

1 **The relevance of functional amino acids to support the health of growing pigs**

2 **Text**

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13 **Abstract**

14 On commercial farms, young growing pigs are frequently affected by health problems from multifactorial
15 origins (e.g. environmental changes, biosecurity, management, and feed) that result in inflammation and
16 activation of body defenses. Inflammation states alter animal metabolism in such a way that nutrients
17 (particularly amino acids) are diverted from the use for growth towards the production of defense-related
18 proteins and low-molecular-weight compounds (e.g., nitric oxide, H₂S, and glutathione) for supporting the
19 activity of rapidly dividing cells such as immune cells and enterocytes. Furthermore, amino acids may act
20 specifically as signaling molecules to regulate metabolic pathways during inflammation. Thus, new
21 knowledge on the specific role and metabolism of each amino acid is needed to refine nutritional
22 recommendations for pigs of different phenotypes and genotypes, with the objective of maintaining animal
23 health and performance under sub-optimal rearing conditions. This paper aims at summarizing recent
24 advances in research on the functional roles of amino acids related to swine health. Specifically, the review
25 highlights current knowledge on the impact of inflammation on the intake and metabolism of amino acids;
26 their relevance for the physical gut mucosal barrier and antioxidant defense, as well as their roles in the

27 syntheses of defense molecules and in the regulation of immune response. Practical implications for feeding
28 strategies adapted to various health conditions of growing pigs are also discussed along with our general
29 perspectives on related research.

30

31 **Keywords:** Amino acid, Gut barrier, Inflammation, Metabolism, Nutrient requirements, Pig

32 *Abbreviations:* AAs, amino acids; APP, acute-phase protein; Arg, arginine, Asn, asparagine; Asp, aspartate;
33 CP, crude protein; Cys, cysteine; ETEC, enterotoxigenic *Escherichia coli* K88; Gln, glutamine; Glu, glutamate;
34 Gly, glycine; GSH, glutathione; His, histidine, Ig, immunoglobulin; Leu, leucine; Lys, lysine; Met, methionine;
35 NO, nitric oxide; Pro, proline; Ser, serine; Thr, threonine; TJ, tight junction; Trp, tryptophan, Val, valine; ZO,
36 *zonula occludens*

37

38 1. Introduction

39 The production performance of pigs is highly variable within a farm and among farms, and their health
40 status is one of the factors explaining such differences (Rojo-Gimeno et al., 2016). As many diseases have
41 multifactorial origins, feeding strategies should be considered to support optimal animal growth and health.
42 To this end, the impact of health on the nutritional and metabolic states of pigs needs to be determined to
43 better adjust feeding programs.

44 Among the dietary nutrients whose intake, digestion, absorption and metabolism by the pig are greatly
45 impacted during stress and inflammation, amino acids (AAs) are good candidates for feeding adjustments
46 because they have key roles in animal metabolism (Wu, 2013). As sanitary or stress challenges may cause
47 deviation from the homeostasis state in pigs, these factors also affect diverse processes involving AAs and
48 proteins. For example, the occurrence of runt pigs is associated with low concentrations of essential and
49 nonessential AAs in plasma during both the nursery and finishing periods (He et al., 2016). During the
50 recovery after a period of poor health, the utilization of sulfur AAs, threonine (Thr) and tryptophan (Trp) for
51 growth is reduced when growing pigs are fed a diet with a low content of these AAs because of competition
52 between skeletal muscle and the immune system (Kampman-van de Hoek et al., 2016). Although reports on
53 AA requirements for pigs under suboptimal sanitary conditions exist (Litvak et al., 2013; Capozzalo et al.,

54 2017; Jayaraman et al., 2017), there is a paucity of information about the potential sources of variation in
55 dietary AA requirements for the synthesis of different proteins, as well as the health and growth of tissues,
56 particularly the small intestine and skeletal muscle. This knowledge is required to further develop more
57 precise feeding programs for growing pigs.

58

59 **2. Impact of inflammation on amino acid intake, digestion, absorption and metabolism**

60 The meta-analysis by Pastorelli et al. (2012) quantified the impact of different experimental health
61 challenges on growth and feed intake of swine. The authors showed that, compared to the growth
62 performance of healthy pigs, all challenges negatively impacted growth rate and feed intake (figure 1).
63 However, the range of the responses to the challenge was variable, indicating that the relative reduction in
64 the average growth rate following a health challenge can be either totally or only partially associated with a
65 reduction in feed intake. Actually, different challenges differentially affect feed intake, as well as the
66 digestion, absorption and metabolism of nutrients, depending on which disturbance contributes to reducing
67 growth rate and feed efficiency. As a result, animal nutritionists should adjust the composition of diets
68 according to practical on-farm conditions and the challenging factors. By one side, a large quantity of several
69 proteins related to inflammation may be synthesized in the body. Among them, the proteins secreted into
70 the gut lumen are particularly relevant (see section 4 for more details), because some of them are neither
71 digested nor recycled, and thus their AAs are definitively lost for the host and should be supplied adequately
72 in the diet. This implies that adjustments to the dietary composition should be considered when nutritionists
73 take into account how AAs support health maintenance and growth. On the other side, when growth
74 restriction results mainly from reduced feed intake, energy supply may be more limiting for growth than a
75 single nutrient. Thus, the adjustments of feed composition may not necessarily favor the restoration of
76 animal health and growth. However, the provision of specific AAs involved in the control of feed intake may
77 help to improve the health status of animals. This view is consistent with the report that dietary
78 supplementation with 1 g L-Trp/kg enhanced the feed intake of pigs challenged with the enterotoxigenic
79 *Escherichia coli* K88 (ETEC), but had no effect on ETEC-resistant pigs (Trevisi et al., 2009).

