been recovered from pig farms (45–79% of tested farms) (Hammerum et al., 2014; Dohmen et al., 2015; Fischer et al., 2016). Therefore, pigs are now regarded as potential 'producers' of ESBL-bacteria, especially ESBL-*E. coli*.

E. coli pathogens are found only in the gastrointestinal tract (Andersen et al., 2015). On the contrary, *Salmonella* spp. can be found in feces (4.9% of 934 slaughter pigs) and distal colonic content (3.9% of 937 pigs) (Bahnson et al., 2006) of weaner and finisher pigs, but also in ileocolic lymph nodes with a prevalence of 12.5 to 25%, in cecal content (17.4%) or in the gall bladder (Burns et al., 2014). This suggests that the gut and its associated contents is a major source of *Salmonella* contamination of pork at slaughter (Bahnson et al., 2006; Li et al., 2016). Therefore, pre-slaughter feed withdrawal has been used to reduce carcass contamination by digestive tract rupturing or spilling with intestinal chyme and feces (Berge and Wierup, 2012).

Table 2.1. Prevalence of *Salmonella* spp. and *E. coli* in pork production

Country & Period	Species	Prevalence (%)	Sample and sampling stage	Reference
EU 2014	*Salmonella*	0.5	Fresh meat at slaughterhouse (68 134)	(EFSA, 2015b)
		0.7	Retail ready-to-eat meat (20 259)	
		10.1	Herd level (4243)	
		7.7	Individual pig level (47 612)	
Ireland 2012–2013	*Salmonella*	90 (9)	Farm level (10)	(Burns et al., 2014)
		14.9	Individual pig at farm (926)	
		7.9	Environmental sample (1474)	
Egypt	*Salmonella*	25.0 (20)	Retail meat sample (80)	(Ashraf et al., 2015)
Taiwan, 2004–2010	*Salmonella*	4.1	Sample at slaughterhouse (649 500)	(Wang et al., 2012)
Denmark 2010–2011	ESBL-*Salmonella*	79	Farm level (19)	(Hammerum et al., 2014)
Nigeria 2013	ESBL-*Salmonella*	5.8 (11)	Individual pig at farm (190)	(Ugwu et al., 2015)
US, 2008	*Salmonella*	10.4 (462)	Individual pig at farm (4426)	(Abley et al., 2013)
2006–2007	*Salmonella*	7.2 (564)	Individual pig at farm (7788)	(Haley et al., 2012)
		52.6 (71)	Farm level (135)	
China 2012–2013	*Salmonella*	41.4 (72)	Individual pig at slaughterhouse (169)	(Li et al., 2016)
		26 (53)	Retail meat samples (204)	(Lin et al., 2014)
EU 2012–2014	VTEC	0.7	Fresh meat at slaughterhouse (274)	(EFSA, 2015a; EFSA, 2015b)
		1.2	All type of meat (841)	
		16.0	Herd level (187)	
		13.5	Individual pig (340)	
Umbria & Italy 2012–2014	STEC	2.8 (19)	All type of meat (675)	(Ercoli et al., 2016)
2013–2014		38.6 (81)	Individual pig at slaughterhouse (210)	
South Africa	Total *E. coli*	35.8 (179)	Individual pig at farms (500)	(Iwu et al., 2016)

Country & Period	Species	Prevalence (%)	Sample and sampling stage	Reference
2014	STEC	18.4 (92)		
Egypt	VTEC	73.8 (59)	Retail meat sample (80)	(Ashraf et al., 2015)
India	Total *E. coli*	100 (782)	Individual pig at farm(782)	(Rajkhowa and
2010–2013	STEC	14.4 (113)		Sarma, 2014)
US, 2008	Total *E. coli*	98.6 (833)	Individual pig at farms (845)	(Abley et al., 2013)
2011–2012	STEC	65.3 (98)	Individual pig at farms (150)	(Tseng et al., 2015)
Argentina	STEC	4.1 (31)	Samples from farm, slaughterhouse or	(Colello et al., 2016)
2012–2014			Boning Rooms (764)	
China, 2015	ESBL-	56.7 (34)	Individual pig at farm (60)	(Zhang et al., 2016)
2011–2012	E. coli	25.4 (255)	Samples at farm, slaughterhouse	(Meng et al., 2014)
2013–2014	STEC	4.4 (14)	(1003)	(Bao et al., 2015)
	STEC		Retail raw meats (318)	
Japan	Total *E. coli*	9.1 (3)	Individual pig at farm (33)	(Hiroi et al., 2012)
2007	ESBL-	25 (3)	Farm level (12)	
	E. coli	3 (1)	Individual pig at farm (33)	
		8.3 (1)	Farm level (12)	
Germany,	ESBL-	61 (31)	Farm level (51)	(Fischer et al., 2016)
2014	*E. coli*	28.3 (155)	Individual pig at farm(547)	(Schmithausen et al.,
2012				2015)
Netherlands	ESBL-	45	Farm level (40)	(Dohmen et al.,
2011	*E. coli*	6.6	Individual pig at farm (2388)	2015)
Nigeria, 2013	ESBL-	2.1 (4)	Individual pig at farm (190)	(Ugwu et al., 2015)
	E. coli			

Several studies have examined the prevalence of *Salmonella*, STEC and ESBL-*E. coli* in pigs. Within the EU, prevalence of *Salmonella*-positive individual pigs on the farms ranges from 7.7% to 14.9% (Burns et al., 2014; EFSA, 2015b) while the prevalence of ESBL-*E. coli* varies between 6.6% and 28.3% (Dohmen et al., 2015; Schmithausen et al., 2015), and between 13.5 and 38.6% for STEC and verotoxin-producing *E. coli* (VTEC). In other pig producing countries, such as China, India, the United States (US), South Africa, and Nigeria, the prevalence of *Salmonella* spp. in individual pigs on the farms varies from 10.4% to 41.4% (Abley et al., 2013; Li et al., 2016), between 2.1 and 56.7% for ESBL- *E.coli* (Hiroi et al., 2012; Ugwu et al., 2015; Zhang et al., 2016), and between 15 and 65% for STEC (Rajkhowa and Sarma, 2014; Tseng et al., 2015; Iwu et al., 2016). It should be noted that the highest *Salmonella* prevalence (41%) is measured for pigs at the slaughterhouse where the rate of prevalence is higher than for pigs on the farms. Although the prevalence of these pathogens is highly variable and difficult to compare between studies because different sampling and detection methods are used (EFSA, 2015b), these comparisons highlight the utmost importance of strict sanitary quality controls and practices as those enforced in the EU to reduce the prevalence of these pathogens.

