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## **Immunomodulating effects of probiotics for microbiota modulation, gut health and disease resistance in pigs**

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## HIGHLIGHTS

- Updated literature overview about probiotics, prebiotics and synbiotics on pig gut health is provided
- Both *in vivo* studies performed in pigs and *in vitro* studies conducted in pig intestinal cell lines are described
- A critical outcome of the described results is provided
- The concept of “proxy measurements” for pig gut health is widely discussed
- The link between gut microbiota and mucosal immune system is described

## Abstract:

Probiotics are live microorganisms that can confer a health benefit on the host, and amongst various mechanisms probiotics are believed to exert their effects by production of antimicrobial substances, competition with pathogens for adhesion sites and nutrients, enhancement of mucosal barrier integrity and immune modulation. Through these activities probiotics can support three core benefits for the host: supporting a healthy gut microbiota, a healthy digestive tract and a healthy immune system. More recently, the concept of combining probiotics and prebiotics, i.e. synbiotics, for the beneficial effect on gut health of pigs has attracted major interest, and examples of probiotic and prebiotic benefits for pigs are pathogen inhibition and immunomodulation. Yet, it remains to be defined in pigs, what exactly is a healthy gut. Because of the high level of variability in growth and feed conversion between individual pigs in commercial production systems, measuring the impact of probiotics on gut health defined by improvements in overall productivity requires large experiments. For this reason, many studies have concentrated on measuring the effects of the feed additives on proxies of gut health including many immunological measures, in more controlled experiments. With the major focus of studying the balance between gut microbiology, immunology and physiology, and the potential for prevention of intestinal disorders in pigs, we therefore performed a literature review of the immunomodulatory effects of probiotics, either alone or in combination with prebiotics, based on *in vivo*, *in vitro* and *ex vivo* porcine experiments. A

consistent number of studies showed the potential capacity in terms of immunomodulatory activities of these feed additives in pigs, but contrasting results can also be obtained from the literature. Reasons for this are not clear but could be related to differences with respect to the probiotic strain used, experimental settings, diets, initial microbiota colonization, administration route, time and frequency of administration of the probiotic strain and sampling for analysis. Hence, the use of proxy measurements of enteric health based on observable immunological parameters presents significant problems at the moment, and cannot be considered robust, reliable predictors of the probiotic activity *in vivo*, in relation to pig gut health. In conclusion, more detailed understanding of how to select and interpret these proxy measurements will be necessary in order to allow a more rational prediction of the effect of specific probiotic interventions in the future.

**Keywords:**

Probiotics; prebiotics; immunomodulation; pig diarrhea prevention; gut microbiota; gut health

**Abbreviations list:**

CFU, colony forming units; EPS, extracellular polysaccharide; GIT, gastrointestinal tract; GM-CSF, granulocyte macrophage colony-stimulating factor; ETEC, enterotoxigenic *E. coli*; FOS, fructo-oligosaccharide; GOS, galacto-oligosaccharide; HSP, heat shock protein; IPEC-1, intestinal porcine epithelial cells-1; IPEC-J2, intestinal porcine epithelial cells-2J, IPI-2I, ileal porcine intestinal-2I; LGG, *Lactobacillus rhamnosus* GG; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; PIE, porcine intestinal epitheliocyte; RV, Rotavirus; SCFA, short chain fatty acids; TEER, transepithelial electrical resistance; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; Tregs, regulatory T-cells; VSV, vesicular stomatitis virus; ZO-1, *zonula occludens-1*.

**1. Introduction**

The value of dietary modulation and nutritional strategies to enhance gut health of pigs is becoming increasingly apparent. While a frequently used term in relation to human and animal health, the precise scientific definition of ‘gut health’ is still lacking. An absolute state of optimal gut health is probably practically impossible to define, as gut health is a dynamic and relative concept. Bischoff (2011) proposed five major criteria for a healthy gastrointestinal tract in humans, being: 1) effective digestion and absorption of food, 2) absence of gastrointestinal illness, 3) normal and stable intestinal microbiota, 4) effective immune status, and 5) a status of well-being. However, it is worth noting that many of the terms used (‘effective’, ‘normal’, ‘well-being’) are, in themselves, relative terms and difficult to define. A definition of a healthy gut has to be accompanied by a measure of the overall health and welfare of the animal. Whereas the interest in immune modulation in relation to human gut health has primarily addressed severe inflammatory diseases such as inflammatory bowel disease and colon cancer, the focus of pig gut health has been both in relation to prevention of infectious diseases and performance of the animals, i.e. nutrient utilization and growth performance (Heo *et al.* 2013; Pieper *et al.* 2016). Weaned piglets commonly suffer from gastroenteritis caused by enterotoxigenic *Escherichia coli* (ETEC). The European legislation has banned the use of in-feed antibiotics as growth promoters since 2006, and the high reduction of antibiotics use has been shown to be effective in limiting the prevalence of antibiotic resistance genes in the gut microbiota of European pigs compared to Chinese pigs (Xiao *et al.* 2016). However, the use of sub-therapeutic antibiotics for prevention of enteric diseases among weaning pigs has continued the concerns regarding the increasing emergence of antibiotic resistant bacteria. There is still a demand for the development of alternatives to antibiotics while preserving health in farm systems. Probiotics, especially, have been primarily used as feed-additives to prevent infectious intestinal diseases and to improve performance of livestock (Guo *et al.* 2006). In their review, Lalles *et al.* (2007) concluded that manipulation of the prebiotic composition of the weaning diet may be the most promising way to improve gut health in weaned piglets, and that positive results have also been produced with probiotics fed to piglets or to sows. The major

responses appeared to be mediated through early changes in the gastrointestinal microbiota, including enhanced number of beneficial bacteria and/or decreased number of potentially pathogenic bacteria together with favorable fermentation products. Measureable, reproducible effects of dietary pre- and probiotics on intestinal physiology and mucosal immunology were limited or difficult to interpret (Lalles *et al.* 2007). However, subsequent and more recent studies have been conducted with probiotics to study the effect on intestinal immune responses under challenge of the pigs (e.g. Yang *et al.* 2016), and more scientific knowledge is available on the fundamental mechanisms of the potential immunomodulatory effects of the feed additives.

The purpose of the present paper was to review the literature in order to synthesize the knowledge concerning the immune modulating effects and mechanisms of action of probiotics, either alone or in combination with prebiotics in relation to gut health, with special emphasis on the fine balance between gut microbiology, immunology and physiology, and the potential prevention of intestinal disorders in pigs. *In vitro* (intestinal pig cell lines) and *in vivo* investigations on pigs were considered in the literature search. General criteria of including peer-reviewed journal articles in English and selectively including book articles or chapters, as well as grey literature such as PhD theses and dissertations were used.

## **2. Definitions**

The widely accepted definition of probiotics was formulated by a FAO/WHO Commission of experts in 2001: “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (FAO/WHO, 2001). Most of the species ascribed as having probiotic properties belong to the genera *Lactobacillus* and *Bifidobacterium*, commonly found in the gastrointestinal tract (GIT) of humans and animals and thus generally regarded as safe. However, also members of other bacterial genera can have probiotic activity, indeed most of the probiotic strains used in pig farms belong to *Bacillus*, *Enterococcus* and *Saccaromyces* genera. Such strains are selected mainly

on the basis of their good producibility on larger scales and high viability and stability during storage and feed preparation (Ohashi and Ushida, 2009).

Amongst various possible mechanisms of action, probiotics are believed to exert their effects by production of antimicrobial substances, competition with pathogens for adhesion sites and nutrients, enhancement of mucosal barrier integrity and immune modulation (O'Hara and Shanahan, 2007; Bermudez-Brito *et al.*, 2012). Thus, the beneficial activities of probiotics are ascribable to three main core benefits: supporting a healthy gut microbiota, a healthy digestive tract and a healthy immune system (Hill *et al.* 2014).

It is widely recognized that the health benefits of probiotics are highly strain-specific, thus different strains belonging to the same species can have different effects. For such reason, multi-strain mixtures may be more effective than single strains by complementing each other's health effects and exerting synergistic activities (Timmerman *et al.* 2004). Prebiotics are "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that have the potential to improve host health" (Gibson and Roberfroid, 1995). Prebiotics can also be fermented in pig large intestine (Jensen, 1998). From that derives the capacity of prebiotics to positively modulate the composition and/or activity of gut microbiota that confer benefits upon host wellbeing and health (Gibson, 2004, Gibson *et al.* 2010). The most commonly used prebiotics are galacto-oligosaccharides (GOS), inulin and fructooligosaccharides (FOS), which are plant storage carbohydrates in vegetables, cereals and fruits (Macfarlane *et al.* 2008). By the early 2000s, the development of next generation, rationally selected prebiotics was proposed (Rastall and Maitin, 2002). In this context, resistant starch, pectin and other fiber components, as well as milk oligosaccharides are now suggested as having prebiotic potential (Coppa *et al.* 2006; Bird *et al.* 2010).

Synbiotics are defined as products containing both probiotics and prebiotics, and this combination is believed to be more efficient compared to probiotics and prebiotics alone in terms of gut health and function (Gibson and Roberfroid, 1995). The synbiotic formulation can be chosen following

two different criteria: (1) Complementary effect: single or multi strain probiotics, selected based on the specific desired host benefits, and prebiotics are independently chosen to stimulate beneficial gut microbial populations. Thus, the prebiotics increase the levels of resident gastrointestinal beneficial microbiota of the host (2) Synergistic effect: specific host beneficial probiotics are selected, and the prebiotic component is chosen to specifically enhance the survival, growth and activity of the selected ingested probiotic strain(s). However, an ideal synbiotic supplement should include both complementary and synergistic effects, containing an appropriate single or multi strain probiotic/s and suitable mixture of prebiotics, where the latter both selectively favors the former and also favors the multiplication of endogenous beneficial bacteria and the reduction of detrimental bacteria (Kolida and Gibson, 2011).

### **3. Immunomodulating effects of pre- and probiotics: *in vitro* and *ex vivo* studies**

The probiotic strains used for *in vitro* immunomodulation studies and their origin (where the information is available), as well as main results obtained, are listed in Table 1. The origins are very diverse, most are from human and pig intestine or faeces, but also strains isolated from human and animal milk have been studied in relation to their immunomodulating properties *in vitro*.

#### ***Pig intestinal cell lines for in vitro studies***

Other than their barrier function, intestinal epithelial cells play an important role in the initiation of the mucosal immune response, as they represent the first line of defense against pathogens and toxic agents eventually reaching the intestinal lumen. Similarly to many immune cells involved in innate immunity, such as macrophages and dendritic cells, intestinal cells express on their surface the toll-like receptors (TLRs) that recognize structural components, widely conserved among different microorganism classes (Akira *et al.*, 2006). Epithelial TLR expression is thought to play a key role in the host defense against pathogens by triggering innate immune responses, through activation of NF- $\kappa$ B and mitogen-activated protein kinase (MAPK) pathways, that ultimately lead to the production of pro-inflammatory cytokines and chemokines (Stadnyk, 2002; Oswald, 2006).



The use of *in vitro* cell lines allows characterization of host/microbe interactions at a basic level, representing a good starting point for further higher-level *in vivo* studies. In particular, four pig intestinal cell lines have been employed to investigate epithelial innate immune responses to many different microorganisms: a) intestinal porcine epithelial cells (IPEC)-1 (Gonzalez-Vallina *et al.* 1996); b) intestinal porcine epithelial cells-jejunum (IPEC)-J2 (Schierack *et al.* 2006); c) ileal porcine intestinal (IPI)-2I cells (Kaeffer *et al.* 1993); d) porcine intestinal epitheliocyte (PIE) cells (Moue *et al.* 2008).

These studies aimed to evaluate the probiotic potential against several pathogen-induced damages, including adhesion to the epithelial cell membrane, alterations of tight junction integrity and induction of inflammatory response.

#### ***Probiotics used in pig intestinal cells challenged with ETEC***

ETEC is a major pathogen of neonatal swine. It attaches to mucosal surfaces to release toxins that induce intestinal inflammation and diarrhea, resulting in reduced growth rate, increased mortality and economic loss (Fairbrother *et al.* 2005). The majority of the *in vitro* studies presented in this review have been conducted using this pathogen as challenge.

*Enterococcus faecium* NCIMB 10415, a probiotic that can reduce diarrhea incidence in piglets (Büsing and Zeyner, 2015; Zeyner and Boldt, 2006), has been investigated in ETEC-infected IPEC-J2 cells, to deepen insight on the mechanisms underlying the bacterial-epithelial crosstalk during innate immune responses triggered by enteric infections. ETEC decreased transepithelial resistance (TEER) and increased IL-8 expression, and this effect could be prevented by both pre-incubation and simultaneous addition with *E. faecium* for up to 4 h (Klingspor *et al.* 2015; Lodemann *et al.* 2015). Similarly, another *E. faecium* strain (HDRsEf1), as well as its cell-free supernatant, could attenuate ETEC K88-induced IL-8 secretion and TEER decrease in IPEC-J2 cells (Tian *et al.* 2016). It is interesting to note that a recent study using jejunal tissue explants in Ussing chambers revealed that increased expression of pro-inflammatory cytokines was accompanied with an initial TEER increase and reduced permeability towards macromolecules upon ETEC challenge when pigs were

fed control but not *E. faecium* strain NCIMB 10415 supplemented diets (Lodemann *et al.* 2017). In this study, changes in tight junction protein expression (i.e. down-regulation of claudin-4 with control but not *E. faecium* diet) were observed at later stages after the *ex vivo* ETEC infection showing the close link between early immune response and protection against a loss of barrier function with this probiotic strain.

Several studies have also been conducted with strains belonging to the *Lactobacillus* genus. Downregulation of ETEC-induced increases in TLR4 and NOD2, receptors involved in pro-inflammatory NF- $\kappa$ B signaling, as well as in TNF- $\alpha$  concentration, was observed with *L. rhamnosus* ATCC 7469. Moreover, this probiotic strain was able to enhance the intestinal barrier function by increasing ZO-1 and occludin protein expression, confirming that the protection from ETEC-induced damage was achieved partly through the anti-inflammatory response and partly through the enhancement of tight junction integrity (Zhang *et al.* 2015). This same strain has been also used in *in vivo* trials, where it was found to positively modulate intestinal lymphocyte subpopulations in pigs challenged with ETEC (Zhu *et al.* 2014). Downregulation of the TLR4-dependent NF- $\kappa$ B and MAPK activation upon ETEC or lipopolysaccharide (LPS) challenge, with consequent reduction of IL-6 and IL-8 expression, was also observed with the the probiotic strain *L. jensenii* TL2937 in PIE cells (Shimazu *et al.* 2012). A new mechanism of counteracting the pathogenic effects of ETEC was demonstrated for two novel porcine isolates, *L. johnsonii* P47-HY and *L. reuteri* P43-HUV, that were able to induce the expression of cytoprotective heat shock protein (HSP)-27 and HSP-72, and to preserve barrier function and tight junction integrity in IPEC-J2 cells (Liu *et al.* 2015). Another interesting study evaluated the immunomodulatory effect of *L. delbrueckii* subsp. TUA4408L and its extracellular polysaccharide (EPS): acidic EPS (APS) and neutral EPS (NPS) against ETEC challenge in PIE cells. ETEC-induced inflammatory cytokines were downregulated when the cells were pre-stimulated with both *L. delbrueckii* or its EPSs. The anti-inflammatory capability of *L. delbrueckii* was diminished when PIE cells were blocked with anti-TLR2 antibody, while APS failed to suppress inflammatory cytokines when the cells were treated with anti-TLR4 antibody,

indicating that TLR2 played a principal role in the immunomodulatory action of *L. delbrueckii*, while the activity of APS was mediated by TLR4 (Wachi *et al.* 2014). Indeed, several studies have demonstrated that TLR2 is required by some probiotic strains to exert their immunomodulatory effects (Rizzo *et al.* 2013; Finamore *et al.* 2014). *L. amylovorus* strain DSM 16698, initially named *L. sobrius* (Konstantinov *et al.* 2006), has been used in both *in vivo* (see Section 5) and *in vitro* studies. This strain was able to prevent the cellular damage induced by ETEC K88 strain in IPEC-1 cells, by strongly reducing ETEC adhesion, inhibiting the alterations of the tight junctions proteins ZO-1 and occludin, and counteracting the F-actin rearrangements and the alterations of IL-1 $\beta$ , IL-8, and IL-10 gene expression induced by ETEC (Roselli *et al.* 2007). Anti-inflammatory activity of four different human-derived *L. reuteri* strains, namely DSM 17938, ATCC PTA4659, ATCC PTA 5289, and ATCC PTA 6475, was evaluated in IPEC-J2 cells challenged with LPS. The four strains differentially affected LPS-induced IL-8 response in IPEC-J2 cells, as the three ATCC strains significantly inhibited IL-8 production, whereas DSM 17938 did not show this ability, highlighting the importance of considering the strain specificity, as responsible of the protective effects (Liu *et al.* 2010a). Another *L. reuteri* strain, I5007, was assayed for its protective activity against LPS, and it was observed that the expression of LPS-induced pro-inflammatory cytokines (TNF- $\alpha$  and IL-6), and TJ proteins (claudin-1, occludin and ZO-1) was reversed by pre-treatment with *L. reuteri* I5007 or its culture supernatant (Yang *et al.* 2015a). Similar results were also obtained with some *Lactobacillus reuteri* isolates, LR1 and CL9, that were able from one side to inhibit the ETEC-induced expression of pro-inflammatory cytokines IL-6, IL-8 and tumor necrosis factor (TNF)- $\alpha$ , as well as to increase the level of anti-inflammatory cytokine IL-10, and from the other side to maintain cell membrane barrier integrity, by preventing tight junction protein *zonula occludens* (ZO)-1 disruption (Wang *et al.* 2016; Zhou *et al.* 2014).

The anti-inflammatory and immunomodulatory effects of different bifidobacterial strains, *Bifidobacterium breve* MCC-117, *B. longum* BB536 and *B. breve* M-16V, were studied in PIE cells challenged with heat-killed ETEC. These strains were shown to activate TLR2 and upregulate some

TLR4 negative regulators, as ubiquitin-editing enzyme A20, thus reducing the activation of MAPK and NF- $\kappa$ B pathways and the subsequent production of pro-inflammatory cytokines (Murata *et al.* 2014; Tomosada *et al.* 2013). Another study by the same authors using PIE cells and swine Peyer's patches immunocompetent cell co-culture system showed that the immunoregulatory effect of *B. breve* MCC-117 was related to its capacity to influence intestinal-immune cell interactions, leading to the stimulation of the regulatory T (Treg) CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cell population among Peyer's patches immune cells, expressing high IL-10 levels (Fujie *et al.* 2011).

The probiotic activity of a yeast strain, *Saccharomyces cerevisiae* CNCM I-3856, against ETEC challenge was explored in IPEC-1 and IPI-2I cells. The CNCM I-3856 strain was able to inhibit the ETEC-induced expression of pro-inflammatory cytokines and chemokines, and such inhibition was associated to a decrease of ERK1/2 and p38 MAPK phosphorylation (Zanello *et al.* 2011a). Moreover, this inhibition was dependent on secreted soluble factors, as the heat-killed yeast did not maintain the protective activity that indeed was maintained by yeast culture supernatant (Zanello *et al.* 2011b).

#### ***Probiotics used in pig intestinal cells challenged with enteropathogenic E. coli (EPEC) and Salmonella enterica***

EPEC infection leads to serious acute diarrhea in weaned pigs, accompanied by high mortality rates (Zhu *et al.* 1994). Infection of intestinal epithelial cells by EPEC is a complex process, where initially EPEC loosely adheres to the epithelial cell membrane and translocates effector molecules into host cells. Subsequently the bacterium binds more tightly, intimately adheres to epithelial cells and forms microcolonies, resulting in histopathological alterations of the host cell surface, known as attaching and effacing (AE) lesions, that finally lead to microvilli destruction and loss of barrier function (Cleary *et al.* 2004). The *E. coli* Nissle 1917, a non-pathogenic *E. coli* strain with beneficial activity, has been employed as probiotic strain in pigs, where it has been shown to prevent the deleterious effects of pathogen-induced secretory diarrhea (Schroeder *et al.* 2006). Moreover, this strain could drastically reduce EPEC infection efficiency *in vitro*, by inhibiting

initial bacterial adhesion, due to strong adhesion capacity and secretion of inhibitory components, able to significantly reduce EPEC virulence-associated proteins (Kleta *et al.* 2014).

Another pathogen primarily associated with systemic invasive infection in swine is *Salmonella enterica* serovar Typhimurium, causing considerable economic losses and public health problems (Berends *et al.* 1996). Similarly to EPEC, *Salmonella* invades epithelial cells by using a specialized mechanism to inject its effector proteins into the cytoplasm through the host membrane surface. The action of injected proteins leads to a dramatic reorganization of the host actin cytoskeleton and to a vigorous epithelial cell membrane protrusion, and as a result the bacterium is engulfed inside the host cell (Ly and Casanova, 2007). *E. coli* Nissle 1917 showed similar inhibitory effects against *Salmonella* as those described for EPEC, and this inhibitory activity always correlated with probiotic adhesion capacity (Schierack *et al.* 2011). The *Salmonella*-induced inflammatory challenge was also used to evaluate the immunomodulatory activity of the two probiotic strains *L. reuteri* ATCC 53608 and *Bacillus licheniformis* ATCC 10716, that were able to significantly inhibit *Salmonella*-stimulated IL-8 basolateral secretion (Skjolaas *et al.* 2007).

Inhibition of adherence of several pathogens, belonging to the genera *Salmonella*, *Clostridium*, and *Escherichia*, has also been studied in a pig intestinal mucosa model, where two probiotic strains, *B. lactis* Bb12 and *L. rhamnosus* GG (LGG), alone and in combination, significantly reduced the adhesion of the tested pathogens (Collado *et al.* 2007).

#### ***Probiotics used in pig intestinal cells challenged with viruses***

Some probiotics have also been explored for their potential antiviral activities. The first study showing that some probiotics could exhibit an antiviral activity has been conducted in IPEC-J2 cells infected with vesicular stomatitis virus (VSV), used as a model virus, as VSV is not a classical intestinal pathogen. Pre-incubation of cell monolayers with two *Bifidobacterium* (*B. breve* DSM20091 and *B. longum* Q46) and five *Lactobacillus* strains (*L. paracasei* A14, *L. paracasei paracasei* F19, *L. paracasei/rhamnosus* Q 85, *L. plantarum* M1.1 and *L. reuteri* DSM 12246) showed that these probiotics reduced viral infectivity through secretion of antiviral substances, and

prevented VSV binding to cell monolayers by interfering with virus attachment or entry into the cells, or by trapping the VSV itself (Botic *et al.* 2007).

Rotaviruses (RV), members of the *Reoviridae* family, are important etiologic agents of viral gastroenteritis in suckling and weaned piglets (Chang *et al.* 2012). Intestinal cells respond to RV infection by activating TLR3, that recognizes viral double stranded RNA. The TLR3-triggered innate immune response induced by RV was evaluated in PIE cells to select probiotic strains with specific anti-viral and immune enhancing properties. The strains *L. casei* MEP 221106 (Hosoya *et al.* 2011), *L. casei* MEP 221114 (Hosoya *et al.* 2013), *L. rhamnosus* CRL1505 (Villena *et al.* 2014), *B. infantis* MCC12 and *B. breve* MCC1274 (Ishizuka *et al.* 2016) were able to significantly reduce RV titers in infected cells and modulate several molecular markers of TLR3-triggered pathway, finally leading to NF- $\kappa$ B activation and proinflammatory cytokine secretion. Liu and coauthors (2010b) used IPEC-J2 cells to compare the probiotic activity of *L. acidophilus* NCFM (LA) to the well known probiotic LGG. Although these two strains were not able to reduce virus replication into IPEC-J2 cells, they could differently modulate cellular immune response. Indeed, LA treatment prior to RV infection significantly increased virus-induced IL-6 response, consistent with the adjuvant effect of this strain, in potentiating immunogenicity of an oral RV vaccine in a gnotobiotic pig model (Zhang *et al.* 2008). On the contrary, LGG treatment post-RV infection downregulated the IL-6 response, confirming the well documented anti-inflammatory effect of LGG.