80 Intestinal inflammation can affect the absorption and transport of AAs by enterocytes into the blood.
81 In mice, a *Trichinella spiralis* infection reduced serum arginine (Arg) concentration and the intestinal
82 expression of the *SLC7A7* gene (Zhou et al., 2015) that encodes for the basolateral y⁺L system transporter
83 with an affinity for lysine (Lys), Arg, glutamine (Gln), histidine (His), methionine (Met) and leucine (Leu). This
84 could be related to an increased use of Arg by the enterocytes. The perfusion of a solution containing ETEC
85 into the jejunal loops from young pigs reduced the expression of genes encoding for apical (*SLC7A9*, *SLC3A2*,
86 *SLC6A19*) and basolateral (*SLC7A7*, *SLC7A*, *SLC16A10*) AA transporters, in association with the general up-
87 regulation of genes related to the induction of inflammation (Priori et al., submitted). This finding could
88 indicate that the acute inflammation induced by ETEC may reduce the intestinal absorption of some AAs as
89 well as their concentrations in the blood.

90 Inflammation-induced changes in nitrogen metabolism have been extensively documented. In growing
91 pigs, inflammation reduces N retention in the body and increases urinary N loss (van Heugten et al., 1994;
92 Litvak et al., 2013; Campos et al., 2014; Rakhshandeh et al., 2014; Kampman-van de Hoek et al., 2015). These
93 changes result from: (a) decreased feed intake, (b) impaired digestion and absorption of nutrients, (c)
94 enhanced catabolism of body proteins, and (d) increased oxidation of AAs, and are orchestrated by
95 inflammatory cytokines and hormones. The rate of protein synthesis decreases in the skeletal muscle, but
96 increases in the liver and other tissues that are involved in the production of acute-phase (APP), defense and
97 immune proteins (Obled et al., 2002). Thus, because of the different AA composition of synthesized proteins,
98 inflammation modifies AA partitioning between the skeletal muscle and other organs or cell types, such as
99 the liver, spleen, and digestive tract, and immune cells (Klasing and Johnstone, 1991; Obled et al., 2002; Le
100 Floc'h et al., 2004). Losses of digestive enzymes and mucosal proteins and cell renewal increase during
101 digestive disturbances, leading to increased losses of endogenous AAs that can be substantial for certain AAs,
102 such as Arg, Leu, Thr, valine (Val), proline (Pro) (Adeola et al., 2016). In minipigs, acute inflammation of the
103 intestine stimulates the uptake of luminal Thr and cysteine (Cys) by enterocytes (Rémond et al., 2009 and
104 2011) to support the synthesis of mucins (see section 3) and glutathione (GSH or L-γ-glutamyl-cysteinyl-
105 glycine), a powerful antioxidant tripeptide (see section 4). During acute inflammation induced by intravenous
106 endotoxin administration, the rate of muscle protein breakdown may exceed the rate of protein synthesis in

107 the liver, resulting in greater AA catabolism and utilization for energy supply (Bruins et al., 2003). Increased
108 expression of myostatin, a negative regulator of protein accretion, in skeletal muscle was reported in pigs
109 infected with the porcine reproductive and respiratory syndrome (PPRS) virus, suggesting that muscle protein
110 synthesis might be decreased by infection (Escobar et al., 2004). Accordingly, inflammation caused by
111 turpentine injection decreased muscle protein synthesis, while hepatic fibrinogen synthesis increased by
112 140% (Jahoor et al., 1999). The liver is involved in the synthesis of APPs, which serve important functions in
113 restoring the cellular homeostasis of the immune system after infection or inflammation (Moshage, 1997).
114 Therefore, the increase in APP synthesis may require muscle protein breakdown to supply AAs, and more
115 specifically Trp and phenylalanine (Phe), because the profile of these AAs in APP differs from that of muscle
116 protein (Reeds et al., 1994). Likewise, the synthesis of immunoglobulins (Ig) would require a great amount of
117 Thr (Li et al., 1999) (see section 4).