Salmonella observations were reported at different stages: at the farm and/or slaughterhouse. The rate of *Salmonella* positive farm is ranged from 10% to 90% (Haley et al., 2012; Burns et al., 2014; EFSA, 2015b). The high prevalence (90% of tested farms) in the study of (Burns et al., 2014) is due to a specific selection of farms with a history of high *Salmonella* sero-prevalence. The rate of *Salmonella* positive individual animals for slaughter pig (from lymph nodes or cecal content, 41.4%) (Li et al., 2016) were higher than that for pig shedding on the farm (from feces, 5.8-14.9%) (Haley et al., 2012; Abley et al., 2013; Burns et al., 2015). Lower prevalences in feces may be explained by low detection rates in fecal samples and no symptoms of disease (Albino et al., 2014). In addition, another explanation for high *Salmonella* prevalence at the slaughterhouse is *Salmonella* cross-contamination due to the poor *Salmonella* control measures taken during organ withdrawal.

As stated before, *E. coli* are ubiquitous commensals of the pig's gastrointestinal tract (GIT) with a prevalence of 100% (Abley et al., 2013; Rajkhowa and Sarma, 2014). Pathogenic strains are however highly prevalent as well. The rate of STEC/VTEC positive individual animals are 13.5-65.3% (Rajkhowa and Sarma, 2014; EFSA, 2015b; Tseng et al., 2015; Iwu et al., 2016) and 2.1-56.7% for ESBL-*E. coli* (Hiroi et al., 2012; Dohmen et al., 2015; Schmithausen et al., 2015; Ugwu et al., 2015; Zhang et al., 2016). Curiously, Japan has a low on-farm prevalence of ESBL-*E. coli (8.3%)* (Hiroi et al., 2012).

4. Multiplication in the host and pathogenicity

In order to understand how to reduce the burden of the pathogens, it is necessary to review how they can invade the host successfully (Fig. 2.1). Several virulence factors are expressed that allow the pathogens to persist in the host and then cause disease (Zhou et al., 2014; Nesterenko et al., 2016) through the attachment, translocation of effector proteins, and replication and spread of the pathogenic bacteria into the host (Bhunia, 2008).

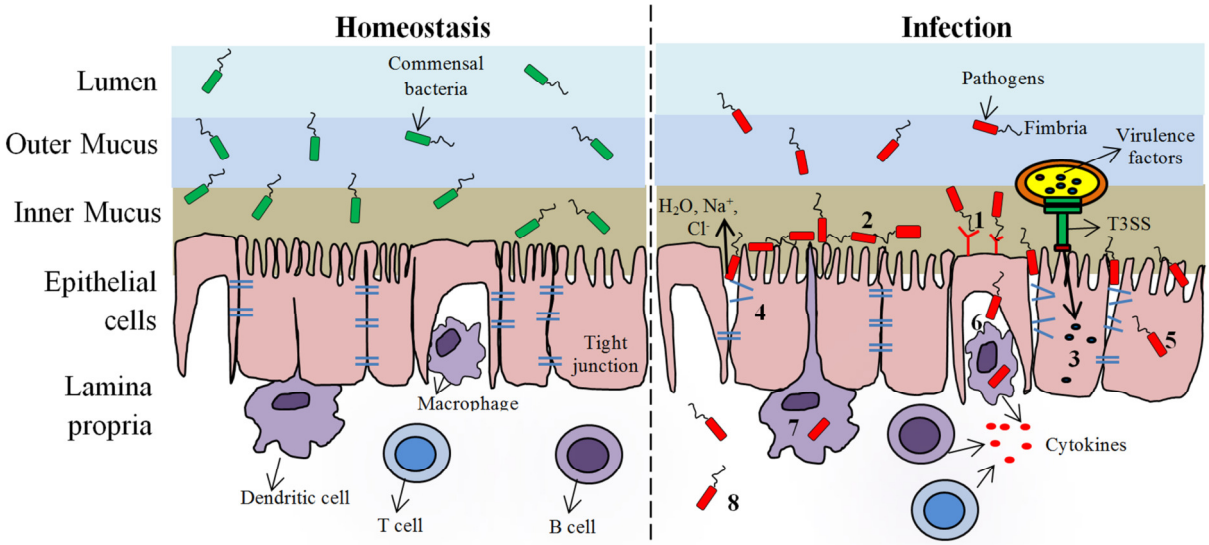

Figure 2.1. Schematic representation of the colonization ways and pathogenicity of *Salmonella* enterica and *Escherichia coli* into animal host

(reproduced from Sansonetti (2004) and Kalita et al. (2014)): bacterial adhesion on apical surface of epithelial cells thank to protein receptors (1); biofilm formation (2); via the type-III secretion system (T3SS), virulence factors of pathogens into the host cells (3), then disruption of tight junctions between intestinal epithelial cells (4); presentation of pathogens in intracellular cells (5), in macrophage (6), in dendritic cell (7) or in lamina propria (8).

4.1.Attachment to host cell surface

Immediately following oral intake, bacteria that survive passage through the acidic stomach environment reach the small intestine in 2 to 3h (EFSA, 2010; Nguyen et al., 2015). There, pathogens must first attach to the intestinal mucosa or intestinal epithelial cell surface to avoid wash-out by mucosal secretion and/or peristalsis (Kalita et al., 2014).