In conclusion, all the studies presented in this section have described how the various probiotic strains can exert immunomodulatory activities and contribute to the maintenance of intestinal homeostasis. These *in vitro* studies are quite consistent and clearly demonstrate that most of the different tested strains are able to counteract the pathogen-induced damages at various levels by virtue of several mechanisms of action, including reduction of pathogen adhesion, downregulation of inflammatory signaling pathways, reduction of pro-inflammatory cytokine secretion, maintenance of tight junction integrity. In some cases it has been demonstrated that the beneficial effects are maintained also by the spent culture supernatant, suggesting that one or more soluble

factors released by probiotic bacteria were responsible for the protective activity. However, it is important to consider that the beneficial effects are highly strain-specific, as clearly demonstrated in some studies observing different results on pathogen protection, by testing different strains from the same bacterial species.

#### *In vitro studies with prebiotics*

Much less *in vitro* studies with prebiotics have been performed to test for direct (microbiota-independent) immunomodulatory effects. In one study the prebiotic  $\beta$ -galactomannan was used to evaluate the protective role against *S. enterica* serovar Typhimurium infection in IPI-2I cells, and interestingly this prebiotic was found to attenuate *Salmonella*-induced secretion of pro-inflammatory cytokine IL-6 and chemokine CXCL8 (IL-8). The authors concluded that the particular oligosaccharide structure of  $\beta$ -galactomannan was able to interfere with pathogen adhesion (Badia *et al.* 2013). In a previous study by the same authors,  $\beta$ -galactomannan was used and compared to *Saccharomyces cerevisiae* var. *boulardii*. Both prebiotic and probiotic were able to inhibit the *in vitro* ETEC adhesion to IPI-2I cells, and to decrease the ETEC-induced gene expression of pro-inflammatory cytokines TNF- $\alpha$ , IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and chemokines CCL2, CCL20 and CXCL8. Very similar results were obtained by using the *S. enterica* serovar Typhimurium challenge model (Badia *et al.* 2012a and 2012b).

#### **4. Influence of supplemental pre- and probiotics on the intestinal microbiota**

The intestinal microbiota composition in pigs is very dynamic and is subject to change over time, especially in early life. As the microbiota composition has a large impact on many aspects of the host's health (i.e., digestion of feed to breakdown products, stimulation of the immune system, competition with pathogens), it plays an important role in maintenance of health. To date, several studies have focused on the possibility of changing the microbiota composition in pigs by particular

feed supplementation with either pre- or probiotics (see Tables 2 and 3 for representative examples).

Frequently investigated prebiotics in pig microbiota composition studies are fermentable carbohydrates, which are linked to improvement of the microbial balance in both the small and large intestines by stimulating the carbohydrate metabolism. Pig microbiota composition and functionality can also be affected by other natural feed additives, such as milk components, proteins and fats (Bauer *et al.* 2006). Positive effects regularly ascribed to prebiotics are a bifidogenic effect (meaning the stimulation of bifidobacteria) or a butyrogenic effect (meaning the stimulation of butyrate producing bacteria). Also, the reduction of enteropathogens growth is often considered when studying the effect of a particular prebiotic as will be addressed below.

It has been observed that a diet high in resistant starch can change the microbiota composition in both the caecum and colon of adult pigs: in the colon, the healthy gut-associated butyrate-producing *Faecalibacterium prausnitzii* was stimulated, whereas potentially pathogenic members of the *Gammaproteobacteria*, including *E. coli* and *Pseudomonas spp.* were reduced in relative abundance (Haenen *et al.* 2013). Furthermore, Loh *et al.* (2006) showed that the addition of inulin to the diet led to an increase in the abundance of colonic bifidobacteria. In addition, total colonic short-chain fatty acid (SCFA) concentrations were lowered due to reduced acetate, although the proportion of colonic butyrate was higher in pigs fed inulin-supplemented diets. In another study, the amounts of both lactobacilli and bifidobacteria were increased in the caecum upon addition of inulin to the diet (4%) (Tako *et al.* 2008).

In two studies carried out by Pierce *et al.* (2006; 2007), it was shown that the addition of lactose increased the amounts of lactobacilli and bifidobacteria in the caecum and colon of weaning piglets, while *E. coli* numbers were decreased. The inclusion of lactose to the diet also resulted in increased SCFA concentrations in the colon (Pierce *et al.* 2006; Pierce *et al.* 2007). Other milk components, i.e, milk oligosaccharides have also been studied. These are major components of mammalian milk, whereas little is known on the oligosaccharide profile of porcine milk, although it has recently been



shown that porcine milk contains 29 distinct oligosaccharides (Tao *et al.* 2010). Several animal studies showed that both FOS and GOS were linked to a bifidogenic effect. For instance, piglets fed a diet supplemented with FOS post-weaning had increased bifidobacteria and reduced *E. coli* in the proximal colon (Gebbinck *et al.* 1999). Similarly in another study, it was shown that piglets fed a diet supplemented with GOS post-weaning had increased numbers of bifidobacteria in the proximal and transverse colon (Tzortzis *et al.* 2005).

Beta-glucans have gained special focus due to their immune-stimulatory properties, since they interact with specific receptors of the innate immune system such as dectin-1 on dendritic cells (Vannucci *et al.* 2013). Next to the immune-stimulatory properties, beta-glucans can modulate the gut microbiota. Oat-derived beta-glucans have been shown to raise the numbers of lactobacilli and bifidobacteria in the colon (Metzler-Zebeli *et al.* 2011), while yeast-derived beta-glucans reduced *Enterobacteriaceae* counts in the colon (Sweeney *et al.* 2012) (Table 2).

Addition of arabinoxylans to the feed resulted in a higher number of *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, *Blautia coccooides-Eubacterium rectale*, *Bifidobacterium* spp. and *Lactobacillus* spp. in the faeces. In conclusion, addition of arabinoxylans to the feed shifted the microbial composition towards butyrate-producing species, and, as an additional effect, the butyrate concentration in the large intestine was increased (Nielsen *et al.* 2014).

The general mode of action by which many probiotics influence the intestinal microbiota is their ability to competitively exclude the growth of pathogens. Table 3 provides representative examples of studies addressing the influence of probiotics on porcine microbiota composition. Production of lactic acid by lactobacilli is responsible for the lowering of pH, which is related to the reduction of growth rates of potential pathogens like *Salmonella* and *E. coli*. Furthermore, many lactobacilli are capable to produce peptide-based molecules known as ‘bacteriocins’ which can inhibit the growth of similar or closely related bacteria (Cotter *et al.* 2005). Using cultivation-dependent methods, studies using oral treatments of piglets with probiotics have shown a positive influence on the

microbiota in terms of decreased numbers of potential pathobionts and increased numbers of beneficial bacteria (Table 3).

### **5. *In vivo* studies with probiotics and intestinal disorders**

The main interests in feeding probiotics, and synbiotics are the health benefit especially in terms of diarrhea reduction and performance improvement in piglets (e.g. Hodgson and Barton, 2009).

#### ***Prevention of diarrhea***

Some of the studies addressing the influence of probiotics on microbiota composition have also included performance and diarrhea (Table 4): in the study by Huang *et al.* (2004), the *Lactobacillus* preparation significantly decreased *E. coli* counts and anaerobe counts while also significantly decreasing diarrhea incidence. When *B. longum* was added to the diet as a supplement, reduced numbers of total anaerobes and clostridia in the faeces were observed, indicating that the administration of bifidobacteria could provide a beneficial effect on pig growth and performance (Estrada *et al.* 2001). Supplementation of the probiotic *L. amylovorus* DSM 16698 could reduce the levels of ETEC expressing K88/F4 *fimbriae*, in the ileum of challenged piglets. In addition, an improved daily weight gain was observed when compared to the control group (Konstantinov *et al.* 2008). In another study it was found that pretreatment with *E. coli* Nissle 1917 completely abolished clinical signs of secretory diarrhea in a model of intestinal infection with an ETEC strain (Schroeder *et al.* 2006). Pigs fed a diet supplemented with the probiotic *E. faecium* strain CECT 4515 showed increased counts of lactobacilli in ileum, caecum and faeces and a reduced number of coliforms in the ileum. In addition, the probiotic resulted in heavier piglets, caused by a significantly improved growth and feed conversion ratio (Mallo *et al.* 2010). Similarly, when *Bacillus subtilis* LS 1-2 fermented biomass was added to the diet, counts of *Clostridium* spp. and coliforms were reduced in the caecum. Additionally, *B. subtilis* fermented biomass resulted in enhanced growth rate and enhanced feed conversion ratio (Lee *et al.* 2014). Hence, several studies amongst others shown in Table 4 have concluded that probiotics supplementation exert a beneficial

effect on diarrhea reduction and growth, however, it should be noted that the studies may not have been experimentally designed to study these parameters. Moreover,, contrasting results can be obtained in the literature. Reasons for this are not clear but could be related to experimental settings, diets, initial microbiota colonization, administration route, time and frequency of probiotic strain administration, differences in strains from the same microbial species, and sampling for analysis. For example, some studies using *E. faecium* NCIMB 10415 showed that diarrhea was reduced and performance increased (Taras *et al.* 2006; Zeyner and Boldt, 2006; Büsing and Zeyner, 2015) whereas others did not (Broom *et al.* 2006; Martin *et al.* 2012). It was initially shown that *E. faecium* NCIMB 10415 could be transferred already from the mother to the piglets during the early postnatal period (Macha *et al.* 2004; Taras *et al.* 2007; Starke *et al.* 2013). Depending on the administration route (daily oral dose or within the feed of sows and piglets), different amounts of the probiotic strain could be recovered (Taras *et al.* 2007). In these studies, it was shown that in suckling piglets receiving no supplemented feed, *E. faecium* reached similar fecal cell counts as compared to weaned piglets receiving probiotic supplemented feed, suggesting that this strain is capable to occupy a niche within the intestinal ecosystem. Feeding *E. faecium* to piglets 1-14 days of age decreased the number of *E. coli* in faeces, decreased pH in the duodenum and increased the concentration of lactic and propionic acid in the colon (Strompfova *et al.* 2006). Interestingly, feeding the strain to sows and their piglets showed effects on intestinal microbial community composition but also showed some animal-specific variability with so-called “responders” and “non-responders” (Starke *et al.* 2013). This phenomenon has not been further clarified yet but should probably be addressed with regard to experimental designs and data interpretation. *In vitro* co-culture experiments and *in vivo* analyses showed that this probiotic strain could reduce the growth of other enterococci and pathogenic *E. coli* (Vahjen *et al.* 2007; Starke *et al.* 2015). Similarly, data from *in vivo* feeding trials showed that *E. faecium* NCIMB 10415 did not affect the diversity and number of luminal enterobacteria but reduced the abundance of *E. coli* virulence factors and the number of pathogenic *E. coli* adhering to the intestinal mucosa (Taras *et al.* 2006;

Scharek *et al.* 2005; Bednorz *et al.* 2013). In contrast, two independent but similar challenge experiments with *S. Typhimurium* showed no protective effect of the probiotic *E. faecium* NCIMB 10415 on pathogen shedding in weaned pigs (Szabo *et al.* 2009; Kreuzer *et al.* 2012). An explanation could be that *Salmonella* has evolved manifold strategies to evade or down-regulate the host immune system and spread beyond intestinal boundaries (Monack, 2013). In this context, it could be possible that – despite the protective effect against pathogenic *E. coli* - some immunomodulating properties of *E. faecium* NCIMB 10415 in pigs could cause a disadvantage for the host upon challenge with *Salmonella*. For example, it was initially shown that feeding *E. faecium* NCIMB 10415 reduced the number of intraepithelial cytotoxic T-cells and fecal IgA in weaned piglets (Scharek *et al.* 2005; Scharek *et al.* 2007). Further analyses confirmed that *E. faecium* likely exerts anti-inflammatory/immuno-suppressive reactions in suckling piglets, which are then intensified during the weaning time (e.g. reduced expression levels of IL-8, IL-10 and no change in T-cell inhibitory molecule CTLA4 in jejunal and ileal Peyer’s patches) and thereby opens a so called “window of opportunity” for *Salmonella* to colonize and persist in the host (Twardziok *et al.* 2014; Siepert *et al.* 2014). A recent study further suggests that this early-life effect on the immune response may be promoted not only through the transfer of the probiotic itself but also through immunoactive compounds (i.e. CD14 expressing epithelial cells) from the probiotic-fed mother via the milk (Scharek-Tedin *et al.* 2015).

Another probiotic, *Bacillus toyonensis* sp. nov. (previously known as *B. cereus* var. *toyoi*, Jimenez *et al.* 2013) has been used for many years in pigs and has been shown to reduce the incidence of diarrhea and improve feed efficiency (Taras *et al.* 2005). *B. toyonensis* has been associated with a decreased ETEC number and morbidity in weaned piglets (Papatsiros *et al.* 2011, Table 4). Feeding of *B. toyonensis* to sows changed their intestinal microbiota, and this probiotic was shown being transferred to the neonatal piglets and significantly alter the intestinal fermentation patterns during the early suckling period and weaning (Kirchgessner *et al.* 1993; Jadamus *et al.* 2002). One of the main modes of action of *Bacillus* spp. in pigs might be through

their immunomodulating properties. Probiotic treatment with spore formers such as *B. toyonensis* have been shown to lead to an increased abundance of intraepithelial cytotoxic T-cells and fecal IgA in weaning pigs, which may confer protection against pathogen colonization (Scharek *et al.* 2007; Schierack *et al.* 2009). In fact, a challenge trial with *S. Typhimurium* showed that piglets in the control group responded to *S. Typhimurium* challenge with reduced growth and high incidence of diarrhea, which was less pronounced in piglets fed with the probiotic (Scharek-Tedin *et al.* 2013).

Several different *Lactobacillus* spp. and strains have been used in past studies in pigs showing effects on the intestinal microbial communities or beneficial activities under ETEC, *Salmonella* or RV challenge conditions. These studies included *L. plantarum*, *L. amylovorus* DSM 16698, *L. reuteri* or LGG. *L. amylovorus* DSM 16698 was also used in an experiment performed on pig intestinal explants, where ETEC induced a higher level of TLR4, P-IKK $\alpha$ , P-I $\kappa$ B $\alpha$ , and P-p65, while *L. amylovorus* completely abolished all these alterations and upregulated the negative TLR4 regulators Tollip and IRAK-M expression, when co-treated with ETEC (Finamore *et al.*, 2014). In particular, *L. amylovorus* was effective in reducing ETEC K88 colonization (Table 3) and could improve the weight gain of infected piglets (Konstantinov *et al.* 2008), while the administration of *L. plantarum* DSM 8862 and 8866 strains at weaning could influence gastrointestinal microbiota in piglets, with positive outcomes on gastrointestinal health (Pieper *et al.* 2009). Several *L. reuteri* strains have been used in pig trials, in particular, the I5007 strain has been shown to have beneficial effects on performance and growth, prevention of diarrhea, altered gut microbiota, and immunomodulation (reviewed by Hou *et al.* 2015). Finally, LGG supplementation could alleviate the diarrhea in RV-challenged weaned piglets through inhibition of virus multiplication, as well as improvement of intestinal mucosal barrier function and of intestinal microbiota composition (Mao *et al.* 2016).

Studies performed with synbiotics in relation to *E. coli* related diarrhea are limited (Table 5), and to date, different synbiotic combinations have been used in weaning piglets after *E. coli* challenge

with variable results. The study by Krause *et al.* (2010) concluded that the use of *E. coli* probiotic strains UM-2 and UM-7 against ETEC K88 in the presence of raw potato starch positively impacted on piglet growth performance and resulted in a reduction of diarrhea and increased microbial diversity in the gut. In another study, the synbiotic combination of lactulose and *L. plantarum* JC1 strain showed a good potential to be used to control post-weaning colibacillosis in piglets (Guerra-Ordaz *et al.* 2014).

## **6. The link between the intestinal microbiota and the mucosal immune system**

It has been demonstrated that axenic or germ-free animals mature physically without the contribution of microbial colonization but remain functionally immature in many systems including mucosal and systemic immunity, development of secondary lymphoid tissues, and susceptibility/response to pathogens (reviewed by Smith *et al.* 2007). These results provide a strong initial basis to demonstrate that the maturation of the immune system depends on both colonization and diversification of the intestinal tract with symbiotic microorganisms. Thus, understanding the role of the host-microbiota interplay for shaping local and systemic immunity is a key issue for identifying efficient strategies to modulate the gut microbiota with the goal of promoting host health and resilience in the face of pathogens. Note also that, despite a known and well-acknowledged strong impact of maternal colonization and environmental parameters for driving the gut microbiota composition, the genetics of the host is also likely to play a role (Goodrich *et al.* 2014; Dabrowska and Witkiewicz, 2016), and questions on how to measure heritability of the microbiomes have been raised (van Opstal *et al.* 2015). Clearly, distinguishing true genetic predisposition from vertical transmission from the sow to her piglets is important when assessing ‘heritability’ of microbiomes. In addition, estimating ‘heritability’ of microbiomes from simple breed or strain differences may also be flawed. Thus, initial studies in rodents suggested that knocking out TLRs and their signaling pathways resulted in changes in the microbiomes: however, recent studies demonstrate that this effect is likely to be a consequence of maintaining separate wild-type and knockout colonies (Ubeda

*et al.* 2012). Despite these difficulties, several recent studies in mice have shown that genetic loci influence the relative abundances of specific microbial taxa in the gut (Benson *et al.*, 2010), and that polymorphisms in the major histocompatibility complex contribute to shape individual's unique microbial patterns that influence the susceptibility to enteric pathogens and thus health (Kubinak *et al.* 2015). Variants of the FUT1 genotype, which has recently been highlighted for its property on controlling expression of the receptor for ETEC F18; may also affect the commensal intestinal microbiota and host metabolism and immune response of non-infected pigs (Poulsen, 2016).

Because of the high level of variability in growth and feed conversion between individual pigs in commercial husbandry systems, measuring the impact of prebiotics, probiotics or synbiotics on gut health defined by improvements in overall productivity requires large experiments. For this reason and as described in the previous sections, many studies have concentrated on measuring the effects of diet or bacteria on proxies of gut health including many immunological measures and changes in the microbiota, in more controlled experiments and physical environments. In model species such as laboratory rodents, colonization with single microorganisms or administration of their products can have profound effects on immunological responses in the intestinal mucosa, and microbiota species have been described with immunomodulatory effects in the GIT. For example, the presence or absence of a single bacterial species, the segmented filamentous bacterium, can result in marked differences in mucosal T-cell numbers and functions, including Th17 and Treg cells (Ivanov *et al.* 2008; Gaboriau-Routhiau *et al.* 2009). Similarly, *Bacteroides fragilis* was shown to direct the development of Foxp3<sup>+</sup> Tregs in the colon (Liu *et al.* 2008; Mazmanian *et al.* 2005; Mazmanian *et al.* 2008; Round *et al.* 2011); members of *Clostridium* cluster IV (*C. leptum* group) and XIVa (*C. coccoides* group) were reported to promote the expansion of colonic and systemic Tregs (Wingender *et al.* 2012); *Sphingomonas yanoikuyae* was shown to modulate the phenotype and response of invariant NKT cells (Atarashi *et al.* 2011; Atarashi *et al.* 2013). Hence, the link between microbiome and mucosal immune system is clear. Similarly, colonization of germ-free mice with microbiota derived from mice or humans with different obesity phenotypes (lean or

obese) can result in recapitulation of the phenotype of the donor, establishing a causal link between the microbiome and metabolism (Turnbaugh *et al.* 2006; Ridaura *et al.* 2013).

Similar administration of single or complex mixtures of probiotic organisms can also have marked effects on immunological measures in pigs. In the most reductionist version of the experiment, colonization of true germ-free pigs recapitulates postnatal development of the mucosal immune system (Laycock *et al.* 2012). In 70-day-old Large White pigs with more complex, naturally establishing microbiomes, the presence of ‘enterotypes’ characterised by *Ruminococcus* or *Prevotella* was associated with differences in luminal secretory IgA concentrations, as well as body weight (Mach *et al.* 2015), higher IgA levels and growth performances being seen in pigs with the *Prevotella*-dominated microbiomes. In this report, there was no antagonism between IgA levels expected to be protective and production traits. The two ‘enterotypes’ were refined with more than 500 60-day-old piglets, the *Prevotella*-enterotype being also dominated by *Mitsuokella* and the *Ruminococcus*-enterotype by *Treponema* (Ramayo-Caldas *et al.* 2016).

Nevertheless, direct intervention with single probiotics has produced conflicting results. In one study, administration of *B. lactis* NCC2818, an organism with established probiotic activity in humans and rodents, resulted in overall reduction in IgA secretion in the intestine, rather than an increase (Lewis *et al.* 2013). This was associated with an increased expression of the enterocyte tight-junction protein ZO-1, suggesting that increased barrier function might have resulted in decreased antigen uptake and reduced stimulation of IgA production. In contrast, other studies have shown an increase in IgA as a consequence of feeding the probiotics *E. faecium* NCIMB 10415, SF68 and *Bacillus cereus* var. *toyoi* NCIMB 40112, and suggested that the probiotic effect may be attributable to increased IgA providing better protection at mucosal surfaces (Scharek *et al.* 2007).

## **7. Conclusions and future perspectives**

The literature review provided here has revealed that many porcine studies have been performed showing that probiotics can influence the gut microbiota, and are having immunomodulatory



effects. These effects have especially been studied with the goal to inhibit pathogens and enteric diseases. However, contrasting results can be observed in the literature. Reasons for this are not clear but could be related to experimental settings, diets, initial microbiota colonization, administration route, time and frequency of the probiotic strain, differences in strains from the same microbial species and sampling for analysis. In addition, our review reports studies showing the influence of prebiotics on the porcine microbiota composition, but only few studies in pigs are available yet on the combination of pre- and probiotics in relation to gut health parameters. The main interests in feeding probiotics or synbiotics are the reduction in diarrhea and improvement in performance in piglets.

However, in this context it should be stressed that the current use of proxy measurements of enteric health based on observable immunological parameters presents significant problems. The immune system functions to provide protection against true pathogens and against ubiquitous organisms with only mild negative effects on enteric health such as the ‘pathobionts’ identified in mouse intestinal microbiomes (Chow *et al.* 2011). However, expression of immunological functions in the intestinal mucosa can result in clearance or exclusion of such micro-organisms (a ‘good’ thing) while mediating inflammation (a ‘bad’ thing). As a result, the overall impact of any of the immunological parameters which we commonly measure on health and performance is, currently, difficult to predict. We strongly suggest that the value of such measures is as explanatory variables: that is, they should be used to understand a mechanism which has been defined either previously or in the same experiment by more robust, meaningful measures of enteric health. These proxy measures are necessary to understand and develop mechanistic models which, in future, will allow rational prediction of the effect of specific probiotic or synbiotic interventions: however, at the moment, they cannot be considered robust, reliable predictors of probiotic, or synbiotic activity and efficacy in relation for diarrhea reduction and improvement in performance.