118 Analysis of plasma AA responses to different challenges provides some indications on the changes in
119 AA metabolism when health is compromised. Lower AA concentrations in plasma have been reported in pigs
120 suffering from chronic lung inflammation, compared to healthy pair-fed pigs (Melchior et al., 2004), and also
121 in pigs with infectious peritonitis (Yoo et al., 1997). Pigs co-infected with *Mycoplasma hyopneumoniae* and
122 H1N1 virus exhibited alterations in the postprandial kinetics of glucose and AAs in plasma, indicating major
123 changes in nutrient metabolism. More specifically, postprandial concentrations of Thr and Arg in plasma were
124 much lower in co-infected pigs, compared to control healthy pigs (Le Floc'h et al., 2014). Tracer studies based
125 on the infusion of labelled AAs revealed many metabolic changes that cannot be assessed by alterations in
126 plasma AA concentrations. Indeed, plasma AA concentrations are greatly influenced by the nutritional status
127 and dietary supply, and by the metabolic fluxes of AAs entering or leaving the plasma pool. Thus,
128 modifications of the two opposite fluxes to the same extent would have no impact on plasma AA
129 concentration. For instance, Val and tyrosine (Tyr) fluxes were decreased by experimentally induced lung
130 inflammation without concomitant changes in their pool size in plasma (Kampman-van de Hoek et al., 2015).

131

132 **3. Amino acids and the physical gut mucosal barrier**

133 The intestine is a complex organ in which epithelial and secreting cells are closely associated to
134 immune cells. This complex cellular network, which includes secreted peptides and proteins and other host
135 defenses, contributes to the intestinal immune defense against invading pathogens, as well as to the
136 digestion and absorption of dietary nutrients. If the integrity of the digestive tract is compromised by
137 inflammation (see section 2), digestive capacities and the mucosal barrier function will be impaired. The
138 intestinal mucosal epithelium is a single layer of cells lining the gut that consists mainly of enterocytes and
139 tight junctions (TJ) between enterocytes (Arrieta et al., 2006), which regulates traffic through this paracellular
140 pathway and prevents the passage of pathogens. The TJ structure is formed by membrane proteins such as
141 occludin, members of the claudin family, and *zonula occludens* (ZO) proteins ZO-1, ZO-2 and ZO-3 (Arrieta et
142 al., 2006). Dysregulation of the intestinal mucosal barrier due to stress (e.g., weaning), along with the invasion
143 of pathogenic organisms and immunological challenges, has been reported to be associated with multiple
144 diseases (Groschwitz and Hogan, 2009; Camilleri et al., 2012; Bergmann et al., 2013). Consequently, early-
145 weaned pigs commonly experience diarrhea caused by impaired mucosal barrier function, as indicated by
146 reductions in jejunal transepithelial electrical resistance and elevations in paracellular permeability up to 40%
147 (Campbell et al., 2013; Wang et al., 2015). An increase in intestinal permeability is associated with villus
148 atrophy and significant reductions in the jejunal expression of occludin, claudin-1, ZO-2, and ZO-3 (Wang et
149 al., 2015).

150 Half of all proteinogenic AAs have been shown to exert positive effects on gut villus morphology when
151 supplied slightly above the estimated requirements. Attenuation of villus atrophy has been described for
152 aspartate (Asp) (Pi et al., 2014; Wang et al., 2016), Arg (Zhu et al., 2013), Gln (Wu et al., 1996b; Yi et al., 2005;
153 Noth et al., 2013), glutamate (Glu) (Rezaei et al., 2013), Pro (Wu et al., 2011), glycine (Gly) (Wang et al.,
154 2014b), Lys (Wang et al., 2009; He et al., 2013), Met (Chen et al., 2014), Thr (Ren et al., 2014) and Trp
155 (Koopmans et al., 2006) (Figure 2). Threonine is the major substrate for synthesis of mucosal glycoproteins
156 (mucins) and, therefore, for maintenance of gut barrier integrity (Bertolo et al 1998; Hamard et al 2007). In
157 addition, a number of AAs, such as Asp (Wang et al., 2016), Gln (Noth et al., 2013), Glu (Jiao et al., 2015), Gly
158 (Li et al., 2016), and Met (Chen et al., 2014), play critical roles in supporting gut integrity and function due to
159 their ability to increase the expression of TJ proteins. Therefore, the intestinal mucosal barrier function could

160 be improved during inflammatory processes, including the post-weaning stress syndrome (Jiao et al., 2015)
161 by supplementing the piglet diet with the aforementioned AAs. Dietary AAs stimulate intestinal cell
162 proliferation via several mechanisms. Under challenging conditions, oral Arg supplementation was shown to
163 activate the mTOR signaling pathway in the intestinal tissue (Corl et al., 2008). Of particular interest, Gln
164 stimulates protein synthesis and inhibits proteolysis in enterocytes by activating the mTOR pathway (Xi et al.,
165 2012), up-regulating ornithine decarboxylase expression to increase the production of polyamines, which are
166 required for DNA and protein synthesis (Wu et al., 2013a). Furthermore, Gln is a precursor for the synthesis
167 of purine and pyrimidine nucleotides, which are essential for DNA synthesis and the proliferation of cells
168 (Wu, 1998). Glutamine enhances the expression of genes for mitogen-activated protein kinases, resulting in
169 the activation of gene transcription, thereby contributing to cell proliferation in the intestinal epithelium
170 (Rhoads et al., 1997). Glutamine metabolism also provides ATP to support intestinal ion transport, cell growth
171 and migration, thereby maintaining intestinal integrity (Curi et al., 2005; Wu et al. 2011).