Two mechanisms involve the adherence of these organisms to the intestinal mucosa and epithelium. First, bacterial adhesins such as fimbriae (i.e., aggregative adherence factors of EHEC), pili (i.e., Saf polyadhesins of *Salmonella enterica)*, or surface antigens (i.e., coli surface antigen of ETEC) interact with their receptor on host cell (Guevara et al., 2013; Zhou

et al., 2013; Berry et al., 2014). *Salmonella* spp. and *E. coli* use a syringe-like type-III secretion system (T3SS, virulence central) to sense the presence of the host cell receptor (Fig. 2.1). Indeed, the adhesion factors of pathogens may be recognized by extracellular matrix proteins of the host located at the surface of the target cells (Farfan et al., 2011; Berry et al., 2014), e.g., pili of *Salmonella* interact with neuraminic acid-containing proteins of the host (Sakarya et al., 2010), pili and fimbriae of AIEC interact with carcinoembryonic antigen (Barnich et al., 2007), or long polar fimbriae of EHEC recognize fibronectin, laminin, and collagen of the host (Farfan et al., 2011). The effacement of enterocyte microvilli and cytoskeletal changes induce ultrastructural lesions in host cells (Nougayrède and Donnenberg, 2004) and a decrease in absorptive surfaces, thereby contributing to a loss in growth performances and diarrhea (Croxen et al., 2013). Secondly, pathogens translocate the bacterial adhesin and their receptor via T3SS in host cells which helps them in the initial attachment. These bacterial subunits are encoded by several mobile genetic elements transferred within a plasmid, chromosome or phage (e.g. pathogenicity islands 1 (SPI-1) of *Salmonella* spp. (Marcus et al., 2000; Knodler et al., 2014; Nesterenko et al., 2016) or the locus of enterocyte effacement (LEE) of EPEC/EHEC (Elliott et al., 2000; Mills et al., 2008)). For example, EPEC translocate the intimin and intimin receptor (Tir, also called EspE) into plasma membrane cells (Frankel et al., 2001). These virulence factors provoke an important mucosal inflammatory response that are associated with the secretion of inflammatory mediators such as interleukins (IL) (Gewirtz et al., 2000). Among those, the pro-inflammatory chemokine IL-8 is responsible for recruiting neutrophils to the epithelial mucosa without mucosal injury, and facilitates intestinal fluid secretion (Kucharzik et al., 2005).

Moreover, biofilm formation on the surface of host's enterocytes is also another important adherence property of these pathogens. Pathogens may aggregate and recruit surrounding cells to form bacterial biofilms associated with the epithelium (P. Stoodley et al., 2002), especially in EAEC and EPEC strains (Kaper et al., 2004). These biofilms are multicellular structures held together by several factors such as fimbriae, pilus, curli, flagella, exopolysaccharide (Danese et al., 2000; Zogaj et al., 2001). Bacteria in biofilms adopt a starved state due to the undernutrition and waste accumulation. This change in physiological state increase their resistance to antimicrobial medication (Stewart and Costerton, 2001) and host innate immune responses. In addition, pathogenic cells can detach from mature biofilms and spread to other organs (P. Stoodley et al., 2002).

4.2.Translocation of effector proteins into host cells

Once established on intestinal surfaces, *Salmonella* spp. and *E. coli* pathogens translocate bacterial effector proteins through T3SS to the extracellular space or the cytosol of target cells (Negrate et al., 2008). These effectors will help them to fight back the immune response of the pig to survive in the intestinal environment or invade tissues by modulating multiple signaling pathways linked to the tight junction proteins and the inflammatory response to finally induce cell lysis through disruption of the tight junctions, weakening of the host response and loss of intestinal homeostasis.

Disruption of intestinal epithelial tight junction (TJ)*:* EPEC and EHEC strains (A/E pathogens *E.coli*) produce *E. coli* secreted protein F (EspF, encoded by LEE) (Mills et al., 2008) which can redistribute TJ proteins such as occludin and claudin from the villous membrane to the cytoplasm in colon epithelial cells (Zhang et al., 2010, 2012). This disruption leads to a loss of trans-epithelial electrical resistance (TER) (Zihler et al., 2011; Badia et al., 2013; Knetter et al., 2015) and an increased paracellular intestinal permeability that cause local and systemic infections including gastroenteritis, bacteremia, endovascular infections or cell inflammation (Croxen et al., 2013; Bao et al., 2015).

Weak host inflammatory response*: Salmonella* secreted factor L (SseL, encoded in SPI-2) and NleB (encoded by LEE) of A/E pathogens *E. coli* inhibit the nuclear factor kappa B (NF-κB) activation in infected macrophages (Negrate et al., 2008; Gao et al., 2015). NleB inhibits the NF-κB activation by disruption of interaction between the receptor of tumor necrosis factor associated factor 2 (TRAF2) and glycolysis enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH)(Gao et al., 2013). On the other hand, SseL produced by *S*. Typhimurium suppress NF-κB activity through degradation and ubiquitination of inhibitory protein kappa B alpha (IκBα) (Negrate et al., 2008). The host innate immune responses and cellular processes such as proliferation and differentiation are thereby negatively modulated (Negrate et al., 2008; Rahman and McFadden, 2011; Gao et al., 2015).