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Table 1. Probiotic strains used in pig intestinal cell lines and main results.

Strains	Origin and source	Results	Reference
<i>Lactobacillus amylovorus</i> DSM 16698 (previously called <i>L. sobrius</i> )	Piglet intestine	Protection against ETEC K88 adhesion and membrane damage	Roselli <i>et al.</i> , 2007
<i>Bifidobacterium breve</i> DSM 20091 <i>B. longum</i> Q 46 <i>L. paracasei</i> A14 <i>L. paracasei</i> F19 <i>L. paracasei/rhamnosus</i> Q 85 <i>L. plantarum</i> M1.1 <i>L. reuteri</i> DSM 12246	Infant intestine <i>n.d.</i> <i>n.d.</i> <i>n.d.</i> <i>n.d.</i> Human babies faeces Pig faeces	Reduction of viral infectivity and adhesion, through secretion of antiviral substances	Botić <i>et al.</i> , 2007
<i>L. reuteri</i> ATCC 53608 <i>Bacillus licheniformis</i> ATCC 10716	Swine intestine isolate <i>n.d.</i>	Inhibition of Salmonella-stimulated IL-8 basolateral secretion	Skjolaas <i>et al.</i> , 2007
<i>L. reuteri</i> DSM 17938 <i>L. reuteri</i> ATCC PTA4659 <i>L. reuteri</i> ATCC PTA 5289 <i>L. reuteri</i> ATCC PTA 6475	Human milk Human milk Human oral cavity Human milk	Inhibition of LPS-induced IL-8 secretion by the three ATCC strains	Liu <i>et al.</i> , 2010a
<i>L. acidophilus</i> NCFM (LA) <i>L. rhamnosus</i> GG ATCC 53103	Human adult faeces Human adult faeces	LA pretreatment: increase of virus-induced Il-6 response LGG post-treatment: downregulation of Il-6 response	Liu <i>et al.</i> , 2010b
<i>L. casei</i> MEP221106	<i>n.d.</i>	Antiviral activity, modulation of TLR3-triggered pathway	Hosoya <i>et al.</i> , 2011
<i>Saccharomyces cerevisiae</i> CNCM I-3856 <i>S. cerevisiae</i> var <i>bouardii</i> CNCM I-3799	<i>n.d.</i> <i>n.d.</i>	Inhibition of ETEC-induced pro-inflammatory cytokines, downregulation of MAPK pathway	Zanello <i>et al.</i> , 2011a and 2001b
<i>Escherichia coli</i> Nissle 1917 DSM 6601	Human	Regulation of <i>Salmonella</i> virulence genes expression and adhesion	Schierack <i>et al.</i> , 2011; Kleta <i>et al.</i> , 2014
<i>B. breve</i> MCC-117	<i>n.d.</i>	Stimulation of Treg cells from Peyer's patches TLR2 activation and TLR4 negative regulators upregulation	Fujie <i>et al.</i> , 2011; Murata <i>et al.</i> , 2014
<i>L. jensenii</i> TL2937	Human faeces	Downregulation of NF-kB pathway, activated by ETEC or LPS	Shimazu <i>et al.</i> , 2012
<i>L. casei</i> MEP221114	Human faeces	Antiviral activity, modulation of TLR3-triggered pathway	Hosoya <i>et al.</i> , 2013
<i>B. longum</i> BB536 <i>B. breve</i> M-16V	<i>n.d.</i>	TLR2 activation and TLR4 negative regulators upregulation	Tomosada <i>et al.</i> , 2013
<i>L. reuteri</i> CL9	<i>n.d.</i>	Inhibition of ETEC-induced pro-inflammatory cytokine expression	Zhou <i>et al.</i> , 2014
<i>L. delbrueckii</i> TUA4408L	Sunki, japanese fermented pickle	Downregulation of ETEC-induced inflammatory cytokines, mediated by TLR2	Wachi <i>et al.</i> , 2014
<i>L. rhamnosus</i> CRL1505	Goat milk	Antiviral activity, modulation of TLR3-triggered pathway	Villena <i>et al.</i> , 2014



<i>Enterococcus faecium</i> NCIMB 10415	Infant faeces	Inhibition of ETEC-induced TEER decrease and IL-8 secretion	Klingspor <i>et al.</i> , 2015; Lodemann <i>et al.</i> , 2015
<i>L. reuteri</i> I5007	Piglet intestine	Reduction of LPS-induced pro-inflammatory cytokines and TJ proteins	Yang <i>et al.</i> , 2015a
<i>L. rhamnosus</i> ATCC 7469	<i>n.d.</i>	Attenuation of ETEC-induced NF- $\kappa$ B signaling; enhancement of barrier integrity	Zhang <i>et al.</i> , 2015
<i>L. rhamnosus</i> GG ATCC 53103 <i>L. johnsonii</i> P47-HY <i>L. reuteri</i> P43-HUV	Human adult faeces Pig ileal digesta Pig ileal digesta	Induction of cytoprotective HSP, preservation of barrier function	Liu <i>et al.</i> , 2015
<i>B. infantis</i> MCC12 <i>B. breve</i> MCC1274	<i>n.d.</i> <i>n.d.</i>	Antiviral activity, modulation of TLR3-triggered pathway	Ishizuka <i>et al.</i> , 2016
<i>L. reuteri</i> LR1	Weaned piglet faeces	Inhibition of ETEC adhesion and pro-inflammatory cytokine expression, maintenance of membrane barrier integrity	Wang <i>et al.</i> , 2016
<i>E. faecium</i> HDRsEf1	Pig faeces	Inhibition of ETEC-induced IL-8 secretion and TEER decrease	Tian <i>et al.</i> , 2016

Table 2. Influence of prebiotics on porcine microbiota composition

Prebiotics	Dose	Age and period of treatment	Analyzing method	Results	Reference
Resistant starch	Diet high in resistant starch (34%)	Adult female pigs received diet for 2 weeks.	16S rRNA V6-8 PCR amplicons on DGGE and PITChip	Stimulation of <i>Faecalibacterium prausnitzii</i> and reduction of <i>E. coli</i> and <i>Pseudomonas</i> spp. in the colon.	(Haenen <i>et al.</i> 2013)
Inulin	Diet supplemented with 3% inulin	Pigs of 9-12 weeks of age received the diet for 3 or 6 weeks.	FISH with 59-Cy3 labeled 16S or 23S rRNA oligonucleotide probes	Inulin supplementation increased the number of pigs harboring Bifidobacteria.	(Loh <i>et al.</i> 2006)
	Diet supplemented with 4% inulin	Anaemic piglets of 5 weeks of age received the diet for 6 weeks.	Amplification of 16S rDNA targeted probes	Inulin supplementation increased <i>Lactobacillus</i> and <i>Bifidobacterium</i> populations in the caecum.	(Tako <i>et al.</i> 2008)
	Diet supplemented with 1.5% inulin	Piglets weaned at 28 days of age, fed the diet for 11 days, experiment performed at commercial and experimental farms	16S rRNA V6-8 PCR amplicons on DGGE, and cultivation-dependent methods	Inulin supplementation increased bacterial diversity, but did not affect metabolites, changes were more obvious under commercial farm conditions	(Janczyk <i>et al.</i> 2010)
Lactose	125 and 215 g/kg	Piglets of 33 days of age were assigned to 12-day period of commercial creep feed, followed by 28 days of experimental diets.	Cultivation-dependent methods	Pigs offered 215 g/kg lactose had a higher Bifidobacteria population in their faeces than pigs offered 125 g/kg.	(Pierce <i>et al.</i> 2007; Pierce <i>et al.</i> 2006)
FOS	Diet supplemented with 5% FOS	Piglets were weaned at 26 to 28 days of age, then fed the experimental diets for 4 weeks.	Cultivation-dependent methods	FOS increased Bifidobacteria and reduced <i>E. coli</i> in the proximal colon.	(Gebbinck <i>et al.</i> 1999)
GOS	Diet supplemented with 4% GOS	35 day old male pigs received the experimental diet for 34 days.	Bacterial enumeration by fluorescence in situ hybridization (FISH)	GOS increased the number of Bifidobacteria in the proximal and transverse colon.	(Tzortzis <i>et al.</i> 2005)
Oat-derived beta-glucans	Diet supplemented with 8.95% of oat beta-glucan concentrate	Piglets were weaned at 21 days of age, then fed the experimental diets for 2 weeks.	Quantitative PCR	Oat beta-glucan raised Lactobacilli and Bifidobacteria numbers in the colon.	(Metzler-Zebeli <i>et al.</i> 2011)
Yeast-derived beta-glucans	Diet supplemented with 250 ppm beta-glucans	49 day old pigs were fed the experimental diets for 28 days.	Cultivation-dependent methods	Yeast beta-glucans reduced <i>Enterobacteriaceae</i> counts in the colon.	(Sweeney <i>et al.</i> 2012)
Arabinoxylans	Diet designed to hold 17% of Arabinoxylans	Adult female pigs received diet for 3 weeks.	Quantitative PCR assays on 16S ribosomal DNA	Higher number of <i>Faecalibacterium prausnitzii</i> , <i>Roseburia intestinalis</i> , <i>Blautia coccoides</i> - <i>Eubacterium rectale</i> , <i>Bifidobacterium</i> spp. and <i>Lactobacillus</i> spp.	(Nielsen <i>et al.</i> 2014)

Table 3. Influence of probiotics on porcine microbiota composition.

Probiotics	Dose	Age and period of treatment	Analyzing method	Results	Reference
Complex <i>Lactobacilli</i> preparation, which included <i>Lactobacillus gasseri</i> , <i>L. reuteri</i> , <i>L. acidophilus</i> and <i>L. fermentum</i>	10 <sup>5</sup> CFU/ml in drinking water	Piglets were weaned at 28 days. Treatment was first week after weaning.	Cultivation-dependent methods	Decrease of <i>E. coli</i> and increase of <i>Lactobacilli</i> numbers in digesta and mucosa of most sections of the GI tract.	(Huang <i>et al.</i> 2004)
<i>Bifidobacterium longum</i> strain 75119	Twice an oral dose of 10 <sup>10</sup> CFU	Piglets were weaned at 18 days. Treatment was on day 1 and day 3 after weaning.	Cultivation-dependent methods	Reduced numbers of total anaerobes and <i>Clostridia</i> , and increased numbers of <i>Bifidobacteria</i> in faeces.	(Estrada <i>et al.</i> 2001)
<i>E. coli</i> Nissle 1917	10 <sup>8</sup> CFU single dose	One week old gnotobiotic pigs were orally mono-associated.	Cultivation-dependent methods	Reduced <i>Salmonella</i> Typhimurium translocation after challenge.	(Splichalova <i>et al.</i> 2011)
<i>L. sobrius/amylovorus</i> DSM 16698	10 <sup>10</sup> CFU/day	Administration throughout entire experiment.	Cultivation-dependent methods	Reduced ETEC levels in the ileum after challenge.	(Konstantinov <i>et al.</i> 2008)
<i>Enterococcus faecium</i> strain CECT 4515	10 <sup>6</sup> CFU/g feed	28 day old piglets were given the diet for 4 weeks.	Cultivation-dependent methods	Increased counts of <i>Lactobacilli</i> in ileum, caecum and faeces and reduced numbers of coliforms at the ileum level.	(Mallo <i>et al.</i> 2010)
<i>Lactobacillus plantarum</i> strains DSM 8862 and 8866	Single dose with 5 x 10 <sup>9</sup> or 5 x 10 <sup>10</sup> CFU	Single oral dose either before (25 days of age) or at the time point of weaning (28 days of age)	Cultivation-independent methods	Increased bacterial diversity and abundance of potentially health-promoting bacteria when probiotics were administered at 28 days of age	(Pieper <i>et al.</i> 2009)
<i>Bacillus subtilis</i> LS 1-2	4,5 g fermentation biomass/kg feed	21 day old piglets were given the diet for 4 weeks	Cultivation-dependent methods	Reduced <i>Clostridium</i> and coliforms counts in the caecum.	(Lee <i>et al.</i> 2014)

Table 4. Influence of probiotics and prebiotics on pathogen adhesion and diarrhea in pigs

Probiotics/prebiotics	Dose	Age and period of treatment	Results	Reference
<i>Bacillus cereus</i> var. <i>toyoi</i> spores	1.9x10 <sup>9</sup> spores/g feed	Pigs weaned at d 28 of age, 5 weeks	Reduced diarrhea score and number of ETEC with probiotics	Papatsiros <i>et al.</i> 2011
<i>Bacillus pumilus</i> WIT 588	5x10 <sup>10</sup> spores per pig and >10 <sup>10</sup> spores per pig (topdress)	Pigs 1-21 days after weaning	Reduction in numbers of <i>E. coli</i> in ileum	Prieto <i>et al.</i> 2014
<i>Enterococcus faecium</i> EK130	2x10 <sup>9</sup> CFU/piglet	Piglets 1-14 days of age	Reduced number of <i>E. coli</i> in faeces	Strompfova <i>et al.</i> 2006
<i>Lactobacillus acidophilus</i> or <i>Pediococcus acidilactici</i>	1.7 x 10 <sup>7</sup> CFU/ml	Weaned piglets 18-21 days old, 5 weeks	Reduction in number of coliforms when fed <i>L. acidophilus</i> compared to <i>P. acidilactici</i>	Wang <i>et al.</i> 2012
<i>Bacillus licheniformis</i> DSM5749. <i>Bacillus subtilis</i> DSM5750	3.9x10 <sup>8</sup> CFU/day (low dose) 7.8x10 <sup>8</sup> CFU/day (high dose)	Weaned piglets 21-36 days of age	Ameliorated pathophysiological changes cause by <i>E. coli</i> F4 infection.	Zhou <i>et al.</i> 2015
<i>Lactobacillus plantarum</i> strains DSM 8862 and 8866	Single dose of 3x10 <sup>9</sup> or 3x10 <sup>10</sup> CFU at 28 days of age, 2h after infection with 3x10 <sup>9</sup> CFU <i>Escherichia coli</i> (O149:K91:F4ac)	Weanling piglets, 28 days of age	No differences in performance, reduced incidence of diarrhea with single dose of 3x10 <sup>10</sup> CFU <i>L. plantarum</i> after infection with <i>E. coli</i>	Pieper <i>et al.</i> 2010
Reuteran and levan (10 g/L) produced by <i>L. reuteri</i> TMW 1.656 and LHT5794		Weanling gilts (n=6), 5-6 weeks of age, challenged with ETEC K88	Tendency for reduced number of adhering ETEC K88 bacteria to the mucosa of intestinal segments infused with ETEC	Chen <i>et al.</i> 2014
Lactulose (10 g/kg) and <i>Lactobacillus plantarum</i> JC1	2x10 <sup>10</sup> CFU/day	Weanling piglets, app. 25 days old	No effect of the treatments on diarrhea	Guerra-Ordaz <i>et al.</i> , 2014
<i>E. coli</i> UM-7 and UM-2 and raw potato starch (C: control, RPS: only raw potato starch, PRO: only <i>E. coli</i> probiotic, PRO-RPS: <i>E. coli</i> probiotic and RPS)		Weanling pigs (n=40), 17 days old, challenged with <i>E. coli</i> K88	Lowest number of <i>E. coli</i> in PRO and PRO-RPS	Krause <i>et al.</i> , 2010
Fermented feed with <i>L. reuteri</i> TMW 1.656 and LHT5794		Castrated male pigs (n=36) (no challenge)	No development of diarrhea – all pigs remained healthy	Yang <i>et al.</i> , 2015b