172 The major products of sulfur AA metabolism are GSH, homocysteine and taurine (Tau). Glutathione,
173 which is synthesized from cysteine (Cys), Gly and Glu, contributes to higher rates of cell proliferation, whereas
174 Tau plays an important role in membrane stabilization (see section 5) and anti-oxidative reactions (Wang et
175 al., 2009, Wu et al., 2013b). Apart from improving the intestinal morphology and cell proliferation, dietary
176 AAs have beneficial effects on the physical gut barrier. For instance, Arg is an essential precursor for the
177 synthesis of important compounds, including nitric oxide (NO), polyamines, and creatine (Wu and Morris,
178 1998). Therefore, this AA mediates vasodilation, intestinal fluid secretion, and whole-body energy
179 metabolism. In addition, Arg is of critical importance for the maintenance of intestinal mucosal barrier
180 function. The roles of Arg are separately described in Chapter 6. Asparagine may also improve the intestinal
181 energy status, as indicated by: (a) increases in the concentrations of ATP, ADP and total adenine nucleotides
182 and in adenylate energy charge; (b) a decrease in the AMP/ATP ratio; and (c) increases in the activities of
183 tricarboxylic acid cycle enzymes (Pi et al., 2014) or ileal diamine oxidases (Wang et al., 2016).
184 Supplementation of Gln promoted cell survival by stimulating the expression of heat shock proteins (Rhoads
185 and Wu, 2009) and anti-oxidative genes (Wang et al. 2008). Furthermore, supplementation of Gln recycled
186 cellular proteins and organelles by promoting autophagy in epithelial cells (Sakiyama et al., 2009), and

187 inhibited the intestinal expression and activation of nuclear factor- κ B (Haynes et al., 2009; Mondello et al.,
188 2010).

189 Based on current knowledge, Arg, Gln, Glu, Gly, sulfur AA, and Thr are promising for the nutritional
190 management of a wide array of inflammatory processes that affect the physical gut barrier and the resultant
191 gut-related disorders in pigs. However, there is a lack of studies about the involvement of AAs, other than
192 the Arg family of AAs, in the regulation of intestinal health. Such research is warranted to identify dietary
193 supplementation with appropriate AAs as an alternative approach to reduce the preventive use of feed
194 antibiotics in animal production in the future.

195

196 **4. Amino acids as primary constituents of defense proteins**

197 Data showing the specific needs of AAs as primary constituents of proteins involved in defense and
198 protection functions at the gut level (antibacterial proteins, IgA and IgM, mucins) are scarce. Nevertheless,
199 ensuring that dietary AAs do not limit the synthesis of specific protective proteins is relevant for gut health.
200 Several of these proteins are structural complexes, and undergo post-transcriptional modifications to fulfill
201 their biological functions, for example conferring the proteins an ability to conjugate pathogens or reducing
202 the intracellular degradation of the proteins. The presence of asparagine (Asn), serine (Ser), and Thr
203 sequences is important for N-glycosylation in the endoplasmic reticulum and O-glycosylation in the Golgi
204 apparatus (Blom et al., 2004). Thus, certain AAs confer specific properties to these functional proteins. The
205 fact that such specific AAs are more abundant in those proteins than in the average body protein is highly
206 relevant for developing dietary interventions to stimulate the synthesis of the aforementioned proteins.

207 The most abundant AAs in some mucins, immunoglobulins, and other intestinal defense proteins are
208 summarized in Table 1. These values were calculated from their AA composition obtained from the gene
209 sequence reported in the Ensemble database for pigs (or for human when the sequence was not available).
210 In general, the amount of Thr in endogenous protein losses, particularly in young pigs (Adeola et al., 2016),
211 agrees with the abundance of this AA, representing 16% of total AAs in mucins (Lien et al., 1997). Data in
212 Table 1 also help to understand some conflicting results about the requirement for Thr during digestive
213 disorders in pigs. In neonate pigs, adequate Thr is critical to maintain the necessary mucin production (Law

214 et al., 2007). Experimentally induced ileitis increased the utilization of arterial-blood Thr by the portal-drained
215 viscera, and ileal mucin synthesis in mini pigs (Rémond et al., 2009). However, when the dietary ratio of
216 Thr:Lys was increased from 65% to 70%, ETEC-susceptible weaned pigs orally challenged with ETEC did not
217 exhibit a change in the total mucin content in jejunal mucosal scrapings (Trevisi et al., 2015a). The lack of an
218 effect of supplementary Thr can be explained, in part, by the fact that mucin-13, which is mainly expressed
219 in the jejunum of pigs, is not rich in Thr, in contrast to other mucins that are dominant in other segments of
220 the gastrointestinal tract.

221 The most abundant AA in the IgA and IgM proteins and in the protein joining multimeric IgA and IgM is
222 Thr (Tenenhouse and Deutsch, 1966). In healthy pigs injected with bovine serum albumin or swine fever-
223 attenuated vaccine (Li et al., 1999) or ovalbumin (Wang et al., 2006), serum IgG concentrations increased
224 with dietary Thr intake, as observed for serum IgG and IgM in healthy weaned pigs, but not for piglets
225 challenged with the porcine pseudorabies live vaccine (Mao et al., 2014a). Supplementation of Thr increased
226 IgG and interleukin-1 β jejunal concentrations in serum after an ETEC challenge (Ren et al., 2014). Increasing
227 dietary Thr content from 8.5 g/kg to 9.0 g/kg (beyond the current requirement) with addition of L-Thr
228 resulted in a higher secretion of IgM in ETEC-challenged pigs susceptible to ETEC, while this was not seen in
229 infected non-susceptible pigs (Trevisi et al., 2015a). In the latter, IgM and IgA concentrations in blood did
230 not rise after the challenge. This result indicates that the effect of dietary provision of Thr on the humoral
231 immune response depends on the health status and genetic background of pigs.

232 The first limiting AA affecting the synthesis of several porcine defense and antibacterial proteins or
233 polypeptides can be predicted from their AA composition. It has been reported that Ser is a major AA for the
234 synthesis of regenerating islet-derived protein 3 gamma (REG3G), a C-type lectin that targets Gram-positive
235 bacteria and is abundantly produced in the porcine small intestine during certain intestinal infections (Soler
236 et al., 2015). Serine is classified as a nutritionally nonessential AA; however, it is the major metabolic source
237 of the one-carbon pool (Kalhan and Hanson, 2012) and the pathway for its synthesis from glucose uses Glu,
238 whose metabolism is in turn affected by Ser requirement. Inadequate provision of Ser can result in impaired
239 synthesis of Gly (Wu, 2013), with consequent nutritional imbalance of other AAs (Wang et al., 2013). Another
240 important antibacterial protein that has not received much consideration for nutritional requirements is

241 lysozyme, which contains a high proportion of Leu. To date, there is no information on the impact of dietary
242 leucine intake on lysozyme production in literature. On the whole, paying attention to AA composition and
243 to the AAs that potentially limit the production of defense proteins would advance studies aimed at meeting
244 AA requirements to sustain animal growth under sub-optimal rearing conditions. Furthermore, these
245 proteins could be considered as potential biomarkers in blood, saliva, feces or other tissue samples to assess
246 intestinal health and function in pigs subjected to different feeding strategies.

247

248 **5. Amino acid involved in the antioxidant defense**

249 Oxidative stress results from an imbalance between the endogenous production of reactive oxygen
250 species (ROS) and antioxidant defenses (Wu et al., 2004a). Endogenous ROS are produced within
251 mitochondria during cell respiration and thus are normal products of cellular oxygen metabolism. Besides,
252 the production of ROS is a mechanism used by some immune cells (e.g., macrophages) to exert their cytotoxic
253 function. Thus, ROS production during inflammation and the activation of innate immune response are
254 defense mechanisms that can generate oxidative stress when antioxidant defenses are overwhelmed (Li et
255 al., 2007). Finally, ROS can be produced after animals are exposed to pollutants and xenobiotics but their
256 impact on farm animals is not fully understood. As previously mentioned in section 2, one of the most
257 powerful endogenous antioxidant components is GSH (Malmezat et al., 1998). In postnatal pigs, the liver and
258 gut seem to be the two major sites for GSH synthesis (Wu et al., 2004a; Bauchart-Thevret et al., 2011;
259 Rémond et al., 2011). In the liver of rats, the synthesis of Cys from methionine during inflammation increases
260 to support the greater demand for GSH (Malmezat et al., 2000), but Met provision does not appear to be
261 sufficient for GSH production. In growing pigs, repeated injections of endotoxin increased the conversion of
262 Cys into GSH and taurine, while decreasing the catabolism of Cys into sulfate (Rakhshandeh and de Lange,
263 2010; Rakhshandeh et al., 2010). In mini-pigs, experimental ileitis increased liver and ileal GSH synthesis
264 during the acute phase of inflammation, and increased the whole-body flux of Cys (Rémond et al., 2011).
265 Cysteine supplementation through organ infusion positively influenced the pool of GSH in the liver (Budzinski
266 et al., 2011). Under practical conditions, feeding strategies based on the addition of stable precursors of Cys
267 (e.g., N-acetyl-cysteine) in feed may be relevant. Likewise, the addition of Met, its hydroxyanalogue HMTBA

268 (2-hydroxy-4-(methylthio)butanoate), total sulfur AAs (Met + Cys), or N-acetyl-cysteine to diets also help
269 maintain the intracellular GSH pool, as well as intestinal redox status and integrity in weaned pigs (Bauchart-
270 Thevret et al., 2009; Chen et al., 2014; Li et al., 2014; Xu et al., 2014; Hou et al., 2015a).

271 Other AAs have been reported to reduce the consequences of oxidative stress in cells, particularly in
272 enterocytes. For instance, supplementation with Glu (Rezaei et al., 2013; Jiao et al., 2015) and Gly (Wang et
273 al., 2014a; Jiao et al., 2015), the two other AAs that constitute GSH, as well as Arg (Zheng et al., 2013) and
274 Asp (Yin et al., 2015; Duan et al., 2016), alleviated the consequences of oxidant-induced oxidative stress on
275 intestinal function, AA transporters, redox status, and growth. In the whole animal, a reduction of oxidative
276 stress may result also from the contribution of these AAs because of their pivotal metabolic roles in immune
277 and intestinal cells besides their direct antioxidant effect (Li et al., 2007; Wu, 2013). Likewise, Trp (see section
278 7) may exert a direct antioxidant effect, and several Trp metabolites, produced through the kynurenine and
279 melatonin biosynthesis pathways, act as free radical scavengers and have antioxidant properties (Christen et
280 al., 1990; Goda et al., 1999). In this way, Mao et al., (2014b) showed that Trp supplied above the
281 recommendation (3.0 g/kg vs 1.8 g/kg) alleviated oxidative stress induced by intraperitoneal administration
282 of diquat (an herbicide) in piglets.

283

284 **6. Amino acids related to the arginine - nitric oxide (NO) pathway**

285 Sow's milk is rich in Gln, Glu and Pro (Wu and Knabe, 1994). Specifically, concentrations of free Gln in the
286 milk increase progressively with advancing lactation, and free- and peptide-bound Gln plus Glu account for
287 20% of total amino acids. In contrast, the concentrations of Arg in sow's milk (free plus peptide-bound) are
288 much lower than those of Gln plus Glu and Pro on all days of lactation. Thus, sow's milk provides at most only
289 40% of the Arg needed for metabolic utilization by young pigs (Wu et al., 2004b). *In vivo* studies involving the
290 cannulation of the jejunal artery and jejunal vein of 14- to 58-day-old pigs have shown that the small intestine
291 actively utilizes dietary and arterial-blood Gln, and releases citrulline and, to a lesser extent, Arg (Wu et al.,
292 1994a) (Figure 3). The only AA in arterial blood that is taken up by the small intestine of pigs in the post-
293 absorptive state is Gln (Wu et al., 1994a). Enterocytes synthesize citrulline and Arg from 0.5 - 5 mM Gln via
294 pyrroline-5-carboxylate synthase (Wu et al., 1994b) and from 0.5 - 2 mM Pro via proline oxidase (Wu, 1997)

295 in a dose-dependent manner. The *de novo* synthesis of Arg is consistent with the conversion of [U-¹⁴C] Gln
296 into [¹⁴C]Arg in the enterocytes of 0- to 7-day-old pigs (Blachier et al., 1993). All substrates required for these
297 synthetic pathways, including ammonia, HCO₃⁻, Glu, Asp, and ATP, are produced from Gln catabolism (Wu
298 and Morris, 1998). Because there is no uptake of arterial- blood Pro by the pig small intestine (Wu et al.,
299 1994a), enteral provision of large amounts of Pro from sow's milk and the postweaning diet is crucial for the
300 compensation of Arg deficiency in the diets (Brunton et al., 1999; Bertolo et al., 2003). In young and adult
301 pigs, Arg synthesis is inadequate for their optimal growth and reproduction primarily because of the reduced
302 expression of N-acetylglutamate synthase in enterocytes (Wu et al., 2004b; Zhang et al., 2014). This enzyme
303 catalyzes the production of N-acetylglutamate (from Glu and acetyl-CoA) that is an allosteric activator of
304 carbamoylphosphate synthase-I for the formation of citrulline and arginine (Wu and Morris 1998).

305 The discovery of the synthesis of NO (a major vasodilator, a signaling molecule, and a mediator of
306 immune response) from Arg has renewed interest in Arg nutrition research over the past 25 years (Hou et
307 al., 2015b, 2016a; Wu et al., 1996a). Based on the results of recent studies which indicate that
308 supplementation with Arg to conventional diets can improve the growth or production performance of
309 modern breeds of pigs during gestation, lactation, nursery, weaning, and growing-finishing periods (Wu et
310 al., 2007; Wu, 2014), the NRC now recognizes that Arg is a conditionally essential AA for pigs in all phases of
311 their production. Thus, NRC (2012) has recommended the requirements of Arg in diets for pigs in all the
312 phases of production, which ranged from 0.17 g/kg for early-gestating sows to 0.68 g/kg for nursing pigs, on
313 the standardized ileal digestible basis. Higher values of dietary Arg requirements than NRC (2012) were
314 suggested by Wu (2014) to maximize the growth performance, milk production, and embryonic/fetal survival
315 of pigs.

316 As noted previously in sections 3 and 5, Arg has many roles: protecting against oxidative stress and
317 inflammation, activating mTOR in intestinal tissue, modulating the intestinal inflammatory response, and
318 attenuating villus atrophy. As a functional AA, Arg has a wide range of applications in swine production (Hou
319 et al., 2016b). For example, in neonatal pigs, dietary supplementation with 0.2% and 0.4% Arg to 7- to 21-d-
320 old milk-fed pigs, artificially reared on a liquid-milk feeding system, dose-dependently enhanced plasma Arg
321 concentrations (30% and 61%), reduced plasma ammonia levels (20% and 35%), and promoted weight gain

322 (28% and 66%) (Kim et al., 2004). Most recently, Yang et al. (2016) reported that supplementing 0.4% or
323 0.8% Arg to a milk replacer diet enhanced the weight gain of 4- to 24-day-old piglets by 19% and 22%,
324 respectively, without affecting feed intake. Of interest, supplementation of the preweaning diet with Arg
325 improved intestinal growth and development after termination of the period of supplementation, in 25- to
326 45-day-old pigs (Yang et al., 2016). In weanling pigs, supplementing 0.6% Arg to a corn- and soybean meal-
327 based diet increased small-intestinal mass by 89 g and daily weight gain by 42 g/d, in 21- to 28-day-old
328 weanling piglets (Wu et al., 2010). Dietary Arg supplementation also increased the splenic expression of IL-8
329 and tumor necrosis factor- α , indicators of the activation of innate immunity, as well as the serum
330 concentrations of IgG and IgM, to prevent infections in weanling pigs (Li et al., 2007; Tan et al., 2009a).
331 Likewise, Arg supplementation prevented the death of porcine enterocytes induced by *E. coli* endotoxin
332 through mechanisms involving the activation of mTOR and the suppression of toll-like receptor-4 signaling
333 (Tan et al., 2010).

334 Owing, in part, to improvements in anti-oxidative response and whole-body health, Arg
335 supplementation enhances feed efficiency, fertility and lactation in swine. For example, supplementing 1%
336 Arg to a corn- and soybean meal-based diet for 60 days reduced whole-body white fat content by 11% in
337 growing-finishing pigs, while increasing the skeletal-muscle content in their whole body by 5.5%, without
338 affecting daily weight gain (Tan et al., 2009b). Furthermore, supplementing 0.5% and 1% Arg to a corn- and
339 soybean meal-based diet containing 0.95% Arg, for growing-finishing pigs dose-dependently reduced lipid
340 peroxidation in skeletal muscle and improves meat quality at 48 h postmortem (Ma et al., 2010). In gestating
341 pigs, dietary supplementation with 1.0% Arg-HCl between days 30 and 114 of gestation increased
342 concentrations of Arg, ornithine, and Pro in plasma by 77%, 53%, and 30%, respectively, as well as the number
343 of live-born piglets by two and litter birth-weight by 24% (Mateo et al., 2007). This effect of Arg is associated
344 with (a) the improved health of the conceptus (embryo/fetus and associated membranes) due to the
345 amelioration of oxidative stress, and (b) enhanced placental angiogenesis and vasculature, which is
346 stimulated by physiological levels of NO (50 – 500 nM; Wu and Meininger 2009; Wu et al., 2013b), to remove
347 oxidants from the fetus. Similarly, dietary supplementation with 1% Arg between days 14 and 28 of gestation
348 enhanced the number of fetuses per litter by 3.7 on day 70 of gestation in superovulated gilts, as well as fetal

349 muscle development (Bérard and Bee, 2010). Of note, Arg supplementation to gestating sows enhanced the
350 production of NO and B lymphocyte-derived antibodies, thereby preventing morbidity and mortality in
351 response to the intestinal infection caused by *Brachyspira hyodysenteria*, the swine dysentery pathogen (Li
352 et al., 2007). In lactating primiparous sows, supplementing 0.83% Arg to the diets augmented average pig
353 weight gain by 0.26 kg in the first week of lactation and by 0.42 kg during a 21-day suckling period (Mateo et
354 al., 2008). This effect of Arg may be mediated, in part, by NO-induced increase in blood flow into the
355 mammary gland (Kim and Wu, 2009). Furthermore, dietary Arg supplementation to sows promoted milk lipid
356 production (Kirchgessner et al., 1991), and improved the sow feed efficiency, particularly under hot
357 environmental temperatures (Laspiur and Trottier, 2001). Taken together, these findings underscore the
358 need to carefully consider dietary Arg intake to improve the health, growth, survival, lactation and fertility in
359 swine. This is particularly noteworthy, because low-protein diets, which are currently used to reduce the
360 production of nitrogenous wastes by swine farms, do not sufficiently supply Arg or its AA precursors (Wu et
361 al., 2014a).

362

363 **7. Tryptophan and the kynurenine pathway**

364 Tryptophan is the precursor of kynurenine, the first metabolite of a complex metabolic pathway ending
365 in the formation of quinolinic acid, niacin and nicotinamide, kynurenic and xanthurenic acids (Le Floc'h et al.,
366 2011). Two enzymes are needed to convert Trp into kynurenine. The first enzyme is Trp 2,3-dioxygenase
367 (TDO) located in the liver and responsible for the degradation of Trp in excess. The second enzyme is
368 indoleamine 2,3-dioxygenase (IDO) found in numerous immune cells like macrophages and dendritic cells.
369 Interferon γ , an inflammatory cytokine, stimulates IDO expression and activity (Popov and Schultze, 2008).

370 Activation of IDO and increased Trp catabolism are known as a mechanism for regulating the immune
371 system during pregnancy and diseases (Munn and Mellor, 2013; Badawy et al., 2016) and for immune
372 tolerance. This notion originates from the discovery of the protective role of IDO during human gestation
373 through the prevention of the fetal rejection by maternal T-lymphocytes (Munn et al., 1998). The activation
374 of IDO and the subsequent production of Trp metabolites with cytotoxic activity would contribute to reducing
375 T-cell proliferation. Moreover, IDO expression by dendritic cells is associated with the acquisition of a

376 regulatory phenotype, leading to immune tolerance (Sharma et al., 2007). The activation of IDO is also
377 thought to be involved in long-lasting immune activation that occurs with some inflammatory diseases
378 (Popov and Schultze, 2008). In pigs, experimental data have confirmed that inflammation increases IDO
379 activity and this induction is concomitant with lower plasma Trp concentrations (Melchior et al., 2004;
380 Melchior et al., 2005; Wirthgen et al., 2014). The impact of inflammation on plasma Trp concentration and
381 tissue IDO activity is greater when Trp is supplied below the nutritional recommendations (Le Floc'h et al.,
382 2008). Additionally, oxidative stress, a mechanism associated with the inflammatory response, also
383 depressed plasma Trp and increased plasma kynurenine in weaned pigs (Lv et al., 2012). Such modifications
384 in Trp metabolism are expected to affect Trp availability for growth. Indeed, repeated LPS injections reduced
385 the availability of Trp for body protein deposition and growth (de Ridder et al., 2012). Accordingly, if
386 additional crystalline Trp did not completely prevent growth restriction caused by poor health status, the
387 improvement of growth through supplementing Trp to a low-Trp diet was greater for pigs with compromised
388 health than for pigs with good health (Le Floc'h et al., 2010). The response to dietary Trp supplementation
389 can be affected by the presence of individual genetic predisposition to *E. coli* infections in pigs. Indeed, the
390 susceptibility to ETEC adhesion to jejunal villi is required for the development of the pathology and it is
391 genetically transmitted. In the first 4 days after experimental infection, dietary supplementation with 1 g
392 Trp/kg beyond the minimal nutrient requirement improved growth response in weaned pigs genetically
393 predisposed to ETEC K88, but not in non-susceptible pigs (Trevisi et al., 2009).

394 The dramatic changes in Trp metabolism induced by inflammation are clearly associated with the
395 functional role of this AA during inflammatory states. Pigs suffering from experimentally induced lung
396 inflammation had lower APP concentrations and had less severe lung lesions when they were fed a diet with
397 a small excess of Trp compared to pigs fed a diet moderately deficient in Trp (Le Floc'h et al., 2008). At the
398 same time, IDO activity was also lower in pigs fed the higher Trp diet, indicating that inflammation was
399 alleviated by dietary Trp supplementation. In a porcine model of induced colitis, Trp supplementation down-
400 regulated inflammation, restored the local immune response and reduced colitis symptoms (Kim et al., 2010).
401 At present, the positive effect of dietary Trp on the inflammatory response remains unexplained. However,
402 it could be speculated that dietary Trp may help to control the inflammatory response. As previously

403 mentioned (see section 5), Trp and some of its metabolites produced along the kynurenine pathway, 3-
404 hydroxy-anthralinic acid and 3-hydroxy-kynurenine, may have antioxidant properties (Christen et al., 1990).
405 This hypothesis is supported by the recent finding that liver TDO activity was increased by oxidative stress
406 (Lv et al., 2012; Mao et al., 2014b). