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Dietary alternatives to in-feed antibiotics, gut barrier function and inflammation in piglets post-weaning: Where are we now?

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Abbreviations: ADFI: average daily feed intake; ADG: average daily (body weight) gain; Akt (or PKB): protein kinase B; CRP: C-reactive protein; FD4: fluorescein isothiocyanate-dextran 4 kDa; ETEC: enterotoxigenic *Escherichia coli*; GalNAc: N-acetyl-D-galactosamine; GIT: gastrointestinal tract; IAP: intestinal alkaline phosphatase; IEC: intestinal epithelial cells; IKK: Inhibitory-κB Kinase; IL: interleukins; IFA: in-feed antibiotics; IPEC: intestinal porcine epithelial cells; IRAK: Interleukin-1 receptor-associated kinase; LFCA: lactoferricin-lactoferrampin; LPS: lipopolysaccharide; LMWC: low molecular weight chitosan; MAPK: mitogen-activated protein kinase; MAPK/p38: MAPK p38 protein subunit; MLN: mesenteric lymph node; mTOR: mechanistic target of rapamycin; MyD88: myeloid differentiation primary response protein; NAC: N-acetylcysteine; NF-κB: nuclear factor enhancing kappa light chain of activated B cell; NLR: Nod-like receptor; NOD (1, 2): nucleotide-binding oligomerization domain-containing protein (1, 2); p-Akt: phosphorylated Akt; pBD-2: porcine beta-defensin-2; PQQ: pyrroloquinoline quinone; PRR: patterns recognition receptor; RelA/p65: NF-kappa-

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B p65 protein subunit; sIgA: secretory immunoglobulin A; TEER: transepithelial electrical resistance; TGF: transforming growth factor; TJ: tight junction; TLR: Toll-like receptor; TNF: tumor necrosis factor; Vit. B6: vitamin B6; ZO: zonula occludens

Highlights

- There is now an important number of diversified in-feed antibiotic alternatives for weaning piglet's diet
- In-feed antibiotic alternatives often target cellular inflammatory pathways
- The anti-inflammatory mechanisms of action differ among in-feed antibiotic alternatives
- There is a lack of a consensus approach to evaluate piglet's intestinal (and general) health

ABSTRACT

Despite that in-feed antibiotics (IFA) are still in use in a number of countries, during the last decade an important number of *in vivo* studies on alternatives to IFA have been conducted for the weaned period. This *in vivo* work complemented with *in vitro* work, carried out with intestinal porcine epithelial cells (IPEC-J2), provide a siginificant amount of knowledge that allow understand the underlying mechanisms of action of the different IFA alternatives. The main innate immune response addressed has been the Toll-like receptors (TLR)-dependent, nuclear factor enhancing kappa light chains of activated B cells (NF-κB) canonical signaling pathway. Gene expression of pro-inflammatory (interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α) and regulatory (IL-10, transforming growth factor (TGF)-β) cytokines, and sometimes their concentrations in intestinal tissues or in blood serum or plasma, as well as systemic markers (e.g., haptoglobin, C-reactive protein (CRP), calprotectin) have been investigated as outcome variables of inflammation. An important gut function, permeability which is particularly affected during weaning has been addressed directly *ex vivo* using Ussing chambers, *in vitro* with cell lines (e.g., Caco2 cells), but most often indirectly using molecular biology techniques for gene expression (and sometimes protein relative concentrations) of key tight junction (TJ) proteins. Literature analysis reveals that, taken collectively these data are rather convincing and promising. They also provide a better understanding of the modes of action of IFA alternatives especially on intestinal, and sometimes systemic inflammation. Many functional nutrients including L-amino acids and derivatives (e.g., arginine, N-acetylcysteine, glutamine, glycine, serine), plant components (e.g., terrestrial and marine polysaccharides, polyphenolic compounds) and essential oils have indeed anti-inflammatory properties when given to piglets at optimal concentrations and durations of treatment. This holds true for many probiotic microorganisms (bacteria, yeasts) that have been carefully evaluated in terms of strains, dosage and dietary or nutritional context. In a few cases, mixtures of such categories of substances (e.g., bioactive nutrients, feed components, probiotics) have been evaluated. The mixtures could be protective as the individual substances alone, but sometimes synergistic or antagonistic effects have

been disclosed. This encourages to pursue studies of interactions among IFA. One potential limitation of many published works is that they are often based only on gene expression approaches, with few studies associating physiological or functional variables (e.g., epithelial permeability). Future work aimed at controlling gut permeability and inflammation in young pigs should continue to propose novel solutions that are sustainable, environment-friendly, economically viable to the producer and acceptable to the consummer.

Keywords

In-feed antibiotics; Inflammation; Intestine; Permeability; Weaned piglet

1. Introduction

The gastrointestinal tract (GIT) is not just a tube for digesting feedstuffs and absorbing nutrients (e.g., amino acids, fatty acids, minerals, vitamins), bioactive compounds (e.g., polyphenolic derivatives) and water, but a neuroendocrine organ in permanent communication with the brain and the other organs of the body. Thus, it integrates a large diversity of stimuli (e.g., nutritional, psychological, environmental) and is highly sensitive to all kinds of stress (Li et al., 2017). The GIT is also a major hosting reservoir of a complex microbiota, which ferment nondigested dietary and endogenous (e.g., mucus) components, releasing amounts of fermentation products (e.g., short chain fatty acids, ammonia) and many other microbe-derived bioactive molecules (e.g., lipopolysaccharide, LPS). The microbiota could represent a potential danger for the host in case of a compromised gut barrier function, making the GIT a unique immune organ for enteric and distant (e.g., lung) defense. Given these anatomical, digestive, neurophysiological, microbial and immune characteristics, the GIT is a key organ determining nutrient and metabolic fluxes, redox balance and inflammatory tone, all parameters contributing to the overall performance, health and wellbeing of the animal.

Function of pig GIT has long attracted attention, especially at the time of weaning because it is highly susceptible to structural alterations (e.g., villus shortening), functional disorders (e.g., diarrhea), inflammation and infection (e.g., colibacillosis), thus hampering body accretion and feed efficiency through productive lifespan (Pluske et al., 1997; Lallès et al., 2007; Heo et al., 2013; Pluske et al., 2018). This has led to the generalised use of in-feed antibiotics (IFA) in weaner diets until microbial resistance to antibiotics in farm animals, the food chain and humans became an issue. A ban on IFA was decided in the European Union in January 2006, and was applied in other countries recently (e.g., in China, July 2020). The ban has continuously stimulated the research on IFA alternative substances for preventing or limiting post-weaning gut disorders. Data available one decade ago on "bioactive substances" as alternatives to IFA in piglets at weaning were reviewed (Lallès et al., 2009), with many promising *in vitro* data (especially on antimicrobial substances) published, but *in vivo* data were scarce. Since then, *in vivo* data have been generated importantly, often supported by complementary investigations with intestinal porcine epithelial cells (IPEC-J2) allowing the disclosure of underlying cellular and molecular mechanisms.

The aim of the present review is to analyse the information on dietary factors and supplements modulating intestinal inflammation in piglets at weaning, considering systemic inflammation biomarkers when available. Experimental challenges with lipopolysaccharide (LPS) or pathogens (e.g., enterotoxigenic (ETEC) or enterohemorragic (EHEC) *Escherichia coli* are mentioned when carried out. However, it is out of scope of the present review to discuss details of the challenge models. When data on intestinal permeability and tight junction (TJ) proteins are provided together with inflammatory biomarkers or indices, they were included in the review, as gut barrier permeability is a strong driver of local and systemic inflammation (Farré et al., 2020). Potential biomarkers have been used to evaluate gut inflammation and barrier function in farm animals (Celi et al., 2019). This review does not focus on these biomarkers nor analyses changes in gut microbiota composition or activity, as these are specific topics by themselves (e.g., on gut microbiota: Gresse et al., 2017).

2. Mechanisms of inflammation at the gut level

Intestinal epithelial cells (IEC) have a major role in the crosstalk between microbes and the host, and the maintenance of mucosal homeostasis. These cells have specialised pattern recognition receptors (PRRs) of pathogens: membrane-bound Toll-like receptors (TLR) and cytoplasmic Nod-like receptors (NLR) (Fukata et al., 2009; Abreu, 2010). In summary (for details see appropriate illustrations by Fukata et al., 2009; Abreu, 2010; Wells et al., 2011), IECs can express such PRRs, though they are normally downregulated in absence of inflammation. Other cells of innate immunity, especially antigen-presenting cells such as dendritic cells and macrophages also express PRRs but are hyporesponsive in the normal situation. Toll-like receptors recognize various microbial components, including peptidoglycan (TLR2), double-stranded RNA (TLR3), LPS (TLR4), flagellin (TLR5), lipoteichoic acid (TLR6) and single-stranded RNA (TLR7 and TLR8), diaminopimelic acid and related molecules (nucleotide-binding oligomerization domain-containing protein 1, NOD1) and muramyldipeptide (NOD2). Many of the microbial components have long been known for their potent inflammatory properties (e.g., LPS), though underlying mechanisms were unknown. The stimulation of PRRs induces immune responses leading to the release of pro-inflammatory cytokines and chemokines. Nod-like receptors triggering activates the mitogen-activated protein kinase (MAPK) pathway. Toll-like receptors triggering involves an adaptor protein (myeloid differentiation primary response protein, MyD88) and activates a major intracellular pro-inflammatory signaling pathway, the so-called canonical nuclear factor enhancing kappa light chains of activated B cells (NF-κB) pathway (for more details see e.g., https://www.cellsignal.com/contents/science-cst-pathways-immunology-inflammation/toll-like-receptor-signaling/pathways-tlr). This induces the phosphorylation of the inhibitor IκB that is then degraded, leaving the NF-κB p50p65 protein complex to translocate into the nucleus where it mediates the transcription of various target genes, including those for pro-

inflammatory cytokines and chemokines, and additional regulatory proteins (e.g., those involved in cell death and survival).

Infiltrating immune cells recruited also participate in the regulation of pro-inflammatory cytokines such as interleukins (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α. Intraepithelial cells also produce anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β, which participate in the regulation of the inflammatory response. Finally, the control of the microbiota is exerted at the mucosal level by PRRs and the subsequent induction of antimicrobial peptides and secretory IgA. The antimicrobial peptides α-defensins are constitutively produced by Paneth cells and neutrophils following NOD-2 signaling, while β-defensins are produced by IECs inductively following TLR2- and TLR4-dependent activation. Additional biomarkers of inflammation include myeloperoxidase and calprotectin, enzymes present in neutrophils infiltrating intestinal tissues, tryptase, a protease from mucosal mast cells, and CRP and haptoglobin, circulating inflammatory proteins produced by the liver. These biomarkers are often used to evaluate the inflammation in farm animals (Niewold et al., 2015; Celi et al., 2019).

3. Intestinal permeability and the tight junction protein complex

Intestinal permeability is regulated by neuro-endocrine and immune factors and is highly sensitive to all kinds of stress: psychosocial, non-infectious, infectious and nutritional. It is relevant to highlight the mechanics of intestinal permeability in this review because any reduction in its tightness would favour the translocation of pro-inflammatory compounds, and sometimes microbes from the gut lumen to the interior milieu. These, in turn would induce inflammation and subsequent host inate immune responses aimed at restoring low intestinal inflammatory tone (Farré et al., 2020). Conversely, strengthening intestinal barrier would limit inflammation.

Although intestinal barrier is a multilayered system, TJ protein complex appears as the rate-limiting factor in paracellular permeability (Landy et al., 2016). In summary, this dynamic TJ complex includes

several transmembrane proteins like occludin and various claudins, and peripheral membrane proteins like zonula occludens (ZO) proteins. Zonula occludens proteins are linked to cell cytoskeleton by F-actin and myosin II. Tight junctions are also targets and effectors of different signaling pathways (e.g., myosin-light chain kinase) involved in the modulation of TJ assembly, maintenance and barrier function. There are 27 different isoforms of claudin proteins but claudin-1 and claudin-2 are considered as the major functional proteins in TJ. Finally, TJ complex is sensitive to inflammation and inflammatory cytokines, thus leading to alterations in epithelial permeability. This explains why researchers often focus on these key TJ proteins and expression of their genes. Tight junction functionality can be assessed through measuring epithelial paracellular permeability of chemical probes (e.g., fluorescein isothiocyanate-dextran 4 kDa, FD4), or transepithelial electrical resistance (TEER) *ex vivo* with Ussing chambers or *in vivo* with soluble probes (chromium-EDTA, mannitol, etc.) (Lallès and Oswald, 2015). In that respect, it must be precised here that permeability and TEER are inversely correlated.

In the following sections, the effect of selected dietary interventions (e.g., amino acid, lipids) on gut inflammation and permeability of weaning piglets are reviewed.

4. Proteins, peptides and free amino acids

Several experiments have been conducted with various protein sources used alone or in combination and either untreated or previously hydrolysed enzymatically or fermented, and the results suggest that the effect is dependent on the protein source or mixture used and the treatment applied to them.