Imbalance in intestinal homeostasis, cell lysis*:* The heat-labile (LT, encoded in plasmids) and heat-stable (ST, encoded in transposons) enterotoxin produced by ETEC strains cause an imbalance in intestinal homeostasis by stimulating the hypersecretion of water and intracellular ion balance with an increase in Ca^{2+} concentration (Croxen et al., 2013). Moreover, LT enterotoxin can also alter the continuity and composition of the intestinal epithelial mucin layer, and then exacerbates *Salmonella* Typhimurium infections (Verbrugghe et al., 2015). Shiga toxin (Stx) of EHEC and the cytotoxins of EAEC can also release the host cell iron into the extracellular environment that can then be captured by the bacterium. The

disappearance of calcium ion gradients increases the intestinal intracellular osmotic pressure resulting ultimetaly in cell lysis (Jacobsen et al., 2008). This local effect results in watery or bloody diarrhea, especially in piglets because of their small size making them particularly vulnerable to severe and rapid dehydration (Toledo et al., 2012; Guerra Ordaz, 2013). Post mortem examination showed that piglets that died from neonatal colibacillosis have often a small intestine full with yellowish watery content and a stomach full with clotted milk. In the worst cases, toxins finally enter the blood stream (Bhunia, 2008).

4.3.Pathogen invasion and replication in the host

As *Salmonella* spp. predominantly colonize cell surfaces, mucus, basal membranes of intestinal mucosa, they cause a mucosal inflammation that provides a localized source of high-energy nutrients (i.e., galactose-containing glyco-conjugates, mucin). Then *Salmonella* can efficiently access these nutrients for their fast replication (Stecher et al., 2008). Thereafter, pathogens invade and replicate within the host cells. Almost all *Salmonella* species are able to survive, proliferate in natural phagocytic cells such as dendritic cells, M cells, monocytes/macrophages and neutrophils (Österberg, 2010). This process is encoded by *Salmonella* pathogenicity islands 2 (SPI-2) (Knodler et al., 2014). The effector proteins produced via its T3SS allow bacteria to modify the vacuole of target cells to a *Salmonella* containing vacuole in order to evade lysosomal dergadation, which supports bacterial survival and multiplication (Sansonetti, 2004; Eswarappa et al., 2010). The *Salmonella* bacteria use a range of chemical nutrients inside the host cell such as lipids, carbohydrates, amino acids, nucleosides, and various pro-vitamins for their growth (Steeb et al., 2013). The ability of *Salmonella* to persist in the tissues can be speculated to be even more important for the virulence than the ability to invade extra-intestinal tissues such as phagocytes and leucocytes (Österberg, 2010). *Salmonella* can rapidly invade the lamina propria (enterocytes) (Österberg, 2010). They can spread throughout the body into gut associated lymphoid tissues such as tonsils as quickly as 30 minutes after oral infection, then jejunal and ileocecal lymph nodes (Hurd et al., 2001). *Salmonella* have also the ability to invade non-phagocytic cells such as mono-macrophages. They can cause an acute inflammatory stimulus: increased cytokines blood concentration and body temperature at 4 h post-infection so that fever and neutrophil influx are considered as hallmarks of *Salmonella* Typhimurium infection (EFSA, 2010; Chirullo et al., 2015; Knetter et al., 2015). However, even in the case of successful colonization, these symptoms are not always observed (Pieper et al., 2012b).

In contrast, most *E. coli* pathogens remain extracellular. The intracellular AIEC is the only intestinal pathogenic *E. coli* strain that can invade and proliferate within host cells (Kaper et al., 2004). The extracellular *E. coli* can replicate outside the cells, i.e. in the interstitial space, in the lumen of the respiratory tract, and, obviously, in the intestinal tract from the mid jejunum to the ileum (Guerra Ordaz, 2013). *E. coli* strain use the monosaccharides released from epithelial cells or mucin (i.e., gluconate, mannose, fucose, ribose), other mucosal glycoproteins, and amino acids for their growth in the intestinal lumen (Chang et al., 2004; Conway and Cohen, 2015). Infections of pathogenic *E. coli* strains can cause hemorrhagic gastroenteritis, congestion, and microvascular fibrinous thrombi and villous necrosis into the intestinal lumen (Guerra Ordaz, 2013) or dysentery, septicemia, pneumonia, and meningitis (Bhunia, 2008).

5. Influence of characteristics of feed on pathogen contamination in pig

The feed can potentially be an important vector to introduce pathogens, especially *Salmonella*, onto the farm. Hence, strategies to reduce the load of pathogens on the farms can target this feed contamination as reviewed by (Berge and Wierup, 2012; Canibe and Jensen, 2012; Missotten et al., 2015). Moreover, the feed composition can also influence the inhost proliferation and transmission between pigs of *Salmonella* and pathogenic *E. coli* strains.

Physical properties and chemical composition of the feed can influence the susceptibility of pigs to *Salmonella* and *E. coli* infection (Funk and Gebreyes, 2004). They influence not only the passage and absorption of nutrients in the GIT, but also the risk of colonization and shedding in the pigs once infected (Berge and Wierup, 2012).

Although feeding a coarse meal to pigs results in lower growth performances, they protect animals against colonization better than pelleted feed (Lo Fo Wong et al., 2004; Mikkelsen et al., 2004; Wilhelm et al., 2012). Coarsely ground feed meals change the physicochemical conditions in the stomach with higher concentration of organic acids and lower pH that promote the growth of anaerobic lactic acid bacteria and decrease the survival of *Salmonella* and *E. coli* during passage through the stomach (Mikkelsen et al., 2004). Moreover, larger feed particle are not digested as extensively as small feed particles. They enter the large intestine where they are fermented to produce short-chain fatty acids (SCFAs) which have beneficial effect on gut health leading to inhibition of pathogen infection (Lo Fo Wong et al., 2004; Wilhelm et al., 2012; Lebel et al., 2016).

(Bahnson et al., 2006) observed that pigs from herds with only dry feed (80.4% of 51 farms) had higher level of *Salmonella* infection compared to those fed mixtures of dry feed

and water. In addition, feeding fermented by-products (5.8% of 42 herds) was associated with a lower *Salmonella* seroprevalence than feeding dry compound feed with water (22.7% of 313 herds). In addition, pigs fed fermented by-products had a lowers counts of *E. coli* and total coliforms compared to normal liquid diet with water (Hong et al., 2009) with improvement of pig gut health (Sugiharto et al., 2015). The protective effect of wet feed is ascribed to its chemical composition, the high concentrations of organic acids and the large numbers of lactic acid bacteria (van der Wolf et al., 2001) as reviewed by (Canibe and Jensen, 2012; Missotten et al., 2015).

Besides organic acids, the provision of high amounts of fibre and a low concentration of high-quality proteins in the diets may reduce the pathogen loads in the feed and the risk for intestinal disease in pigs. For example, low protein diets can reduce the growth of ETEC and then the incidence of PWD in piglets (Heo et al., 2008; Heo et al., 2009; Opapeju et al., 2009; Heo et al., 2010; Kim et al., 2011; Heo et al., 2015). Indeed, diets made of poorly digestible proteins result in higher levels of undigested dietary proteins reaching the distal parts of the GIT. The inclusion of some fibre such as cellulose, lignin, arabinoxylans or pectin into pig diets can increase mucus production and then increase the flow of undigested endogenous proteins to the large intestine (Jha and Berrocoso, 2016). Undigested proteins are fermented into harmful metabolites (BCFAs, NH_3…) (Heo et al., 2008; Heo et al., 2009) by proteolytic bacteria such as Firmicutes, Proteobacteria and Bacteroidetes. In turn, these putrefactive compounds can irritate the colonic epithelium, compromise the intestinal barrier function, and the absorption capacity of electrolytes and fluids. Thereby, as explained earlier, it may selectively favour the growth of ETEC and then the incidence of post-weaning diarrhoea (PWD) in piglets (Heo et al., 2008; Heo et al., 2009; Opapeju et al., 2009; Heo et al., 2010; Kim et al., 2011; Heo et al., 2015). Understanding the factors influencing intestinal bacterial protein fermentation, the formation of toxic metabolites and subsequent influence on the host to maintain GIT health is well reviewed by (Paeschke and Aimutis, 2011; Jha and Berrocoso, 2016; Pieper et al., 2016).

As mentioned above, the inclusion of some carbohydrate molecules can increase amount of proteins in the large intestine of pigs. Some carbohydrates can also become as ‘anchors’ for pathogens (Kato and Ishiwa, 2015) because they can use these substrates for their growth (Martín-Peláez et al., 2008; Petersen et al., 2009) (Table 2.2). For this reason, *in vitro* investigation with in co-culture fermenter with complex faecal microbiota showed that there was no inhibition of FOS, XOS, gentiooligosaccharides (GEO), mixture of FOS/inulin, lactulose (Martín-Peláez et al., 2008), corn, sugar beet, wheat (Martín-Peláez et al., 2009),

barley and oat fibre residues after pepsin and pancreatin hydrolysis (Pieper et al., 2009a) on the growth of *S.* Typhimurium, compared to a control (without no added carbohydrate) (Table 2.2). Regarding *E. coli*, when FOS or a mixture of FOS/XOS were added to a batch fermenter, any decrease in EHEC was observed (Fooks and Gibson, 2003).

In contrast, pathogens such as *Salmonella spp. are* not able to metabolize some carbohydrates such as orange peel, orange pulp (Callaway et al., 2008), *apple pectin, xylo-oligosaccharides (XOS), inulin, or polydextrose,* or lactulose (Martín-Peláez et al., 2008). *This might explain the in vivo results displayer in* Table 2.2 *showing that the presence of fructo-oligosaccharides (FOS) in the drinking water reduced the fecal excretion of S. Typhimurium in swine* (Letellier et al., 2000). *In another example, t*he addition of ß-galactomannan-oligosaccharides (ß-GMO) to the diet was associated with a reduction in *Salmonella* spp. prevalence, shedding and seroconversion in fattening pigs (Andrés-Barranco et al., 2015). On the other hand, fermentable fiber can shift bacterial metabolism from proteins toward carbohydrates as the main energy source, and then reduce harmful protein-derived metabolites from the protein feed. Indeed, proteolytic activity in the intestine decreases when the availability of indigestible carbohydrate sources increases (Pieper et al., 2012c). More interestingly, carbohydrates can also be involved in mechanism of the host's defense against pathogenic infections. The mode of action of carbohydrate diet, especially carbohydrates having prebiotic properties, on *Salmonella* and *E. coli* infection in pig animals will be highlighted in the following section.

6. Potential mechanisms of action of prebiotics on the pathogen infections in pigs

Prebiotics are defined as non-digestible carbohydrates (NDCs) including oligosaccharides, resistant starch, and non-starch polysaccharides that are resistant to hydrolysis by digestive secretions. In species without fore-stomach, including humans and pigs, these NDCs resist digestion in the upper gastro-intestinal tract (GIT) and reach the ileum and the colon where they usually undergo fermentation by resident microbes. Currently, prebiotics are more broadly defined as any type of food ingredient that has a favorable direct and/or indirect impact on the beneficial GIT microbiota and the intestinal homeostasis (Hutkins et al., 2016) and consequently inhibit pathogenic infections.

Table 2.2. Effects of some prebiotics on *Salmonella* and *Escherichia coli* in pigs

Prebiotic	Pathogens	Experimental object	Observations	Reference
β –galactomannan MOS, Manose	ST ETEC	*In vitro* cell-culture	↘ *Salmonella* adhesion on porcine ileum intestinal epithelial cells ↘ expression of proinflammatory mRNA of pathogen, ↘ secretion of proinflammatory cytokine IL6 and chemokine CXCL8	(Badia et al., 2013) (Badia et al., 2012a) (Badia et al., 2012b)
Wheat bran, Casein-glycomacropeptide, Locust bean, EPS	ETEC	*In vitro* mucus or cell-culture	↘ number of ETEC on porcine intestinal mucus or intestinal epithelial cell-line IPEC-J2	(González-Ortiz et al., 2014) (González-Ortiz et al., 2013: 2)
Reuteran EPS from *L.* reuteri	ETEC	*In vitro* porcine jejunal segment perfusion model	↘ adhesion of ETEC	(Chen et al., 2014)
Soluble non-starch polysaccharide from plantain bananas	*ST* ETEC	*In vitro* cell culture	↘ adhesion of pathogen to Caco-2 cells, block bacterial translocation into M-cells	(Roberts et al., 2013)
Lactulose	ETEC	Weaning piglets	↗ *Lactobacillus* counts and colonic butyrate ↗ ileum villous height No reduction of ETEC	(Guerra-Ordaz et al., 2014)
Carob seed	ETEC	Weaning piglets	↘ adhesion of ETEC in ileal mucus	(Guerra Ordaz, 2013)
Chito-oligosaccharide	EPEC	*In vitro* cell-culture	↘ adhesion of pathogens on surface of a human HEp-2 cell line	(Quintero-Villegas et al., 2013)
GOS, Inulin, lactulose, raffinose, galactose, FOS	ETEC	*In vitro* cell culture	↘ adhesion of ETEC to Caco-2 and Hep-2 cells	(Shoaf et al., 2006)
Lactulose	*ST*	*In vitro* pure culture	↘*Salmonella* numbers	(Martín-Peláez et al., 2008)
Inulin, dextran, Levan EPS	ETEC	*In vitro* porcine	No anti-adhesive effect	(Chen et al.,

from *L.* reuteri		jejunal segment perfusion model		2014)
Soybean hulls, Sugar beet pulp, Locust gum, FOS, Inulin, Mushroom, MOS	ETEC	*In vitro* cell-culture	No anti-adhesive effect	(González-Ortiz et al., 2013: 2)
FOS	*ST*	Early-weaned piglets	No reduction of *S.* Typhimurium in feces	(Letellier et al., 2000)
β - GMO	*ST*	Fattening pigs	↘*Salmonella* in feces, mesenteric lymph nodes, and in serum	(Andrés-Barranco et al., 2015)
β-glucan hulless barley	*ST*	Weaning piglets	No prevention of *Salmonella* colonization ↘ *Salmonella* persistence	(Pieper et al., 2012b)
FOS, XOS, Lactulose, Oat fibre, GOS, Inulin, Mixture of FOS/inulin, Corn, Sugar beet, Wheat barley	*ST*	*In vitro* co-culture system with intestinal microbiota	No reduction of *Salmonella* numbers Inulin: ↗*Bifidobacteria* growth ↗SCFA production	(Martín-Peláez et al., 2008; Martín-Peláez et al., 2009; Pieper et al., 2009a; Zihler et al., 2011);
FOS+*L. plantarum*, Mixture FOS/XOS+*B. bifidum*	EHEC	*In vitro* batch culture system	↘EHEC numbers	(Fooks and Gibson, 2003)
FOS, Mixture of FOS/XOS	EHEC	*In vitro*	No decrease in EHEC numbers	(Fooks and Gibson, 2003)
MOS	ETEC	Piglets	↗ IgG No decrease in ETEC numbers	(White et al., 2002)

ST: Salmonella Typhimurium; GOS, galacto-oligosaccharides; FOS, fructo-oligosaccharides; EPS, exopolysaccharide; MOS, mannan-oligosaccharides; SCFA, short-chain fatty acids

As shown in Table 2.2, a wide range of molecules is nowadays under scrutiny as they could potentially serve as prebiotics to limit pathogen infections and their consequences on pig performances and transmission to the food chain. However, investigations on the effect of the inclusion of prebiotics in the feed of pigs on pathogens are scarce. Then a deeper understanding of the mechanisms (Fig. 2.2) by which prebiotics potentially act by various ways against *E.coli* and *Salmonella spp is necessary. These mechanisms include an inhibition of adhesion sites, a modulation of the intestinal environment, and a reinforcement of the pig's immunity.*

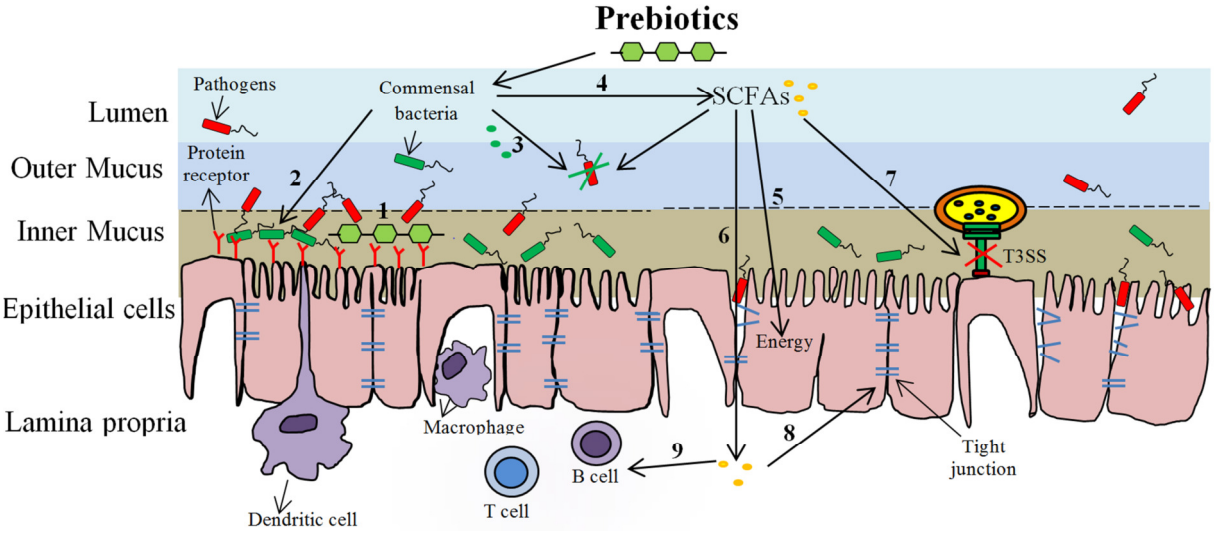

Figure 2.2. Schematic representation of the mechanisms of prebiotics against pathogen infection

coating of the host surface receptors by adhesin analogs (1), or by commensal bacterial biofilm formation (2); bacteriocins (3) or short-chain fatty acids (SCFAs) (4) produced by favourable bacteria (3); use of SCFAs as energy source for epithelial cells (5) and metabolic regulation (6); inhibition of the type-III secretion system (T3SS) (7); improvement of tight junction, mucin production (8) or immunomodulation (9) (based on the figures in reviews of Sansonetti (2004) and Kalita et al. (2014)).

6.1.Inhibition of pathogens adhesion sites

Prebiotics can inhibit pathogen adhesion via several mechanisms. These are a coating of the host epithelial surface, the promotion of beneficial bacteria and the down regulation of adhesion in pathogens. Their potential efficiency was tested in several *in vivo* and *in vitro* studies summarized in Table 2.2

Promoting beneficial bacteria: Prebiotics such as lactulose (Table2) regulate the intestinal microbiota by stimulating selectively the growth of a limited number of beneficial colonic bacteria, especially lactic acid bacteria (Guerra-Ordaz et al., 2014). These beneficial bacteria can form biofilms attached to the intestinal epithelial cells (González-Ortiz, 2013) locking out the adhesion of pathogens to the host's cells (Hopkins and Macfarlane, 2003; Das et al., 2013). Moreover, these bacteria can also display an acute antimicrobial action against invading foodborne pathogens by producing receptor analogs such as exopolysaccharides (e.g. reuteran produced by *Lactobacillus* reuteri) luring the pathogens such as ETEC (Chen et al., 2014; Y. Yang et al., 2015) or by producing antibiotic-like bacteriocin compounds of *Lactobacillus* plantarum or nonpathogenic *E. coli* selectively killing *Salmonella* bacteria (Zihler et al., 2009; Das et al., 2013).

Coating the host surface: Some prebiotics do not enrich beneficial bacteria as lactobacilli or enterococci but they can act by blocking the attachment of pathogens and keeping them from the gut wall due to similar structures to the glycosylated radical of the host's receptors. By the adsorption of prebiotics on the pathogen surface, they saturate the glycan-binding domains of pathogenic lectins and thus prevent binding to host glycoproteins, resulting in their excretion from the intestine (González-Ortiz, 2013; Molist et al., 2014). For example as showed in Table 2.2 casein glycomacropeptides, soluble extracts obtained from wheat (*Triticum aestivum*) bran, locust bean (*Ceratonia siliqua*), locust bean gum, and guar (*Cyamopsis tetragonoloba*) gum (González-Ortiz et al., 2014), chito-oligosaccharides (Quintero-Villegas et al., 2013) or galacto-oligosaccharides (GOS) (Shoaf et al., 2006) were used as anti-adhesives candidates effective against the attachment of ETEC or EPEC to the surface of porcine ileal mucus IPEC-J2 or human HEp-2 cells in *in vitro* experiments. In another example, the presence of β -galactose in β-galactomannan isolated from locust bean gum reduced the adhesion of *E. coli* K88 or *Salmonella* Typhimurium on cell surface of porcine intestinal IPI-2I cells by binding to their adhesion (Badia et al., 2012b; Badia et al., 2012a; Badia et al., 2013). Soluble non-starch polysaccharide from plantain bananas (*Musa paradisiaca*) hampered the adherence of *Salmonella* Typhimurium to Caco-2 cells, and has been suggested to block bacterial translocation into M-cells (Roberts et al., 2013). In contrast, no indication of reduced ETEC colonization in porcine ileal mucus was reported with soybean (*Glycine max*) hulls, sugar beet pulp (*Beta vulgaris*), cranberry (*Vaccinium* sp.), FOS, inulin, exo-polysaccharides (EPS), mannan-oligosaccharides (González-Ortiz, 2013; Chen et al., 2014). However, inulin and FOS reduced the adherence of EPEC to Caco-2 and Hep-2 tissue

culture cells (Chen et al., 2014). This suggests that different cell types and pathogens respond differently to prebiotic exposure.

Down-regulating the expression of adhesin factors or virulence genes: End-products of fermentation, namely short chain fatty acids (SCFA, including acetic, propionic, and *n*-butyric acid) can inhibit the expression of adhesin factors or the invasion genes of *Salmonella* Typhimurium. For example, *n*-butyrate and propionate down-regulate the *Salmonella* pathogenicity island 1 (SPI-1) of *Salmonella* Typhimurium (Lawhon et al., 2002; Sun and O'Riordan, 2013) or the type-1 fimbriae of EHEC (Spring et al., 2000), resulting in inhibition of pathogenic invasion of the tissue. In agreement, lower butyrate concentrations have been shown to enhance the expression of virulence-associated genes required for cell adherence of EHEC (Vogt et al., 2015). Finally, the accumulation of SCFA anions in the cytoplasm alter the osmotic balance of pathogens (Sun and O'Riordan, 2013) and then strongly inhibit the growth of *Salmonella*.

6.2.Modulation of ecology and physiology of the intestinal tract

As mentioned above, prebiotics also stimulate selectively the growth of beneficial intestinal bacteria and then regulate the intestinal microbiota. This microbial community affects host physiology and host health through the fermentation of indigestible carbohydrates to release the SCFA products. As for organic acids added in the diet explained in a previous section, SCFA production can lead to a decrease in pH, especially when lactate is produced because of the low pKa of this acid (Fooks and Gibson, 2002). If the pH is below the optimal for the pathogen, it will inhibit its growth. For example, loss of biofilm formation and diffuse adherence pattern was observed in EAEC at pH 4.0 whereas at pH 7.4, typical aggregative adherence pattern was observed (Kaur and Chakraborti, 2010). (Fooks and Gibson, 2002) observed the inability of *E. coli* and *Salmonella* Enteritidis to support an acidic pH (≤ 5) in bifidobacteria and lactobacilli cultures fermenting inulin, FOS, XOS, mixtures of inulin:FOS or FOS:XOS (Table 2.2). This lowering pH effect of SCFA production contributes also to some extent to the protective effect of many lactic acid bacteria (Hopkins and Macfarlane, 2003). Although the effect of SCFAs on pathogen invasion depends also on the medium pH (Sun and O'Riordan, 2013). But one cannot state that a low pH always correlates with the inhibition of *pathogens* (Fooks and Gibson, 2002). For example, even when the pH in a co-culture of pathogens with human faecal microbes is kept neutral thanks to pH-probes and the addition of NaOH, symbiotics (*L. plantarum* combined to FOS and *Bifidobacterium bifidum* combined with a mixture of FOS/XOS) showed an ability to reduce the growth of *E. colias*

showed in Table 2.2 (Fooks and Gibson, 2003). In contrast, in a monoculture of *E. coli*, despite the pH decrease due to XOS fermentation by *Bifidobacterium bifidum* (Fooks and Gibson, 2002), there was no reduction in pathogen growth (Table 2.2), probably because pathogens can also compete with beneficial bacteria to use the carbohydrate source and reduce the pH themselves (Fooks and Gibson, 2003; Martín-Peláez et al., 2008; Petersen et al., 2009). *Another suggestion is that the presence of pathogens stimulates other* resident *gut microbes to be more efficient at fermenting NDCs and producing the SCFAs*. These increases may be a response of other gut bacteria to the presence of the pathogen (Fooks and Gibson, 2003; Petersen et al., 2009). For example, as displayed in Table 2.2, butyrate accumulation produced by the fermentation of mixture of FOS/inulin, gentio-oligosaccharides *(GOS)*, and lactulose in the presence of *Salmonella* was lower than in the absence of *Salmonella* (Martín-Peláez et al., 2008; Le Blay et al., 2009) probably because of an increase in the *C. cocoides–E. rectale* group which are butyrate producers (Le Blay et al., 2009). Similarly, supplementation with inulin at the end of the fermentation period stimulated *Bifidobacteriae* growth and SCFA production but did not induce any inhibitive effect on *S.* Typhimurium growth in the distal intestine. Moreover, mixtures of *L. plantarum* 0407 and FOS and *B. bifidum* Bb12 and a combination of FOS and XOS added to an *in vitro* model in the absence of pathogens did not increase the levels of SCFAs, whereas an increase in SCFA only occurred when *E. coli* were present (Fooks and Gibson, 2003). As a consequence, at low concentrations, pathogens may use these by-products as a carbon source for their own growth (Petersen et al., 2009).

6.3.Reinforcing the host immune system

Prebiotics have been shown to increase SCFA concentrations that can reinforce the host immune system. They increase the proliferation of epithelial cells and have stimulatory effects on both endocrine and exocrine pancreatic secretions in pigs. Butyrate acts as an energy source of colonocytes enhancing the barrier function of the colonic epithelial cells and helping in preventing the tissue breakdown and reducing oxidative DNA damage (Wang et al., 2012; Molist et al., 2014; Suiryanrayna and Ramana, 2015). Prebiotics that can change the physiology of epithelial cells have been associated with probably reductions in bacterial attachment without affecting the viability of pathogens. For example, as showed in Table 2.2, in weaning piglets fed lactulose or inulin that although had an increase in *Lactobacillus* and *Bifidobacterium* counts, SCFA concentration especially colonic butyrate, and ileum villous height, no effect of treatment were seen on ETEC (Guerra-Ordaz et al., 2014) or on *S.*

Typhimurium counts (Martín-Peláez et al., 2008; Martín-Peláez et al., 2009; Pieper et al., 2009a; Zihler et al., 2011). Addition of sunflower (*Helianthus annuus*) hulls or wheat straw in diet might enhance the maturation of the GIT and restore intestinal transit time, preventing initiation of infection in weaning piglets (Molist et al., 2014). Prebiotics may also enhance the cell - mediated immune response in early weaned piglets by modulating the production of antibodies. (White et al., 2002) described that the administration of mannan-oligosaccharides (MOS) from the brewers dried yeast increased serum levels of immunoglobulin G (IgG) in piglets challenged with *E. coli* K88, associated with lower coliform counts.

7. Conclusion

Salmonella enterica subsp. *enterica* and diarrhoeagenic *E. coli* strains are major intestinal pathogens in pigs causing foodborne infections in humans. Some potential prebiotics appear to be relevant to use in the feed for controlling these pathogens on the farms. From Table 2.2, it seems that inulin, lactulose, exopolysaccharide from probiotic bacteria or dietary fibre such as wheat bran, locust bean are efficient against *Salmonella*. spp and pathogenic *E. coli*. These fermented carbohydrates can be included in diets of weaning piglets and fattening pigs at 0.2–1% for simple carbohydrate molecule (Letellier et al., 2000; Andrés-Barranco et al., 2015) or 14–18% for fibre (Pieper et al., 2012b; Pieper et al., 2012c). Mechanisms by which these prebiotics might help pigs struggling against the pathogenic invasion are changes in intestinal ecology by SCFA production, inhibition of their adherence on gut epithelium and improvement of the host's immune system gene expression regulation by mainly *n*-butyrate. However, many results come from *in vitro* models, while the animal's physiological state and its immune response play a significant part in the mechanisms. Thus, future studies that combine *in vitro* and *in vivo* experiments to examine interactions between *Salmonella* or pathogenic *E. coli* and intestinal host will increase our understanding of the role of both the host and the bacterium in pathogenesis. In addition, most effect seems associated with a limitation in colonization of the pathogens. Hence, acting as early as possible on the intestinal microbiota of piglets and not only around weaning through early-life modulation strategies should also be considered using prebiotics. Finally, there are currently very few studies that have examined toxin production during these infections. Thereby, studies related to downregulating the expression of virulence-associated genes required for toxin production of pathogens is an important point to find potential prebiotics.

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