4.1. Protein sources and peptides

Whole bovine colostrum alone or in association with a complex dietary supplement mixture (including yeast products and various vitamins) displayed limited effects on LPS-induced

148 inflammation in weaner piglets (Bissonnette et al., 2016). In another study in weaned piglets, two
149 weeks supplementation with hyperimmune egg yolk antibodies against *E. coli* (500 mg/kg diet),
150 compared to basal diet and basal diet supplemented with antibiotics (1 g colistin sulphate and 0.15 g
151 enramycin/kg diet) had no effect on markers of inflammation (IL-1β, IL-6, IFN-γ, TNF-α), but it
152 decreased ileal *E. coli* population (as assessed by quantitative real time polymerase chain reaction) as
153 did the antibiotic-supplemented diet (Tan et al., 2019). Performance parameters were similar across
154 experimental treatments. A trial was conducted with weaning diets containing porcine spray-dried
155 plasma protein (30 g/kg) or dried egg protein (2 g/kg) and supplemented or not with IFA (622 mg
156 tetracyclin/kg for first 2 weeks followed with 28 mg carbadox/kg for additional 2 weeks) (Ruckman et
157 al., 2020b). The diet with dried egg protein only reduced jejunal IL-1β relative mRNA abundance.
158 Complex interactions were observed between antibiotic and protein source supplementation (e.g.,
159 for the pro-inflammatory cytokine IL-18 in ileal tissue) (Ruckman et al., 2020b). Gene expression of
160 analysed TJ proteins remained unaffected by dietary spray-dried plasma protein or dried egg protein.
161 Part of the benefical effects observed with the tested supplemental proteins, in absence of IFA, may
162 have resulted from increased feed intake. Growth performance was similar between IFA and
163 supplemental protein sources (Ruckman et al., 2020b).

164 Diets differing in the number and source of proteins were formulated. A simple diet (mixture of corn,
165 wheat and soybean meal) and a complex diet (simple diet supplemeted with fish meal, plasma
166 protein and whey) were given for two weeks to weaned piglets. Gene expression of TNF-α tended to
167 be higher with the simple diet (Koo et al., 2020). Jejunal permeability as measured ex vivo in Ussing
168 chambers was not affected by diet complexity, though gene expression of TJ ZO-1 and occludin-1
169 tended to be higher with the complex diet (Koo et al., 2020). Though being limited, these effects are
170 difficult to assign solely to diet complexity as plasma protein and dried whey are known for their
171 protective effects on piglet intestine, and soybean meal proteins for their antigenic properties (Dréau
172 and Lallès, 1999; Lallès et al., 2009). Piglet performance was not influenced by dietary treatments.

The effects of the balance between untreated vs. enzymatically treated (0, 70, 140 and 210 g/kg, at the expense of untreated) soybean meal on gut health was evaluated in a trial with weaned piglets fed these dets for five weeks (Ruckman et al., 2020a). The two highest levels of incorporation of the hydrolysed soybean meal resulted in reduced feed intake and piglet performance, possibly due to increased feed bitterness (Seo et al., 2008). The impact of such diets on markers of intestinal inflammation and barrier function was limited, and could be abscribed to different factors such as faster digestion and absorption or released of bioactive peptides.

In one study, weaning diet supplementation with Lactic acid bacteria-fermented rapeseed (100 g/kg at the expense of soybean meal) stimulated the intestinal immune system and reduced inflammatory cell density in jejunal epithelium and jejunal and colonic stroma, in comparison with negative (soybean meal) and positive (ZnO, 250 mg/kg) controls (Satessa et al., 2020). Piglet performance was little affected by dietary treatment (Satessa et al., 2020).

Finally, anti-inflamatory properties of dietary peptides have been extensively tested *in vitro* and in some porcine models of intestinal inflammation *in vivo*, but only in a few studies at weaning. Several tested peptides displayed anti-inflammatory properties in the different *in vitro* models (Nosworthy et al., 2016; Xu et al., 2020c; Young et al., 2012), and similar responses in weaning piglets could be expected. Considering other bioactivities reported for peptides (e.g., antimicrobial, antioxidant) (Fernández-Tomé and Hernández-Ledesma, 2020), peptide supplementation could have important implications at the weaned period.

4.2. Amino acids and derivatives

The anti-inflammatory effects of L-arginine (0.5 mM) were tested in porcine IPEC-J2 cells challenged by LPS (Qiu et al., 2019). L-arginine conteracted LPS-induced inflammation by downregulating gene expression for TLR4, MyD88, CD14, NF-κB p65 and IL-8, according to an Arg-1 mediated signaling pathway (Qiu et al., 2019). L-arginine at higher doses (1-8 mM) was shown to enhance resistance of IPEC-J2 cells to LPS-induced inflammation through inhibiting the TLR4/NF-κB and MAPK pathways

198 and increasing intestinal β-defensin expression through an mTOR-dependent pathway (Lan et al.,
199 2020).

200 A trial with a basal diet supplemented with N-acetylcysteine (NAC, 500 mg/kg diet) was conducted
201 for three weeks with weaned piglets that were challenged three times (at days 10, 13 and 20 of the
202 trial) with LPS (Hou et al., 2012). The study revealed that this molecule was able to restore growth
203 rate (feed intake unaffected) and reverse LPS-induced gut disturbances in piglets. Blood plasma
204 concentration of the gut permeability probe D-xylose was reduced and intestinal permeability was
205 improved due to higher protein abundance (relative concentration) of claudin-1 (along the entire
206 small intestine) and occludin (ileum) (Hou et al., 2012). Dietary NAC (500 mg/kg diet) attenuated the
207 effects of i.p. administration of LPS on IL-6 and TNF-α concentrations in blood plasma and duodenal,
208 jejunal and ileal tissues (Hou et al., 2013) (Figure 1). The relative expression of mRNA and protein
209 levels were also reduced in ileal tissues for TLR4 receptor and NF-κB signaling pathway (Hou et al.,
210 2013).

211 In another study, NAC was added to a diet (500 mg/kg) and fed to weaner pigs for three weeks prior
212 to being challenged once with LPS (Yi et al., 2017). N-acetylcysteine supplementation did not
213 influence piglet performance but it downregulated intestinal activation of many inflammatory
214 signaling pathways (TLR4/NF-κB and MAPK but also PI3K/Akt/mTOR, EGFR, type-I IFN) activated by
215 LPS (Yi et al., 2017). More recently, the anti-inflammatory effects of NAC were deeply evaluted *in*
216 *vitro* and *in vivo* (Lee and Kang, 2019). Pre-treatment of IPEC-J2 cells with NAC (0.5 mM) significantly
217 reduced LPS-induced TNF-α production and downregulated gene expression for NF-κB, TNF-α, IFN-γ
218 and IL-6. This treatment also alleviated epithelial cell barrier function *in vitro* through upregulation of
219 TJ ZO-1 and occludin proteins. The supplementation of NAC (500 mg/kg diet) for three weeks to
220 miniature growing pigs prior to being challenged with LPS altered the signals of several pathways in
221 the small intestine (Lee and Kang, 2019). For instance, the transcriptomic profiling of small intestinal
222 tissue revealed that nearly one thousand genes involved in immune responses, inflammation,

223 oxidation-reduction, cytokine-cytokine receptor interaction, and cytokine-mediated signaling and
224 signaling pathways (TLR, Jak-STAT, TNF) were differentially regulated (665 genes upregulated and
225 294 downregulated) by NAC supplementation (Lee and Kang, 2019). Epithelial barrier function of
226 IPEC-J2 cells and gene expression of TJ ZO-1 and occludin were also restored after NAC treatment
227 (Lee and Kang, 2019) (Figure 2). In addition, many genes involved in intestinal wound healing and
228 repair following LPS-induced adverse effects were responsive to NAC treatment.

229 L-glutamine is well known for its supportive properties to intestinal biology and function in piglets at
230 weaning (Ji et al. 2019). L-glutamine oral supplementation (1.52 g/kg BW/day) for two weeks of
231 suckling piglets reduced protein concentrations of IL-1β, IL-8 and TNF-α in jejunal tissue three days
232 after weaning in comparison to their counterpart orally supplemented with L-alanine (1.84 g/kg
233 BW/day) (He et al., 2019). Piglet performance after weaning was not influence by L-glutamine
234 supplementation before weaning.

235 The anti-inflammatory properties of L-glycine were tested in weanling piglets fed a supplemented
236 diet (10 or 20 g/kg) for four weeks and then challenged with LPS (Xu et al., 2018). L-glycine
237 supplementation decreased jejunal and ileal phosphorylation of adenosine monophosphate kinase α
238 while increasing that of mechanistic target of rapamycin (mTOR) in the ileum, and downregulated
239 different proteins of the NOD2 and TLR4 receptor activation pathways (TLR4, MyD88, TRAF6, NF-κB;
240 NOD2 and RIPK2) (Xu et al., 2018). Piglet performance was not affected by L-glycine
241 supplementation.

242 Supplementation of L-serine in a diet (2 g/kg) for weaned piglets reduced serum concentrations of IL-
243 1β, IL-6, IL-8 and TNF-α, and corresponding mRNA levels and those of pNF-κB and IκB in jejunal and
244 ileal tissues (Zhou et al., 2018). Similarly, intestinal barrier was restored as suggested by increased
245 concentrations of TJ proteins claudin-1, occludin and ZO-1 (Zhou et al., 2018). The authors suggested
246 L-serine to have beneficial effects on epithelial (cell) differentiation, TJ set up and to reduce the

activation of inflammatory pathways. L-serine supplementation increased growth rate without affecting feed intake, and decreased diarrhea (Zhou et al., 2018).

L-Threonine was provided to cover piglet nutritional requirements or slightly in excess (115% of requirement) in weaner diets fed for two weeks (Koo et al. 2020). The piglets fed the diet with higher in L-threonine displayed higher occludin gene expression and tended to have lower IL-6 mRNA levels in the jejunum (Koo et al., 2020). These (limited) beneficial effects of supplemental L-threonine may have resulted from better epithelial nutrition as suggested with longer intestinal villi and higher goblet cell densities since functional permeability was unaffected by the dietary treatment. L-threonine supplementation did not influence production parameters.

L-amino acids, especially aromatic ones are activators of the calcium-sensing receptor which is a key anti-inflammatory pathway by uptake of the danger signal extracelluar calcium (Conigrave et al., 2007; Rossol et al., 2012). An experiment was carried out to test this hypothesis in piglets and to investigate underlying mechanisms with IPEC-J2 cells (Li et al. 2018). Briefly, a basal diet was supplemented with a mixture of three aromatic amino acids (L-tryptophan, L-phenylalanine and L-tyrosine at 1.6, 4.1 and 2.2 g/kg diet, respectively) and fed for three weeks to weaned piglets challenged with LPS at the end of the trial. The main results include in LPS-challenged piglets a downregulation of intestinal and colonic inflammation through the NF-κB pathway elicited by the activation of the calcium-sensing receptor, a decrease in mRNA abundances of pro-inflammatory cytokines associated with an increase of those of two anti-inflammatory cytokines (IL-4 and TGF-β) in the gut, and reduced blood plasma concentrations of systemic markers of inflammation (cytokines and myeloperoxidase) (Li et al., 2018). Piglet performance was not influenced by diet supplementation with aromatic amino acids, except feed to gain ratio that was reduced.

Collectively, these data demonstrate that bioactive amino acids, used alone or in combination can alleviate intestinal inflammation in weaned piglets.

5. Animal and vegetal oils and fatty acids

Fish oil is rich in poly-unsaturated fatty acids (PUFAs), including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), while corn oil is rich in linoleic acid, an omega-6 fatty acid. A trial was conducted with diets supplemented with fish oil or corn oil (50 g/kg diet) and fed to weaner piglets for three weeks prior to being challenged with LPS (Liu et al., 2012). As expected, a strong anti-inflammatory effect of fish oil was evidenced, with reduced intestinal TNF-α concentration and downregulation of mRNA levels for TLR4 and downstream signal molecules MyD88, IRAK1, TRAF6 and NOD2, and its adaptor molecule RIPK2. Fish oil also decreased protein expression of intestinal NF-κB p65. Intestinal barrier function was improved with the fish oil diet as suggested by enhanced claudin-1 and occludin protein relative concentrations (Liu et al., 2012). Piglet performance was not affected by dietary treatments.

Flaxseed (*Linum usitatissimum*) oil is a source of α-linolenic acid, which is the precursor of the long-chain n-3 PUFAs DHA and EPA. Flaxseed oil was compared to corn oil (50 g/kg diet) in a trial with weaner piglets for three weeks and then challenged with LPS (100 mg/kg BW) (Zhu et al., 2018). Flaxseed oil downregulated mRNA expression of intestinal TLR4 and its downstream signals (MyD88, NF-κB), and NOD1 and NOD2 and RIPK2 (Zhu et al., 2018). Protein expression of TJ protein claudin-1 was higher in the jejunum and the ileum of piglets fed flaxseed compared to corn oil (Zhu et al., 2018). Piglet performance was not affected by dietary treatments.

6. Minerals

Higher concentrations of calcium and phosphorus (125 and 115% of respective requirements, vs. 65 and 65%) and their ratio (1.09 vs 1.00) in a weaning diet reduced IL-1β (duodenum) and tended to reduce IL-6 (duodenum and ileum) gene expression in intestinal tissue of piglets (Metzler-Zebeli et al., 2012). Of note, calcium and phosphorus stimulate intestinal alkaline phosphatase (IAP) activity

296 and this might be one important mechanisms for anti-inflammatory effects of higher dietary calcium
297 and phosphorus (Lallès, 2010, 2014).

298 Zinc oxide has long been used as a feed additive to limit post-weaning diarrhea in piglets with very
299 good results at high dosages, but concerns about soil pollution by metallic elements have imposed
300 focusing on more sophisticated zinc associations for positive results at much lower concentrations,
301 with subsequent reductions of zinc excretion in manure and soil pollution (Debski, 2016). Zinc oxide
302 used at high level (2,200 mg/kg diet) in weaning diets is known for its anti-inflammatory effects in
303 the gut (Hu et al., 2014). These authors documented decreased mRNA relative abundance of pro-
304 inflammatory cytokines IFN-γ and TNF-α in jejunal mucosa. This effect was mediated through the
305 TLR4/MyD88/IRAK-1/TRIF6 activation pathway. Zinc oxide adsorbed on the aluminosilicate clay
306 diosmectite (DS-ZnO) was added to a diet (2 g DS-ZnO providing 500 mg Zn/kg) and fed for 7 or 14
307 days to 21-day-old weaner piglets (Hu et al., 2013). This treatment was compared to the addition of
308 ZnO alone (2,250 mg Zn/kg) or of diosmectite plus ZnO added separately (2 g DS and 500 mg Zn/kg,
309 same amounts as for the DS-ZnO group) (Table 1). Tumor necrosis factor-α, IL-6 and IFN-γ gene
310 expressions in the jejunal mucosa were reduced with DS-ZnO after one week of treatment, with no
311 difference after two weeks (Hu et al., 2013). Jejunal epithelial permeability (FD4) at one and two
312 weeks of treatment was also reduced, and this was associated with increased gene expressions of TJ
313 proteins claudin-1, occludin and ZO-1 after 1 week, and occludin and ZO-1 after two weeks of
314 treatment (Hu et al., 2013). Importantly, DS and ZnO ingredients added separately, compared to DS-
315 ZnO did not confer intestinal protection nor improved performance as observed with DS-ZnO. Part of
316 the beneficial effects observed at the intestinal level with DS-ZnO may have been due to a large
317 increase in feed intake, after one week of treatment (+16% compared to the control). A recent trial
318 compared coated zinc (CZ: 500, 750 or 1,000 mg Zn/kg diet) to uncoated zinc oxide used at high
319 dosage (2,500 mg/kg diet) in weaned piglets submitted or not to ETEC challenge (Lei and Kim, 2020).
320 The results showed that blood plasma levels of IL-6 and TNF-α were reduced with CZ750 and

CZ1,000, respectively compared to the negative challenged control, with no difference with high uncoated zinc oxide treatment (Lei and Kim, 2020).

A mixture of copper and zinc adsorbed on montmorillonite (Cu/Zn-Mt) was tested for its capacity to reduce LPS-induced inflammation in 21-day-old weaner piglets when incorporated into the diet (2 g/kg corresponding to 39 and 75 mg Cu and Zn, respectively) and fed for three weeks (Jiao et al., 2017). Supplemental Cu/Zn-Mt increased gene and protein expressions of the anti-inflammatory cytokine TGF-β1, and decreased gene expression of pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α in jejunal tissue when compared with the unsupplemented LPS group. Regarding signaling pathways, Cu/Zn-Mt supplementation decreased gene expression of TLR4 receptor and its downstream signals MyD88, IRAK1, TRAF6 and downregulated protein (but not gene) expression of NF-κB p65, and increased TβRII, Smad4 and Smad7 gene expressions of the TGF-β1 canonical Smad signaling pathway in jejunal tissue (Jiao et al., 2017). Intestinal barrier permeability was also restored. In another trial with weaned piglets, the same authors incorporated either Cu/Zn-Mt (39 mg Cu and 75 mg Zn/kg) or the same amounts of mineral (Cu, Zn, Mt) ingredients separately to the diet and fed piglets for three weeks (Jiao et al., 2018). There was no LPS challenge in this trial. The adsorbed Cu/Zt-Mt, compared to the separate mineral supplementation improved piglet performance, increased IL-10 protein concentration and decreased IL-1β, IL-6 and TNF-α concentrations in the jejunal and ileal tissues (Jiao et al., 2018). The minerals added separately to the diet improved inflammation in the duodenum only when compared to the protected form (Jiao et al., 2018). This suggests that the beneficial effects of Cu and Zn on inflammation can be modulated in different parts of the small intestine when those minerals are protected.

7. Vitamins and cofactors

There are two studies reporting data on vitamin B6 supplementation during the weaning period and intestinal inflammation. The diets were either low or high in crude protein content and were

346 supplemented with vitamin B6 (4 and 7 mg/kg), and were fed for two weeks to weaned piglets (Li et
347 al., 2019a; Yin et al., 2020). Both studies indicated immunomodulatory effects of vitamin B6,
348 regardless of the crude protein content (Table 2). Piglet performance was not influenced by dietary
349 treatment.

350 Pyrroloquinoline quinone (PQQ) is a coenzyme of oxidoreductase. It is not considered as a vitamin
351 but as key nutrient, being critical in mammalian growth, development and immunity. A study was
352 carried out with PQQ provided alone (0.8 mg/kg) or with vitamin C (200 mg/kg) in the diet of weaned
353 piglets for four weeks (Hang et al., 2020). When PQQ was given alone, it drastically reduced diarrhea
354 and mRNA relative levels of pro- and anti-inflammatory cytokines (IL-6, TNF-α and IL-10) in the
355 jejunal mucosa, and increased that for occludin (Hang et al., 2020). Importantly, the protective
356 effects observed with PQQ disappeared in presence of vitamin C, indicating an antagonism between
357 these two molecules. Addiotionally, PQQ supplementation resulted in decreased sirtuin-1 and
358 acetylated-NF-κB protein expressions and increased NAD+ tissular concentrations. In terms of
359 mechanisms, the authors proposed that PQQ-induced reduction in NAD+ leading to sirtuin-1
360 activation and then to the downregulation of acetylated-NF-κB, thus resulting in reduced
361 transcription of inflammatory factors (Hang et al., 2020).

362

363 **8. Short- and medium-chain fatty acids**

364 *8.1. Butyric acid, sodium butyrate and tributyrin*

365 Butyric acid is well known for its anti-inflammatory properties on the gut and systemically. Sodium
366 butyrate (2 mM) reduced the release of pro-inflammatory cytokine IL-8 by IPEC-J2 cells in culture
367 (Farkas et al., 2014) (Table 3).

368 Piglet intestinal explants were treated with various treatments, including butyrate (5 mM) and
369 exposed (or not) to LPS challenge (Melo et al. 2016). Butyrate treatment displayed reduced

370 inflammatory responses, as suggested by the downregulation of NF-κB signaling through RelA/p65
371 pathway The anti-inflammatory effect of butyrate may have been indirect through its stimulatory
372 effect on IAP in this trial (Melo et al., 2016). This enzyme has indeed extremely potent anti-
373 inflammatory properties (Lallès, 2010, 2014, 2019). In a weaned piglet model of enterohemorrhagic
374 *E. coli* (EHEC, O157:H7) infection, butyrate (2 g/kg diet) was provided two days before the infectious
375 challenge and was shown to reduce mortality, the severity of the disease and circulating levels of the
376 pro-inflammatory cytokines IL-β, IL-6 and TNF-α (Xiong et al., 2016). The anti-inflammatory effects of
377 butyrate appeared to be related to the upregulation of gene expression of (porcine) β-defensins
378 pBD2 and pBD3 in ileal and colonic tissues (Xiong et al., 2016). Other genes that were upregulated in
379 cultured porcine macrophages (3D4/2 cells) included those of PG1-5, PMAP37 and PR39 defensins
380 (Xiong et al., 2016). Underlying epigenetic modifications of histone H3 (H3K9Ac) by inhibition of
381 histone deacetylases by butyrate were demonstrated (Xiong et al., 2016). In this work, butyrate also
382 increased antibacterial properties of and bacterial clearance by 3D4/2 macrophages. Butyrate
383 supplementation tended to increase body growth of EHEC-challenged piglets while it did not affect
384 body growth in unchallenged animals.

385 In another study, piglets supplemented with butyrate (450 mg/kg diet) for two weeks displayed
386 lesser jejunal mast cell degranulation and lesser IL-6 and TNF-α release and mRNA levels (Wang et al.,
387 2018). This was mediated through a JNK signaling pathway. Encapsulated sodium butyrate was
388 provided in the diet (960 mg/kg) of 4-week-old weaned piglets for three weeks (Grilli et al., 2016).
389 Gene expression of IFN-γ and TNF-α was increased in duodenal tissue and decreased in ileal (TNF-α)
390 and colonic (both cytokines) tissues, with no changes in the jejunum following protected butyrate
391 supplementation (Grilli et al., 2016). Reasons for longitudinal differences were unclear. Growth
392 performance was not influenced by dietary treatment. Butyrate also downregulated TLR4 gene
393 expression and increased IL-8 secretion and gene expression in porcine intestinal IPEC-J2 cells (Yan
394 and Ajuwon, 2017). The latter, surprising result was interpreted as a small pro-inflammatory action
395 of butyrate necessary for its whole anti-inflammatory and protective capacity locally (Yan and

396 Ajuwon, 2017). Importantly, butyrate improved intestinal permeability and inflammation induced by
397 LPS through specific upregulation of TJ proteins claudin-3 and claudin-4 brought about by protein
398 kinase B (Akt) signaling pathway (Yan and Ajuwon, 2017). In another study, tributyrin (2 g/kg diet;
399 52.4% of butyrate), in association or not with a mixture of antibiotics (10 mg olaquindox, 20 mg
400 colistin sulfate, and 8 mg enramycin/kg diet) was fed to weaner piglets for four weeks. Butyrate
401 supplementation itself had very limited effects on inflammation (Gu et al., 2017). Interestingly,
402 butyrate stimulated feed intake and animal growth only in presence of IFA (interaction), possibly
403 reflecting a mediation role by the gut microbiota (Gu et al., 2017).

404 *8.2. Medium-chain fatty acids*

405 The medium-chain fatty acid, capric acid (or decanoic acid; 500 µM) was shown to alleviate
406 cyclophosphamide-induced inflammation in IPEC-J2 cells in culture (Lee and Kang, 2017). More
407 precisely, capric acid decreased cell production of IL-6 and TNF-α and downregulated gene
408 expression of TNF-α and IFN-γ cytokines and also NF-κB signaling pathway, and upregulated those for
409 IL-4 and IL-10 (Lee and Kang, 2017). Improvements in epithelial barrier were associated with gene
410 expression upregulation for occludin and ZO-1 (Lee and Kang, 2017). Part of the positive effects of
411 capric acid on IPEC-J2 cells may have also been related to the upregulation of porcine antimicrobial
412 peptides pDB2 and pDB3 (Zeng et al., 2013). *In vivo*, anti-inflammatory findings for capric acid, similar
413 to those documented in IPEC-J2 cells were also observed in cyclophosphamide-treated miniature pigs
414 (Lee and Kang, 2017).

415

416 **9. Bacteria and yeast probiotics**

417 *9.1. Bacteria*

418 A trial was conducted with dietary *Lactobacillus acidophilus* supplementation (0.5, 1 or 2 g
419 corresponding to 0.25, 0.5 or 1.0x10^{11} CFU/kg diet), together with negative (basal diet) and positive

(250 mg flavomycin/kg) control treatments (Qiao et al., 2015). The two highest doses of *L. acidophilus* were as effective as flavomycin in enhancing piglet growth rate (without influencing feed intake) (Qiao et al., 2015). A second trial without antibiotic but with an infectious challenge (ETEC K88 given orally) was set up for investigating underling mechanisms (Li et al., 2016). The probiotic was found to downregulate gene expression of various cytokines (IL-1β, IL-8 and TNF-α) and receptors (TLR2 and TLR4) in piglet mesenteric lymph node (MLN) and spleen, that were upregulated with ETEC K88 challenge (Li et al., 2016). In the spleen, *L. acidophilus* also restored IL-10 gene expression and altered signaling pathways through reducing phosphorylation of NF-κB/p65 and MAPK/p38 and increasing gene expression of various negative regulators of TLRs signaling (Tollip, IRAK-M, A20 and Bcl-3) (Li et al., 2016) (Table 4).

Lactobacillus amylovorus (DSM 16698T, $1.25x10^8$ CFU/ml) was shown to suppress the activation of TLR4 signaling in piglet jejunal explants previously challenged with ETEC ($5x10^6$ CFU/ml) (Finamore et al., 2014). This was achieved by inhibiting ETEC-induced TLR4 and MyD88 phosphorylation of many proteins of the NF-κB signaling pathway (IKK α and β, IκBα, NF-κB/p65) and reducing IL-8 and IL-1β gene expressions. These effects also involved the upregulation of genes of negative regulators (Tollip and IRAK-M) (Finamore et al., 2014).

The protective effects of the probiotic *L. casei* (strain Zhang) against infection with ETEC K88 was evaluated in 2-week-old piglets fed a diet supplemented with *L. casei* (10^7 CFU/kg diet) for four weeks (Wang et al., 2019c). *Lactobacillus casei* was provided to piglets as preventive and/or curative treatment, being given before or after two weeks of ETEC challenge. *Lactobacillus casei* was able to downregulate ETEC-induced TLR4, TLR2, IL-17 and TNF-α gene expressions in jejunal tissue, irrespective of the mode of treatment (preventive, preventive and curative or curative alone) (Wang et al., 2019c). Permanent (preventive or curative) *L. casei* administration drastically boosted jejunal tissue secretory immunoglobulin A (sIgA) concentration. Jejunal barrier function was also improved

444 with relative protein concentrations of ZO-1 and occludin being increased, except with the curative
445 alone treatment (Wang et al., 2019c). Performance data were not reported in that study.

446 Three Japanese publications reported on the protective effects of the probiotic *L. jensenii*, strain
447 TL2937 on weaned piglet intestine, and investigated underlying mechanisms in a primary culture of
448 intestinal epithelial cells collected from unsuckled piglet neonates (Shimazu et al., 2012; Suda et al.,
449 2014; Kobayashi et al., 2016). *Lactobacillus jensenii* TL2937 reduced the inflammation caused by
450 ETEC or LPS challenge in TLR4-dependent NF-κB and MAPK activation pathways also involving
451 upregulation of various TLR negative regulators (Shimazu et al., 2012). A complementary
452 transcriptomic study provided an overview of the immunomodulatory effects of this probiotic,
453 notably revealing differential expression regulation of many chemokines, complement and
454 coagulation factors (Kobayashi et al., 2016). The growth trial carried out with 3-week-old weaned
455 piglets lasted for 24 weeks until pig slaughter at a commercial body weight (Suda et al., 2014).
456 Briefly, *L. jensenii* TL2937 supplementation (6.10^{10} cfu/d), compared to no spupplementation
457 resulted in decreased blood plama CRP concentration and improved growth performance, slaughter
458 age and meat quality (Suda et al., 2014).

459 The probiotic *Lactococcus lactis*, strain pAMJ399-LFCA/LLMG1363, which actively produces the
460 molecules lactoferricin and lactoferrampin (LFCA) was tested in a trial with piglets fed a
461 supplemented diet ($5x10^9$ CFU/kg) for four weeks (Song et al., 2019a). The basal diet (negative
462 control) was supplemented with the antibiotic aureomycin (75 mg/kg) for the positive control group.
463 The *L. lactis* strain not producing these molecules (pAMJ399/LLMG1363) was also provided at the
464 same bacterial concentration to another group of piglets. Administration of pAMJ399-
465 LFCA/LLMG1363 improved piglet performance, increased serum IgG, sIgA, IL-2, IL-10 and TGF-β
466 concentrations and drastically (-50%) reduced serum endotoxin levels (Song et al., 2019a). Intestinal
467 permeability was also improved, with gene expression of TJ proteins claudin-1, occludin and ZO-1
468 being upregulated (Song et al., 2019a). On the other hand, the LFCA non-producing *L. lactis* strain,

469 although having similar effects on piglet performance as the LFCA-producing one, was unable to
470 modulate inflammatory responses or barrier function. These comparisons support a specific role for
471 lactoferricin and lactoferrampin combination in these anti-inflammatory effects. In terms of
472 performance, LFCA supplementation was as effective as aureomycin in sustaining animal growth and
473 feed efficiency and reducing diarrhea incidence (Song et al., 2019a).

474 *Clostridium butyricum* is a butyrate-producing, Gram-positive anaerobe found in the gut and other
475 environments. *Clostridium butyricum,* strain CGMCC13951 was incorporated into a diet ($5x10^8$
476 CFU/kg) and fed to piglets for two weeks before oral challenge with ETEC K88 (Li et al., 2018).
477 *Clostridium butyricum* was shown to reduce the ETEC-induced increase in pro-inflammatory
478 cytokines (IL-1β and IL-18) and to increase that of anti-inflammatory cytokines (IL-10) in blood
479 plasma. Cytokine gene expression in jejunal tissues followed the same pattern (Li et al., 2018).
480 Jejunal permeability altered by ETEC challenge was also improved *by C. butyricum* treatment, with
481 protein relative tissue concentrations of ZO-1, claudin-3 and occludin being increased. Piglet
482 performance was similar across treatments. In a second trial by this research group, *C. butyricum*
483 (strain UCN-12, 10^8 CFU/kg diet) was anti-inflammatory with decreased blood plasma TNF-α and
484 increased ileal mucosa IL-10 and TLR2 mRNA expression in challenged ETEC K88 piglets (Chen et al.,
485 2018b). Piglet performance did not differ across treatments, including between the basal diet and
486 the positive control (75 mg chlortetracycline and 20 mg enramycin/kg diet), but it is important to
487 note that all the diets were supplemented with 3,000 mg ZnO/kg for the first two weeks of the trial,
488 which could have lead to similar performance. In a third trial with weaned piglets challenged with
489 LPS, *C. butyricum* ($6x10^9$ CFU/kg diet) decreased blood serum levels of IL-1β and TNF-α and increased
490 that of IFN-γ (Wang et al., 2019a). Jejunal expression of inflammation signaling pathway-related
491 genes (TLR4, MyD88 and NF-κB) was also downregulated (Wang et al., 2019a). Piglet performance,
492 including feed intake and diarrhea scores were improved with *C. butyricum* supplementation. In a
493 fourth trial with a *C. butyricum*-based mixture containing two additional probiotics (*B. subtilis* and *B.*
494 *licheniformis*) 100 mg probiotics per kg (*C. butyricum* CGMCC 9386 at 5.0×10^{10} CFU/g, *B. subtilis*

CGMCC 9383 at 5.0×10^9 CFU/g, and *B. licheniformis* CGMCC 9385 at 5.0×10^9CFU/g) reduced piglet blood serum and ileal mucosa concentrations of TNF-α, IL-1β and IL-6 (Cao et al., 2019). Piglet performance was (or tended to be) increased, and diarrheoa decreased to the level of the positive control (100 mg colistin sulphate/kg).

The probiotic *Enterococcus faecalis* ($2x10^{10}$ CFU/kg diet) in LPS-challenged waened piglets reduced blood serum levels of IL-1β and TNF-α and increased those of IFN-γ, and gene expression was downregulated for different proteins of the NF-κB signaling pathway (TLR4, MyD88, NF-κB) (Wang et al., 2019a). Piglet performance, including feed intake and diarrhea scores were improved with *E. Faecalis* supplementation. The effects of *E. faecium* (strain HDRsEf1, $5x10^7$ CFU/ml) and its cell-free supernatant were investigated with IPEC-J2 cells (Tian et al., 2016). The results showed a reduced release of IL-8 by cultured cells and an improvement in epithelial permeability with both treatments (Tian et al., 2016). This suggests a role for soluble bioactive compounds from the probiotic. Another *E. faecium* strain, NCIMB 415 displayed similar effects on IPEC-J2 cells challenged with ETEC (Klingspor et al., 2015). However, this probiotic had no protective effects against enteropathogenic *E. coli* in this model.

9.2. Yeast and yeast derivatives

Live yeast (*Saccharomyces cerevisiae,* strain CNCM I-4407, 10^{10} CFU/kg diet) was fed for two weeks to weaned piglets challenged with ETEC K88, in comparison with a basal diet and the same diet supplemented with antibitoics and zinc oxide (20 mg colistin + 75 mg aureomycin + 2,100 mg ZnO /kg diet) (Che et al., 2017). Live yeast supplementation reduced inflammation in piglets challenged with ETEC K88, IL-1β and NF-κB gene expressions were downregulated in MLN, but not in ileal tissues. Intestinal permeability was improved and claudin-1 protein relative ileal tissue concentration was increased, and diarrhea score was lower with yeast supplementation (Che et al., 2017). Piglet performance was not improved to the level observed with the antibiotic-zinc-containing diet. However, in another study with *E. coli* F4 challenge in ETEC-sensitive piglets, systemic inflammation,

520 measured with blood haptoglobin and CRP concentrations, was not influenced by this live yeast
521 supplementation ($5x10^{10}$ CFU/kg diet) (Trevisi et al., 2017).

522 *In vitro*, bakers' yeast *S. cerevisiae* β-glucan at low dose (5 µg/ml, vs. 50 µg/ml) increased gene
523 expression of IL-8 (but not IL-6) in IPEC-J2 cells pre-treated with LPS (Palócz et al., 2019). When yeast
524 β-glucan was added at 250 mg/kg in the diet of growing pigs (49 days of age and 18.8 kg BW at the
525 initiation of the trial), gene expression of four pro- and anti-inflammatory cytokines (IL-1α, TNF-α, IL-
526 10 and IL-17A) in colonic (but not intestinal) tissues decreased (Sweeney et al., 2012). This was
527 associated with a reduction in numbers of colonic Enterobacteriaceae, as also observed with β-
528 glucans from seaweeds (Sweeney et al., 2012). These data demonstrate the anti-inflammatory
529 properties of yeast β-glucan on gut inflammation. Dietary treatment effects were minimal on pig
530 performance in this study.

531 Yeast nucleotides were added to a basal diet (50, 150, 250 and 500 mg/kg) and fed for three weeks
532 to 19-day-old weaned piglets (Jang and Kim 2019). Jejunal mucosa IL-6 concentrations were lower
533 with nucleotides provided at 50 and 150 mg/kg diet. Finally, a mixture of yeast culture, cell wall
534 hydrolysates and yeast extracts were added to a diet (1.2 g/kg) fed for three weeks to 21-day-old
535 piglets (Yang et al., 2016). The yeast mixture increased IL-10 protein concentration in jejunal and ileal
536 tissues and downregulated gene expression of the TJ proteins ZO-1 (jejununal and ileal tissues) and
537 occludin (jejunal tissue) (Yang et al., 2016).

538

539 **10. Exogenous enzymes**

540 Many dietary components are poorly digested, especially in weaning piglets where feed component
541 digestion is compromised due to gut histological and enzymatic alterations. Enhancing nutrient
542 digestion with exogenous enzyme supplementation might be a valuable way to improve feed
543 efficiency and piglet performance, and to decrease the growth of potentially harmful microorganisms
544 (Kiarie et al., 2013) as less specific substrates could be available for fermentation.

545 Exogenous carbohydrases (xylanase, β-glucanase, and pectinase; 0.1, 0.1 and 0.01 g/kg diet,
546 respectively) were added to weaner diets enriched in either insoluble or soluble fiber and fed for two
547 weeks to weaned piglets (Li et al., 2019b). Three days after infection with ETEC, blood plasma
548 concentrations of haptoglobin and CRP were lower with the carbohydrase-supplemented diet with
549 soluble fiber and insoluble fiber, respectively (Li et al., 2019b). The relative abundances of TNF-α and
550 occludin mRNA were lower in ileal and colonic tissues of ETEC-challenged piglets fed the diet
551 enriched in insoluble fiber and carbohydrases (Li et al., 2019b). Piglet performance was enhanced
552 with exogenous carbohydrase supplementation.

553 A trial was conducted with diets supplemented (0.1 g/kg) with xylanase, an enzyme mixture
554 (cellulase and β-glucanase with a small xylanase activity) or both and fed to weaned piglets for four
555 weeks (Li et al., 2019c). Colonic mRNA levels of IL-17, occludin and claudin-3 were higher in piglets
556 supplemented with the combined enzymes, but not with the separate preparations (Li et al., 2019c).
557 Combining both sources of enzymes also decreased blood plasma concentrations of IL-1β and TNF-α.

558 Exogenous (bovine) IAP (4 IU/ml) was shown to downregulate LPS-induced, NF-κB-dependent
559 inflammatory response via RelA/p65 in piglet intestine explants (Melo et al., 2016). Intestinal alkaline
560 phosphatase is physiologically produced by the enterocyte in the small intestine, is still partially
561 active along the large intestine and is a potent anti-inflammatory molecule (Lallès, 2010, 2014, 2019).
562 Therefore, similar properties are expected for IAP when provided exogenously.

563

564 **11. Animal- and plant-derived biochemical components**

565 *11.1. Carbohydrates from animal products and terrestrial plants*

566 N-acetyl-D-galactosamine (GalNAc) is an essential amino sugar derived from lactose. As some
567 oligosaccharides have shown health properties, the effects of GalNAc (0 to $2x10^{-4}$ mmol/L) on
568 inflammation were evaluated in IPEC-J2 cells challenged by soybean agglutinin lectin (Zhao et al.,

2019). N-acetyl-D-galactosamine was shown to alleviate alterations induced by soybean agglutinin, especially epithelial permeability according to a quadratic dose-dependent manner. This was associated with gene upregulation and higher protein expression of TJ claudin-3 and occludin (Zhao et al., 2019). Importantly, the anti-inflammatory enzyme IAP produced by IPEC-J2 cells responded also quadratically to GalNAc (IAP nadir for $0.5x10^{-4}$ mmol GalNAc/L). This led to a linear and negative correlation between TEER and IAP activity (Zhao et al., 2019). These data look odd because IAP is a potent anti-inflammatory intestinal enzyme (Lallès, 2010, 2014, 2019) which has been documented to control intestinal permeability through stimulating TJ proteins in cell cultures and mice (Liu et al., 2016; Plaeke et al., 2020).

Oat β-glucan added (90 g/kg, at the expense of starch) to a diet fed for 14 days to piglets had contrasted effects on inflammatory gene expression: tendency for a reduction for IL-1 in the duodenum and an increase in the ileum, and an increase for IL-6 in the caecum (Metzler-Zebeli et al., 2012) (Table 5). These results cast some doubt on the anti-inflammatory properties of oat β-glucan in piglets, though no proper biomarkers of inflammation or functional tests (e.g., permeability) were used in this study. Piglet performance was not influenced by dietary treatments in this study.

A mannan-rich fraction (Actigen®, 800 mg/kg diet) was fed to weaning piglets for four weeks (Song et al., 2019b). The positive control diet was supplemented with a mixture of antibiotics (100 mg colistin sulfate, 100 mg olaquindox and 50 mg kitasamycin/kg diet). Actigen® reduced TNF-α and increased IL-10 in blood plasma, but had no effect on intestinal tissue TLR4 pathway activation (Song et al., 2019b). Piglet performance was unaffected, but diarrhea incidence was equally reduced in the actigen and positive control groups (Song et al., 2019b).

Lentinan, a mushroom polysaccharide present as commercial preparation (50% lentinan, with polyphenol, protein, amino acid, fat, etc. in the other 50%) added to a weaning diet (200 mg/kg) and fed to piglets for three weeks displayed anti-inflammatory properties as supported by reduced gene expression and protein concentration in jejunal (TNF-α) and ileal (IL-1β, IL-6 and TNF-α) tissue (Wang

et al., 2019b). There was also a downregulation of genes involved in the pro-inflammatory NF-κB signaling pathway and TLR4 and NOD1 receptor activation (MD2, MyD88, IRAK1, TRAF6, NF-κB; NOD1, RIPK2) especially in the ileum (Wang et al., 2019b). Intestinal barrier function was also strenghtened as suggested by lentinan-dependent increase in claudin-1 protein relative concentration in the ileum (Wang et al., 2019b). Part of the anti-inflammatory effects may have been due to increased short-chain fatty acids, especially butyrate concentration in ileal digesta, and subsequent inhibition of histone H3 acetylation which was higher with the lentinan-supplemented diet (Wang et al., 2019b). Piglet performance was similar between the two treatment groups.

The immunomodulatory effects of wheat bran were investigated in IPEC-J2 cells and in weaned piglets fed experimental diets for four weeks (Hermes et al., 2011; Chen et al., 2017). Wheat bran was shown to be anti-inflammatory, downregulating gene expression of IL-8 and TNF-β *in vitro* (Hermes et al., 2011), and of IL-1β, IL-6 and TNF-α *in vivo*, through the TLR4/MyD88/NF-κB pathway (Chen et al., 2017). The anti-inflammatory effects were associated with reduced *E. coli* and increased *Lactobacilli* and *Bifidobacteria* in the intestine (Chen et al., 2017). In another study testing the interactions between dietary fermentable fiber (mixture of wheat bran and sugar beet pulp) and fermentable protein (high vs. low protein diet: 200 vs. 147 g CP/kg diet) with weaned piglets, fermentable fiber improved dietary high fermentable protein-induced colonic protein fermentation and microbial ecology but also increased gene expression of IL-1β, IL-10 and TGF-β (Pieper et al., 2012). The authors concluded that gene expression upregulation of the anti-inflammatory cytokines IL-10 and TGF-β was probably sufficient to prevent colonic tissue inflammation. Piglet performance was unaffected by dietary treatments in this study.

11.2. Marine-derived polysaccharides

The anti-inflammatory properties of seaweed β-glucans in piglets have been demonstrated at the University College Dublin. These researchers had previously shown that seaweed extracts fed to weaned piglets stimulated body growth and feed efficiency (O'doherty et al., 2010). In a subsequent

study, they showed that laminarin (300 mg/kg diet) from brown seaweeds (*Laminaria* spp.) downregulated gene expression of three proinflammatory cytokines, namely IL-1β, IL-6, IL-17A, and one anti-inflammatory (IL-10) cytokine in proximal colonic tissues of (unchallenged) weaned piglets after 1 week of treatment (Walsh et al., 2013). The anti-inflammatory effects of laminarin were related to a reduction in numbers of pathogens, in particular attaching and effacing *E. coli* in the colon (Walsh et al., 2013). The downregulation of IL-10 was explained as the result of decreased inflammation. Interestingly, the beneficial effect of laminarin disappeared in presence of another seaweed component, fucoidan (240 mg/kg diet) indicating an antagonism between these two components on gut inflammation. In another study with older and heavier piglets (49 days of age; 19 kg BW at the beginning of the trial), the same results were confirmed (Sweeney et al., 2012). Additionally, they showed that laminarin purified from two different brown seaweeds (*L. digitata* and *L. hyperborea*) had distinct modes of actions on inflammation and different impacts on the colonic microbiota. The *L. digitata* soluble laminarin anti-inflammatory mechanisms might have involved GPR-43 receptors trigged by short-chain fatty acids whose concentration increased with supplemented diets (acetic and propionic acids in the ileum and acetic and valeric acids in proximal colon) and a reduction in Enterobacteriaceae numbers. By contrast, those of the insoluble *L. hyperborea* laminarin, which had no effects on Enterobacteriaceae and on colonic concentrations of short-chain fatty acids appeared to be independent from the GPR-43 receptor activation pathway (Sweeney et al., 2012). Another group of piglets supplemented with laminarin from *L. digitata* and challenged with LPS displayed a pro-inflammatory response, as suggested by increased IL-8 gene expression (Sweeney et al., 2012). These important studies demonstrate the potential of seaweed β-glucans in modifying the gut microbiota and alleviating inflammation. They also illustrate negative interactions between feed additives.

Chitosan, chemically close to plant fiber cellulose is a polymer of α-(1-4)-D-glucosamine present in the shell of various marine organisms (prawns, krill, oysters, etc.) (Xiao et al., 2013). A weaner diet was supplemented with low molecular weight chitosan (LMWC, MW < 5 kDa, degree of deacetylation

645 >90%; 300 µg/kg diet) and fed to 21-day-old piglets for three weeks and then challenged with ETEC
646 (Xiao et al., 2014) (Table 5). Although LMWC treatment did not influence circulating cytokine
647 concentrations, it reduced jejunal tissue inflammation as assessed using calprotectin and tissue TLR4
648 protein relative concentration, and gene expression of the pro-inflammatory cytokines IL-1β and IL-6
649 (Xiao et al., 2014). In another study, a weaner diet was supplemented with LMWC (300 µg/kg diet)
650 and fed to piglets for three weeks (Huang et al., 2016). After i.p. challenge with LPS on days 14 and
651 21 of the study, the piglets were slaughtered. Low molecular weight chitosan treatment alleviated
652 the growth and inflammation observed in LPS-challenged piglets (Huang et al., 2016). These piglets
653 displayed at day 14 lower blood serum concentrations of TNF-α, IL-6, and IL-8 associated with lower
654 intestinal mRNA expression of genes coding for these pro-inflammatory cytokines, and higher gene
655 expression for the anti-inflammatory cytokine TGF-β1 (Huang et al., 2016). Other pro-inflammatory
656 cytokines with downregulated gene expression included IL-1α and IL-1β, IL-2, IL-8 and IFN-γ (Huang
657 et al., 2016). Inflammation repression was through NF-κB signaling transduction pathway inhibition,
658 with reduced p-NF-κB p65, IKKα/β, and IκB protein expressions (Huang et al., 2016). Inflammation
659 inhibition involved calcium-sensing receptor stimulation, thus suggesting LMWC to act as a calcium-
660 sensing receptor agonist (Huang et al., 2016). The dietary treatment had no effect on piglet
661 performance in this experiment.

662 In another study, a diet was supplemented with LMWC (20-30 kDa, 50 mg/kg diet) obtained from
663 high MW (1,000 kDa) carbohydrate chitosan by radiation pyrolysis technology (Hu et al., 2018). The
664 diet was fed to 21-day-old piglets for four weeks. Low molecular weight chitosan supplementation
665 displayed anti-inflammatory effects as shown by reduced gene expression of IL-1β and TNF-α, and
666 tendency for increased gene expression of IL-10 in jejunal mucosa of chitosan-supplemented piglets
667 (Hu et al., 2018). Tight junction protein ZO-1 gene expression was also enhanced in this tissue (Hu et
668 al., 2018). Part of this beneficial effect of LMWC may have been due to parallel increase in feed
669 intake (+9%). Piglet growth rate also tended to be increased in the LMWC group. In another study,
670 LMWC (100 mg/kg diet) was fed for 12 days to weaned piglets then challenged with ETEC and killed

671 three days later (Wan et al., 2019). Low molecular weight chitosan treatment downregulated gene
672 expression of the pro-inflammatory cytokines IL-6 and TNF-α, reduced mucosal mast cell tryptase
673 content and increased TJ occludin protein concentration in jejunal tissue (Wan et al., 2019). It also
674 downregulated gene expression of TLR4, TNFR1 anti-apoptotic protein and nuclear NF-κB p65 protein
675 in jejunal and ileal mucosa (Wan et al., 2019). Piglet performance was not influenced by dietary
676 treatment or ETEC challenge. *In vitro*, LMWC with degree of polymerization varying between 2 and 7
677 (concentrations used: 1, 10 and 100 µg/ml) was shown to downregulate gene expression of IL-8 and
678 MCP-1, through protein kinase A (PKA) signaling in IPEC-J2 cells previously challenged with TNF-α as
679 an inducer of inflammation (Yang et al., 2018). Tight junction protein claudin-1 gene expression was
680 also downregulated, but this did not affect epithelial barrier function (as measured by TEER) (Yang et
681 al., 2018). Chitosan nanoparticle (400 mg/kg diet) added to a weaner diet fed for four weeks to
682 piglets, then challenged with LPS reduced circulating levels of IL-1β and IL-6 and slightly increased
683 that of TNF-α (Xu et al., 2020b).

684 *11.3. Polyphenolic compounds*

685 The protective effects of various phenolic compounds alone or in mixtures (e.g., in plant extracts) on
686 the gut of piglets after weaning have been investigated in a number of experiments.

687 Eleutheroside B present in the herbal plant *Acanthopanax senticosus* is a phenylpropanoid glycoside
688 with anti-inflammatory and immunomodulatory properties, among others. Its effects were
689 investigated with increasing concentrations (varying from 0 to 0.20 mg/ml) in IPEC-J2 cells (Che et al.,
690 2019). Eleutheroside B improved epithelial cell permeability and increased the relative concentration
691 of TJ proteins (claudin-3, occludin and ZO-1), decreased gene expression of pro-inflammatory
692 cytokines (IL-6, IFN-γ, TNF-α) and increased that of anti-inflammatory cytokines (IL-10 and TGF-β). By
693 contrast, the activity of IAP, a potent anti-inflammatory enzyme was reduced (Che et al., 2019), a
694 result that is intriguing. No data are presently available *in vivo* with eleutheroside B.

Chlorogenic acid makes part of a complex family of polyphenols present in plants and which display antioxidant and anti-inflammatory properties (Liang and Kitts, 2015). Supplementing weaner diet with chlorogenic acid (1 g/kg) reduced gene expression of pro-inflammatory cytokines in the jejunum (IL-1β, TNF-α) and ileum (IL-6 and TNF- α), and NF-κB inflammatory pathway was also downregulated (Chen et al., 2018a). Intestinal permeability was improved in line with increased gene expression of various TJ proteins (ZO-1, occludin and claudin-1 in the jejunum; ZO-1 and claudin-1 in the ileum) and reduced tissue density of mucosal mast cells (Chen et al., 2018a). These data are consistent with those obtained on the anti-inflammatory effects of chlorogenic acid (25-50 µM) on IPEC-J2 cells in culture (Palócz et al., 2016). Piglet performance was not reported in the study by Chen et al. (2018a).

The polyphenol curcumin, extracted from curcuma (*Curcuma longa*) is known for its anti-inflammatory, antioxidant and anti-microbial properties (Ghosh et al., 2018). A trial conducted in 21-day-old weaner piglets reported many beneficial effects of curcumin added at 300 mg/kg in the diet (Gan et al., 2019). Protein concentration and gene expression for pro-inflammatory cytokines and TLR4 were decreased in intestinal tissues (Table 6) (Gan et al., 2019). Similar effects were recorded for diet supplementation with the polyphenol resveratrol (Table 6) (Gan et al., 2019). Synergistic effects of curcumin combined with resveratrol were observed only for TNF-α concentration, which decreased in ileal tissue. Piglet performance was not reported in this study.

Holly polyphenols were evaluated (65.5% polyphenols in the extract included at 250 mg/kg diet) as possible protective substances in piglets submitted or not to a LPS challenge after 16 days adaptation to the diet (Xu et al., 2020a). The results revealed a strengthening of intestinal barrier (claudin-1 mRNA) in the jejunum of holly polyphenols LPS-challenged piglets (but not in controls, interaction) and in the ileum (claudin-1 and occludin mRNA), of supplemented unchallenged piglets (Xu et al., 2020a). Although blood plasma pro-inflammatory cytokines IL-6 and TNF-α levels were reduced, cytokines or their gene expression in intestinal tissues were not conducted. Investigating local

719 inflammatory pathways did not provide a clear picture. Piglet performance was unaffected by this
720 polyphenol extract.

721 *Agrimonia procera* is an herbal plant containing bioactive tannins like agrimoniine. Two studies were
722 conducted with *A. procera* supplementation in piglets (Gräber et al., 2014, 2018). In the first
723 investigation, porcine peripheral blood mononuclear cells displayed lower IL-1β and TNF-α six hours
724 after challenge with LPS *in vitro* when *A. procera* extract (0.1%/ml) was added to the culture medium
725 (Gräber et al., 2014). In the second study, *A. procera* was provided for six weeks at two dosages (0.87
726 and 8.7 g/kg diet) to piglets weaned at four weeks of age (Gräber et al., 2018). Little anti-
727 inflammatory effects of this plant were observed at both concentrations (Gräber et al., 2018). The
728 treatment had no impact on piglet performance.

729 The protective effects of guava (*Psidium guajava L.*) leaf extract were recently evaluated in weaned
730 piglets (Wang et al., 2020b). Briefly, the extract was characterised by mass spectrometry and
731 comprised 323 compounds including 91 phenolic molecules. This extract was incorporated at varying
732 levels (50, 100 and 200 mg/kg) in diets fed *ad libitum* for four weeks to weaned piglets. Compared to
733 a negative control, this extract improved jejunal barrier as supported by increased jejunal ZO-1,
734 occludin-1 and occludin relative protein concentrations, and reduced jejunal mRNA expression of
735 three pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) (Wang et al., 2020b). This extract brought
736 the level of these barrier and inflammatory biomarkers to those observed with a positive control (50
737 mg quinocetone/kg diet) even at the lowest dose of leaf extract. Piglet performance was unaffected
738 by treatments but they reduced diarrhea compared to the negative control (but not the positive
739 control).

740 *11.4. Essential oils*

741 Essential oils are very diverse molecules with an “oily” concistency when they are concentrated.
742 However, it is to be emphasized they have nothing in common with proper organic (as opposed to
743 mineral) oils.

744 A diet supplemented with cinnamon essential oil (50 mg/kg diet) was fed for four weeks to 28-day-
745 old piglets (Wang et al., 2015). Cinnamon oil treatment upregulated gene expression of TJ protein
746 claudin (and occludin in the ileum) and IAP all along the gut (Wang et al., 2015). Given this, IAP may
747 have been the main cause for the anti-inflammatory effects observed.

748 The essential oil tea tree oil is produced by the Australian plant *Melaleuca alternifolia* belonging to
749 the Myrtacea family. It is rich in 4-terpilenol, p-cimene, γ-terpinene, and α-terpineol, among other
750 chemicals. A study with different doses of tea tree oil (50, 100, 150 mg/kg diet) was conducted in 21-
751 day-old piglets for three weeks (Dong et al., 2019). Tea tree oil increased linearly protein
752 concentrations of IL-1β, IL-10, IL-12 and TNF-α (Ileum) and IL-2 (jejunum and ileum) (Dong et al.,
753 2019). Gene expression for IL-2 and IL-10 increased linearly in the jejunum, while those for IL-1β
754 (jejunum and ileum), IL-2 and TNF-α (ileum) varied quadratically, with the highest expressions
755 observed for the intermediate tea tree oil dose (100 mg/kg) (Dong et al., 2019). Gene expression for
756 TJ proteins increased linearly for occludin in the jejunum and varied quadratically for occludin in the
757 ileum, with a maximum expression at the intermediate tea tree oil dose too (Dong et al., 2019).
758 Finally, piglet growth rate and feed intake increased linearly with tea tree oil supplementation,
759 suggesting that part of the inflammation inhibition could be related to increased feed intake.

760 The essential oil thymol (50 µM), extracted fom thyme (*Thymus vulgaris*) was shown to reduce
761 inflammation and physiological alterations caused by LPS in IPEC-J2 cells (Omonijo et al., 2019).
762 Thymol tended to reduce LPS-induced IL-8 secretion by epithelial cells and reduced mRNA
763 abundance for IL-8 and TNF-α. It also improved epithelial barrier (TEER) and ZO-1 protein
764 concentration, this without affecting gene expression of TJ proteins ZO-1 and claudin-3. One study
765 investigated the protective effects of thymol (0 to 510 mg/kg) added to weaning diets and fed to 28-
766 day-old piglets for two weeks (Toschi et al., 2020). Piglet performance was unaffected by dietary
767 treatments. Thymol increased duodenal gene expression of the inflammatory cytokine TNF-α but this
768 was not confirmed at the protein level (Toschi et al., 2020). Complementary work is probably

769 required. Thymol has been investigated in different *in vivo* settings, but usually in bioactive
770 substance blends so that it is difficult to report on the effect of thymol *per se* on inflammation and
771 barrier function in piglets.

772 *11.5. Miscellaneous*

773 Diets supplemented with the algeae spirulin (brackish water) or chlorella (fresh water) (385 mg/kg
774 BW/day) did not show significant effects on gene expressions of pro-inflammatory cytokines in
775 piglets, except for IL-1β (jejunum) and IL-8 (ileum), which were higher two weeks after weaning in
776 the chlorella and spirulin groups, respectively (Furbeyre et al., 2018). Piglet performance was not
777 influenced by the treatment. More investigation with these promising algeae is required.

778

779 **12. Other factors influencing gut inflammation and permeability**

780 Several studies have shown promising results when weaned piglet diet was supplemented with a
781 range of commercial products. However, the reponse to these dietary interventions could be
782 compromised by other non-dietary factors (e.g., bodyweight at birth) that can affect the response of
783 a piglet to the dietary intervention. Thus, in the following sections, some of these relevant factors are
784 highlighted with other potential substances.

785 *12.1. Sow's nutrition*

786 Since the formulation of the "developmental origin of health and diseases" concept in the 80's, it is
787 now clear that nutrition and environment in early life of mammals impact many of their future body
788 growth and metabolic trajectories (Suzuki, 2018), and this hold true for the porcine species (Lallès,
789 2012). Sows' nutrition is a powerful lever for reducing inflammation and improving health in their
790 offspring (Gonzalez-Bulnes et al., 2016).

791 Inflammation-related parameters in sow's milk and in offspring were highly influenced in sows
792 receiving diets enriched in sugar beet pulp (SBP, 200 g/kg in gestation and 100 g/kg in lactation) or

793 wheat bran (WB, 300 and 150 g/kg) (Table 7, Shang et al., 2019). These fiber sources were
794 incorporated in the diets at the expense of corn. Sows supplemented with SBP ate more and had
795 colostrum richer in IgA and IL-10. Milk IL-10 was increased by both supplementations. Offspring born
796 to SBP sows were heavier at weaning. Serum levels of endotoxin and inflammatory cytokine (IL-6,
797 TNF-α) were reduced, and ileal tissue secretory IgA concentrations increased with both treatments,
798 while IL-10 plasma levels were higher in offspring born to SBP sows only. Ileal mRNA relative levels
799 were decreased for TNF-α, and increased for IL-10 and TJ ZO-1 with both treatments, though more
800 with SBP. Finally, gene expression was lower for IL-6 and higher for TJ occludin with the SBP
801 treatment. Overall, SBP given to sows had the strongest effects on anti-inflammatory indices in
802 offspring (Shang et al., 2019). However, it should be mentioned that the SBP diet had more digestible
803 energy (+7% during gestation) and more soluble fiber (+120 and +60% during gestation and lactation,
804 respectively) than the WB diet, differences that might have contributed to the overall larger effect
805 with SBP.

806 Mannan oligosacharides added to diets for sows and their offspring (400 and 800 mg/kg diet) were
807 shown to have anti-inflammatory effects both in the small intestine and systemically (Duan et al.,
808 2019). Supplemented sows had offspring displaying reduced gene expression of the pro-
809 inflammatory cytokine IL-8 and of NF-κB p65 protein in their intestinal lymphatics. The
810 concentrations of pro-inflammatory cytokines IL-2 and IL-4 in serum were also lower in offspring
811 born to mannan-supplemented sows, and were even lower when offspring also consumed it (Duan et
812 al., 2019). Conversely, serum concentrations of IL-10 were enhanced in piglets supplemented with
813 mannan oligosacharides.

814 The polyphenol resveratrol was anti-inflammatory in offspring when fed (500 mg/kg diet) to
815 pregnant sows for 20 days (Meng et al., 2019). In particular, offspring displayed intestinal tissues with
816 lower IL-6 and TNF-α concentrations.

817 *12.2. Piglet birth weight*

Bissonnette et al. (2016) reported that lighter weaner piglets were more sensitive to i.p. LPS challenge than heavier ones and developed stronger inflammatory responses (e.g., blood plasma TNF-α). This was confirmed in a model of low birth-weight pigs. Feeding sows with low-energy diet during the entire gestation period resulted in low birth weight offspring that were more sensitive to LPS challenge at weaning (Chen et al., 2017). At birth, offspring displayed higher blood plasma concentrations of IL-1β, and higher ileal gene expressions for IL-6 and TNF-α. After LPS challenge at weaning at four weeks of age, they displayed ileal gene expressions that were higher for TLR-4, IL-β and NF-κB and lower for TJ ZO-1 (Chen et al., 2017).

12.3. Glucagon-like peptide 2

Glucagon-like peptide 2 is an endogenous intestinotrophic hormone with anti-inflammatory properties (Connor et al., 2016). In weaner piglets, it was shown that microsphere-protected glucagon-like peptide 2 administered (i.p., 100 mg/piglet) the day before weaning reduced ileal tissue concentrations of IL-8, TNF-α and IFN-γ, while increasing epithelial cell proliferation (Wu et al., 2016). Intraperitoneal injection of unprotected glucagon-like peptide 2 (20 nM/kg BW/day) had more limited and contrasted effects. Piglet performance was not reported.

12.4. Glucocorticoids

Glucocorticoid hormones are potent anti-inflammatory agents. One study reported that i.m. injection of glucocorticoid (0, 0.2 and 0.6 mg/kg BW) the day before weaning reduced blood serum concentrations of IL-1β and haptoglobin at weaning (Wooten et al., 2019). Gene expressions of IL-18, IL-1β and TJ claudin-4 in jejunal tissue also tended to be lower (Wooten et al., 2019). Piglet body weight and feed efficiency were increased in the glucocorticoid treatment.

12.5. Milk exosomes

Milk exosomes that can transfer nucleic acid (RNA) materials improve intestinal and immune development of newborns and may have therapeutic applications (Galley and Bessner, 2020).

Exosomes purified from sow's milk were shown to reduce LPS-induced inflammation in porcine IPEC-J2 cells in culture (Xie et al., 2019). They reduced IL-1β, IL-6, TNF-α concentrations upon cell LPS challenge (Xie et al., 2019). A reduction in relative mRNA concentration of MyD88, but not TLR4 was also recorded. Basal phosphorylation of the NF-κB proteins IκBα and p65, increased by LPS challenge was restored with milk exosomes (Xie et al., 2019). Co-transfection experiments demonstrated that two of the three purified milk exosomes (miR-4334, miR-219) downregulated the pro-inflammatory NF-κB signaling pathway while the third one (miR-338) protected IPEC-J2 cells from apoptosis by inhibiting p53 protein pathway (Xie et al., 2019). *In vivo* data on milk exosomes are presently lacking.

12.6. Antimicrobial peptides

Antimicrobial peptides fall within two large families: defensins and cathelicidins. The protective effects of synthetic porcine beta-defensin-2 (pBD-2) were tested in a trial with 21-day-old weaned piglets (Tang et al., 2016). Piglets were administered orally with saline (negative control), 0.5 mg pBD-2 or 3 mg neomycin sulfate daily for three weeks. Jejunal levels of mRNA for IL-1β, IL-8, TNF-α and TLR-4 were downregulated by both pDB-2 and neomycin, suggesting a TLR-4-mediated anti-inflammatory action (Tang et al., 2016). This protective effect was associated with decreased rectal hemolytic *E. coli* scores and mRNA relative abundances of *E. coli, Bacteroides fragilis* and *Streptococcus*, and increased of *Lactobacilli* and *Bifidobacteria* in cecal digesta (Tang et al., 2016). Both pDB-2 and neomycin stimulated equally piglet performance.

The anti-inflammatroy properties of cathelicidin-BF, purified from snake (*Bungatus fasciatus*) venom were evaluated (i.p. injection of 0.5 mg/kg BW at day 2, 6, 10 and 14) in a trial with 21-day-old piglets for two weeks and then submitted to LPS challenge (Zhang et al., 2017). Cathelicidin-BF was able to alleviate LPS-mediated tissue inflammation as indicated by reduced jejunal mucosa density of neutrophils and concentrations of the inflammatory marker myeloperoxydase, IL-6 and TNF-α (Zhang et al., 2017). Lipopolysaccharide-induced intestinal barrier alterations were also improved through intestinal upregulation of gene expression of TJ proteins ZO-1 (duodenum, jejunum), ZO-2

(duodenum), claudin-1 (duodenum, jejunum) and occludin (jejunum) by cathelicidin-BF (Zhang et al., 2017). Cathelicidin treatment improved piglet growth rate.

These two studies support the anti-inflammatory effects of antimicrobial peptides in the gut of weaned piglets.

13. Conclusions and future prospects

More than 60 trials with weaner piglets and a dozen of investigations with IPEC-J2 cells in culture have been reported on IFA alternatives and their effects on inflammation and gut barrier over the last decade. Taken together, these data are convincing and provide a better understanding of the modes of action of the tested substances. Different intracellular signaling pathways have been evidenced, among which the canonical NF-κB. Many functional nutrients and plant components have anti-inflammatory properties when given to piglets at the optimal concentration and duration. This also holds true for a large variety of probiotic microorganisms (bacteria, yeasts) that need to be carefully evaluated in terms of strains, dosage and dietary context. In a few cases, mixtures of such categories of substances (e.g., nutrient, feed components, probiotics) have been evaluated. They can be protective as the individual substances are, but sometimes there is no effect or even negative effects. This raises again the question of interactions between individual bioactive substances that must be evaluated thouroughly every time in every situation (Lallès and Guillou, 2015). One potential limitation of many mechanistic investigations is that they are often based on gene expression rather than physiological and functional approaches. It must be reminded here that gene expression and functions are not always correlated (see e.g., Richter et al., 2014). This review highlights the need for additional investigations (i) with purified fatty acids in order to evaluate their specific actions compared to oils, and (ii) on long-term effects of early (e.g., sow's and neonatal offspring) nutritional interventions. New experimental techniques *in vitro*, such as intestinal organoids will be valuable in the future as complementary tools for investigating the effects and mechanisms of action of

892 nutrients, minerals, vitamins and novel feed compounds on intestinal health in weaned piglets (e.g.,
893 Wang et al., 2020a). Future work for reducing inflammation and gut barrier dysfunction should
894 continue to unravel new solutions that are environment-friendly, economically viable to the
895 producer and acceptable to the consummer. These studies may be relevant to human health as the
896 pig is a valuable biomedical model (Roura et al., 2016).

897

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901

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903 None.

904

905 **Author contributions**

906 JP Lallès: paper conceptualization; literature review; paper writing; illustration making; validation;
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908 CA Montoya: literature review; paper writing; validation; review and editing.

909

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Table 1. Influence of zinc oxide (ZnO) provided in different forms on growth performance parameters, jejunal barrier function and mucosal inflammation on weaned piglets (data expressed in percentage of a control diet without ZnO) (adapted from Hu et al., 2013).

	0-7 days post-weaning			7-14 days post-weaning		
Treatment	DS + ZnO	DS-ZnO	ZnO alone	DS + ZnO	DS-ZnO	ZnO alone
			Production data			
ADFI	105	116*	115*	102	109*	108*
ADG	105	117*	115*	103	112*	110*
Faecal scores	94	46*	41*	92	51*	56*
			Barrier function in the jejunum			
TEER	105	111	107	104	112	113
FD4 flux	89	65*	59*	90	61*	65*
Occludin protein	124	171*	176*	116	161*	146*
Claudin-1 protein	129	152*	162*	111	143*	125
ZO-1 protein	138	219*	200*	114	152*	166*
			Jejunal cytokine mRNA relative concentration			
TNF-α	69	20*	28*	69	72	56
IL-6	81	34*	30*	124	112	144
IFN-γ	77	41*	32*	113	146	88

DS + ZnO: zinc oxide and diosmectite provided separately (500 mg Zn/kg diet); DS-ZnO: zinc oxide adsorbed on diosmectite (500 mg Zn/kg diet); ZnO alone: zinc oxide added alone at a high dose (2,250 mg Zn/kg diet).

ADFI: average daily feed intake; ADG: average daily (body weight) gain; FD4: fluorescein isothiocyanate-dextran, molecular weight 4,000 Da; IFN-γ: interferon-gamma; IL: interleukin; TEER: transepithelial electrical resistance; TNF-α: tumor necrosis factor-alpha; ZO-1: zonula occludens-1.

** Different from control treatment ($P < 0.05$).*

Table 2. Influence of supplementation of a low- or high-crude protein diet with vitamin B6 on gene expression of cytokines in intestinal tissues of weaned piglets after two weeks of treatment (variations relative to unsupplemented respective basal diets) (adapted from: Li et al., 2019a; Yin et al., 2020).

	Low-protein diet (142 g/kg)		High-protein diet (220 g/kg)	
	+4 mg vit. B6/kg	+7 mg vit. B6/kg	+4 mg vit. B6/kg	+7 mg vit. B6/kg
Jejunum	IL-10↘, TGF-β↘	IL-10↘, TNF-α↘(*t*)	*ns*	*ns*
Ileum	IL-1β↗, TNF-α↗, IL-10↗, TGF-β↗	IL-1β↗	*ns*	IL-1β↗(*t*), TNF-α↗(*t*), TGF-β↗

IL: interleukin; Vit. B6: vitamin B6; TGF: transforming growth factor; *TNF-α: tumor necrosis factor-alpha.*

ns: non-significant; (t): tendency (P < 0.10).

Table 3. Summary of the various anti-inflammatory effects (relative to challenge or weaning alone in studies without infectious challenge, depending on the study) and modes of action of butyrate (Bu) and tributyrin (TBu) on IPEC-J2 cells or pig intestine.

	Cell/Animal/ Challenge	Markers of inflammation – Molecular mechanisms	Reference
Bu 2 mM	IPEC-J2, LPS	IL-8 secretion ↘	Farkas et al. (2014)
Bu 5 mM	Pig intestinal explants, LPS	RelA/p65 protein expression ↗ NF-κB signaling ↘	Melo et al. (2016)
Bu 0.1 – 1 mM	IPEC-J2, LPS	IL-8 secretion and mRNA ↗ TLR4 mRNA ↘ Akt/mTOR signaling ↗ (p-Akt ↘)	Yan and Ajuwon (2019)
Bu 2 g/kg diet	Pig, entero-hemorraghic infection	Blood plasma IL-β, IL-6 and TNF-α ↘ Epigenetic modifications of histone H3 by inhibition of histone deacetylases	Xiong et al. (2016)
Bu 0.45 g/kg diet	Pig, weaned	Mucosal mast cell degranulation ↘ Mast cell IL-6 and TNF-α and mRNA ↘ JNK signaling (protein and phosphorylation) ↘	Wang et al. (2018)
Bu 0.96 g/kg diet (protected)	Pig, weaned	Duodenal IFN-γ and TNF-α mRNA ↘ Ileal TNF-α mRNA ↘ Colonic IFN-γ and TNF-α mRNA ↘	Grilli et al. (2016)
TBu 2 g/kg diet	Pig, weaned	No effects reported	Gu et al. (2017)

Akt (or PKB): protein kinase B; IFN-γ: interferon-gamma; IL: interleukin; IPEC: intestinal porcine epithelial cell; LPS: lipopolysaccharide; mTOR: mechanistic target of rapamycin; NF-κB: nuclear factor enhancing kappa light chains of activated B cells; RelA/p65: NF-kappa-B p65 protein subunit; p-Akt: phosphorylated Akt; TNF-α: tumor necrosis factor-alpha; TLR, Toll-like receptors.

Table 4. Summary of the various anti-inflammatory effects and modes of action of probiotic bacteria on IPEC-J2 cells or piglet intestine (and mesenteric and spleen).

Probiotic bacteria	Cell/Animal/ Challenge	Markers of inflammation – Molecular mechanisms	Reference
L. acidophilus (10^{11} CFU/kg diet)	Pigs, ETEC K88	IL-1β, IL-8 and TNF-α mRNA ↘ (MLN & spleen) TLR2 and TLR4 mRNA ↘ (MLN & spleen) IL-10 mRNA ↗ (spleen) NF-κB/p65 and MAPK/p38 phosphorylation ↘ (spleen) Tollip, IRAK-M, A20 and Bcl-3 mRNA ↗ (spleen)	Li et al. (2016)
L. amylovorus (DSM 16698T, 1.25x10^8 CFU/ml)	Pig jejunal explants, ETEC K88	IL-8 mRNA ↘ TLR4 and MyD88 phosphorylation ↘ NF-κB (IKK α and β, IκBα, NF-κB/p65) ↘	Finamore et al. (2014)
L. casei (strain Zhang) (10^7 CFU/kg diet)	Pigs, ETEC K88	Jejunal TLR4, TLR2, IL-17 and TNF-α mRNA ↘	Wang et al. (2019c)
L. plantarum (strain 2142, 10^9 CFU/ml)	IPEC-J2, LPS	IL-6, IL-8 and TNF-α mRNA ↘	Palócz et al. (2016)
L. jensenii (strain TL2937, 5x10^7 CFU/ml)	PIE, LPS or ETEC	TLR4-dependent NF-κB and MAPK activation pathways ↘ TLR negative regulators ↗	Shimazu et al. (2012)
L. jensenii (strain TL2937, 6x10^{10} CFU/day, orally)	Pigs, weaned	Plasma CRP ↘	Suda et al. (2014)

Lactococcus lactis ($5x10^9$ CFU/kg)	Pigs, weaned	No effects (compared to the strain pAMJ399-LFCA/LLMG1363, see below)	Song et al. (2019a)
L. lactis, strain pAMJ399-LFCA/ LLMG1363 ($5x10^9$ CFU/kg)	Pigs, weaned	Plasma IL-2, IL-10 and TGF-β ↗ Plasma endotoxin (LPS) ↘	Song et al. (2019a)
Clostridium butyricum ($5x10^8$ CFU/kg diet)	Pigs, weaned, ETEC K88	Plasma IL-1β and IL-18 ↘ Plasma IL-10 ↗ Jejunal IL-1β and IL-18 mRNA ↘ and IL-10 mRNA ↗	Li et al. (2018)
C. butyricum UCN-12 (10^8 CFU /kg diet)	Pigs, weaned, ETEC K88	Plasma TNF-α ↘ Ileal IL-10 and TLR2 mRNA ↗ (no effects at 1 and 8 g *C. butyricum*/kg diet)	Chen et al. (2018b)
C. butyricum ($6x10^9$ CFU/kg diet)	Pigs, weaned, LPS	Serum IL-1β and TNF-α ↘ Serum IFN-γ ↗ Jejunal TLR4, MyD88 and NF-κB mRNA ↘	Wang et al. (2019a)
Enterococcus faecalis ($2x10^{10}$ CFU/kg diet)	Pigs, weaned, LPS	Serum IL-1β and TNF-α ↘ Serum IFN-γ ↗ Jejunal TLR4, MyD88 and NF-κB mRNA ↘	Wang et al. (2019a)
E. faecium (strain HDRsEf1, $5x10^7$ CFU/ml) or its cell-free fraction	IPEC-J2, LPS	IL-8 secretion ↘	Tian et al. (2016)
E. faecium (strain NCIMB 415, $5x10^8$ CFU/ml)	IPEC-J2, ETEC	IL-8 secretion and mRNA ↘	Klingspor et al. (2015)

CRP: C-reactive protein; ETEC: enterotoxigenic Escherichia coli; IFN-γ: interferon-gamma; IKK: Inhibitory-κB Kinase; IL: interleukin; IPEC: intestinal porcine epithelial; IRAK: Interleukin-1 receptor-

associated kinase; LFCA: lactoferricin-lactoferrampin; LPS: lipopolysaccharide; MAPK: mitogen-activated protein kinase; MAPK/p38: MAPK p38 protein subunit; MLN: mesenteric lymph node; MyD88: myeloid differentiation primary response protein; NF-κB: nuclear factor enhancing kappa light chains of activated B cells; PIE : Porcine intestinal epitheliocyte primary cells fron unsuckled piglet neonate; TNF-α: tumor necrosis factor-alpha; TLR, Toll-like receptor.

Table 5. Summary of the anti-inflammatory effects and modes of action of fractionated or purified terrestrial and marine carbohydrates on IPEC-J2 cells or pig intestine.

Carbohydrate	Cell/Animal/ Challenge	Markers of inflammation – Molecular mechanisms	Reference
Oat β-glucan (90 g/kg diet)	Pigs, weaned	Duodenal IL-1β ↘ (t) Ileal IL-1β ↗; Caecal IL-6 ↗	Metzler-Zebeli et al. (2012)
S. cerevisiae β-glucan (5 µg/ml)	IPEC-J2 cells, LPS	IL-8 mRNA ↗	Palócz et al. (2019)
S. cerevisiae β-glucan (250 mg/kg diet)	Pigs, weaned, *ex vivo* LPS	Ileum: No effect Colonic IL-1α, IL-10, TNF-a and IL-17A mRNA ↘	Sweeney et al. (2012)
Seaweed β-glucan (*Laminaria digitata or L. hyperborean*, 250 mg/kg diet)	Pigs, weaned, *ex vivo* LPS	Ileum: No effect Colonic IL-1α, IL-10, TNF-a and IL-17A mRNA ↘ Colonic IL-8 mRNA ↗ (with *L. digitate*)	Sweeney et al. (2012)
Mannan-rich fraction (800 mg/kg diet)	Pigs, weaned	Blood plasma IL-10 ↗ and TNF-α ↘	Song et al. (2019b)
Mushroom lentinan (100 mg/kg diet)	Pigs, weaned	Jejunal TNF-α mRNA ↘ Ileal IL-1β, IL-6 and TNF-α mRNA ↘ Ileal NF-κB, TLR4 and NOD1 activation ↘	Wang et al. (2019b)
Low MW chitosan (300 µg/kg diet)	Pigs, weaned, ETEC	Jejunal TLR4, IL-1β and IL-6 mRNA ↘ Jejunal calprotectin ↘	Xiao et al. (2014)

Low MW chitosan (300 µg/kg diet)	Pigs, weaned, LPS	Serum TNF-α, IL-6, and IL-8 ↘ Intestinal IL-1α, IL-1β, IL-2, IL-6, IL-8, IFN-γ, TNF-α, and IL-8 mRNA ↘ Intestinal TGF-β1 mRNA ↗ p-NF-κB p65, IKKα/β, and IκB protein ↘	Huang et al. (2016)
Low MW chitosan (50 mg/kg diet)	Pigs, weaned	Jejunal IL-1β and TNF-α mRNA ↘ Jejunal IL-10 mRNA ↗(t)	Hu et al. (2018)
Low MW chitosan (100 mg/kg diet)	Pigs, weaned	Intestinal mast cell tryptase ↘ Intestinal IL-6 and TNF-α mRNA ↘ TLR4 and NF-κB p65 protein ↘	Wan et al. (2019)
Low MW chitosan (1, 100, 100 µg/ml)	IPEC-J2 cells, TNF-α	IL-8 mRNA ↘ Protein kinase A activation	Yang et al. (2018)
Chitosan nanoparticles (400 mg/kg diet)	Pigs, weaned, LPS	Blood plasma IL-1β and IL-6 ↘ Blood plasma TNF-α slight ↗	Xu et al. (2020b)

ETEC: enterotoxigenic Escherichia coli; IFN-γ: interferon-gamma; IKK: Inhibitory-κB Kinase; IL: interleukin; IPEC: intestinal porcine epithelial; LPS: lipopolysaccharide; MW: molecular weight; NF-κB: nuclear factor enhancing kappa light chains of activated B cells; NOD1: nucleotide-binding oligomerization domain-containing protein 1; (t): tendency (P < 0.10); TNF-α: tumor necrosis factor-alpha; TLR, Toll-like receptors.

Table 6. Influence of diet supplemented with curcumin or resveratrol (300 mg/kg diet) and fed for 28 days on changes (relative to the unsuplemmented control) in intestinal inflammatory markers in piglets weaned at 21 days of age (adapted from Gan et al., 2019).

	Curcumin		Resveratrol	
	Protein	mRNA	Protein	mRNA
Jejunum	IL-1β ↘, TNF-α ↘, TLR4 ↘	IL-1β ↘, TLR4 ↘	IL-1β ↘, TNF-α ↘, TLR4 ↘	IL-1β ↘, TNF-α ↘, TLR4 ↘, IL-10 ↗
Ileum	TNF-α ↘, TLR4 ↘	IL-1β ↘, TNF-α ↘, TLR4 ↘, IL-10 ↗	TNF-α ↘, TLR4 ↘	IL-1β ↘, TNF-α ↘, TLR4 ↘, IL-10 ↗

IL: interleukin; TNF-α: tumor necrosis factor-alpha; TLR, Toll-like receptor.

Table 7. Inflammation indices (expressed as percentages of values in the unsupplemented control) on colostrum, milk and offspring in pregnant sows fed diets supplemented with sugar beet pulp (SBP) or wheat bran (WB) (adapted from Shang et al., 2019).

	SBP	WB
Sows		
Average daily feed intake	**114**	108
Colostrum IgA	**115**	109
Colostrum IL-10	**162**	123
Milk IL-10[1]	**136**	**131**
Offspring[1]		
Weight at 21 days of age	**109**	104
Serum endotoxin	**78**	**75**
Serum IL-6	**86**	**91**
Serum IL-10	**113**	108
Serum TNF-α	**78**	**84**
Ileal tissue sIgA*	**189**	**133**
Ileal tissue IL-6 mRNA	**65**	94
Ileal tissue IL-10 mRNA*	**134**	**121**
Ileal tissue TNF-α mRNA	**64**	**75**
Ileal tissue occludin mRNA	**134**	121
Ileal tissue ZO-1 mRNA*	**152**	**129**

[1] *Milk and offspring samples were collected at three weeks postpartum.*

IL: interleukin; sIgA: secretory Immunoglobulin A; sIgA: secretory immunoglobulin A; TNF-α: tumor necrosis factor-alpha; ZO: zonula occludens.

Values in bold are different from control (P < 0.05).

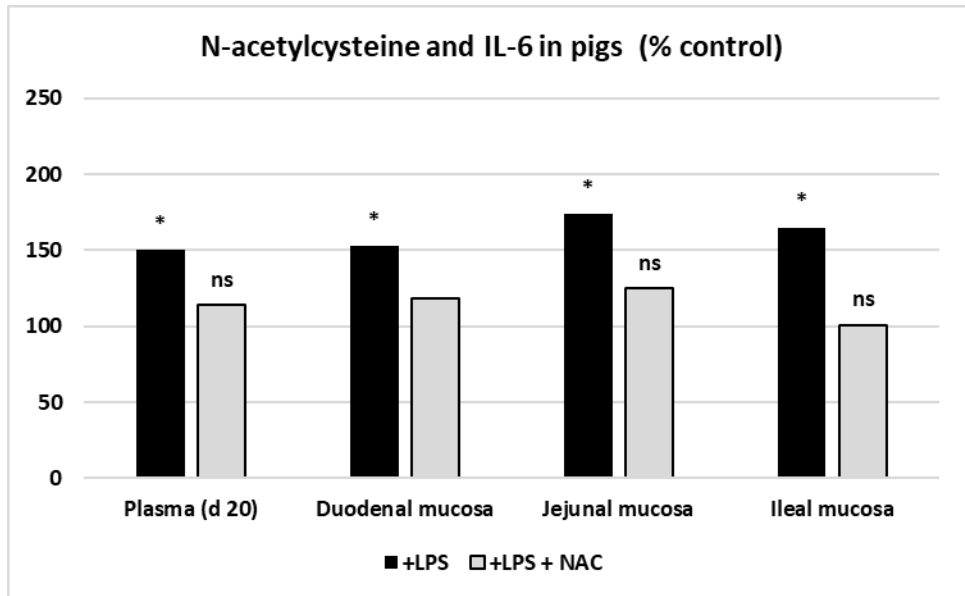
**Difference between SBP and WB treatments (P < 0.05).*

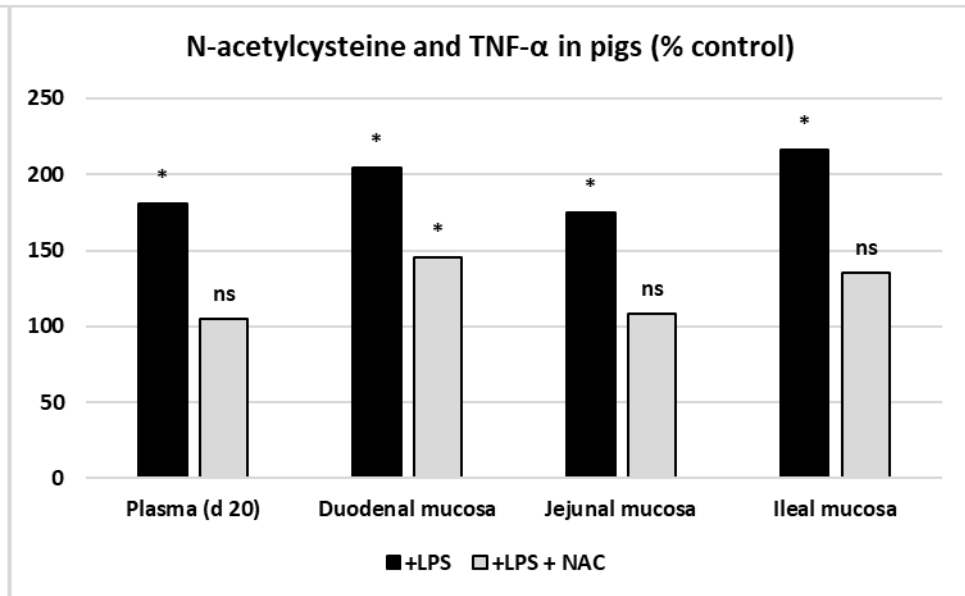
Figure legends:

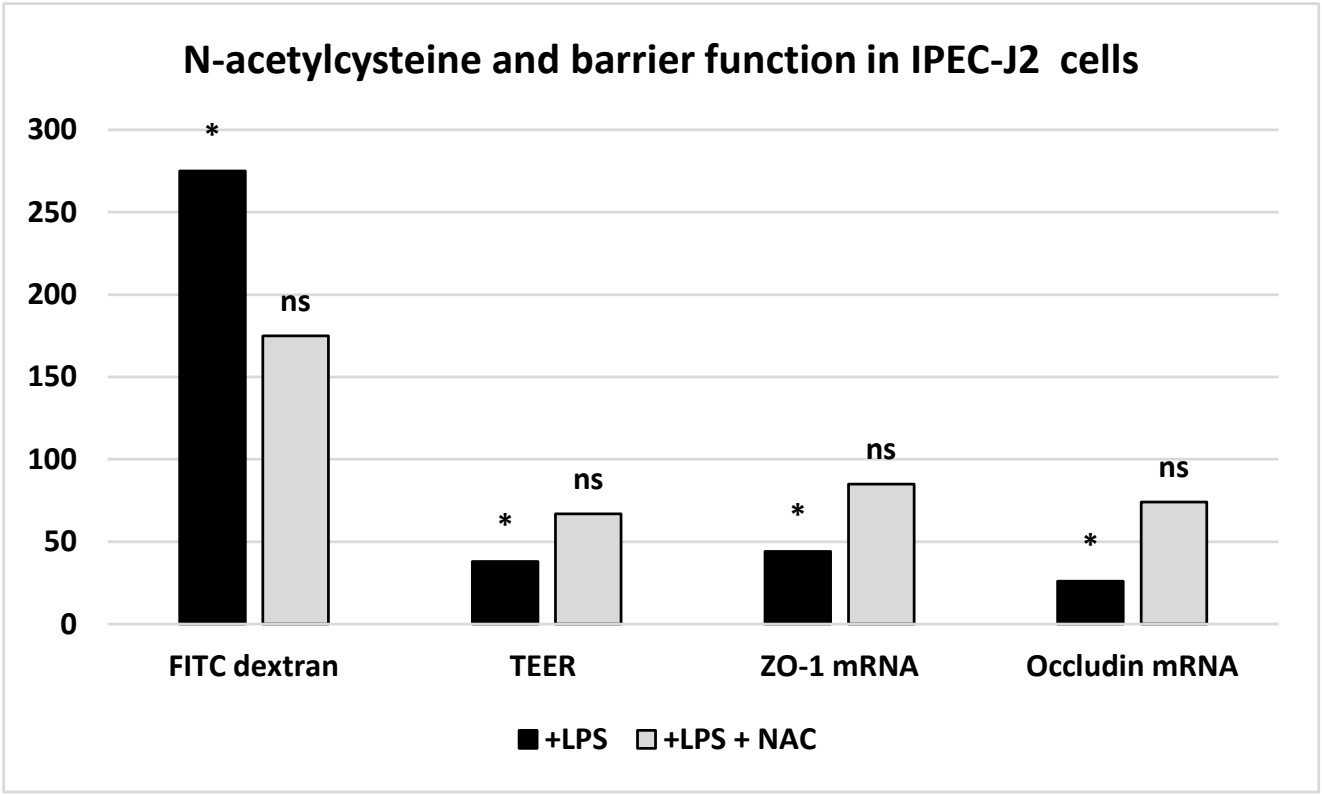
Figure 1. Influence of N-acetyl cysteine (NAC) supplementation (500 mg/kg diet) in piglets challenged with LPS on IL-6 and TNF-α in blood plasma and along the small intestinal mucosa of weaned piglets aged 28 days and slaughtered after three weeks of treatment (data expressed in percentage of control values for unchallenged animals). Blood plasma was collected at day 20 of the study, while the other samples collected at day 21. * $P < 0.05$, ns: non-significantly different from control (adapted from Hou et al., 2013).

IL: interleukin; LPS: lipopolysaccharide; TNF-α: tumor necrosis factor-alpha.

Figure 2. Influence of N-acetyl cysteine (NAC) supplementation (0.5 mM) on epithelial permeability to fluorescein isothiocyanate (FITC) dextran, trans-epithelial electrical resistance (TEER) and mRNA relative concentration of tight junction proteins zonula occludens-1 (ZO-1) and occludin in intestinal porcine epithelial cells (IPEC)-J2 cells previously challenged with lipopolysaccharide (LPS) (data expressed in percentage of control values for unchallenged cells). * $P < 0.05$, ns: non-significantly different from control (adapted from Lee and Kang, 2019).


N-acetylcysteine and IL-6 in pigs (% control)
250
200
150
100
50
0
*
ns
*
*
ns
*
*
ns
Plasma (d 20)
Duodenal mucosa
Jejunal mucosa
Ileal mucosa
+LPS
+LPS + NAC


N-acetylcysteine and TNF-α in pigs (% control)
250
200
150
100
50
0
*
ns
*
*
ns
*
*
ns
Plasma (d 20)
Duodenal mucosa
Jejunal mucosa
Ileal mucosa
+LPS
+LPS + NAC


N-acetylcysteine and barrier function in IPEC-J2 cells
300
250
200
150
100
50
0
*
ns
*
ns
*
ns
*
ns
FITC dextran
TEER
ZO-1 mRNA
Occludin mRNA
+LPS
+LPS + NAC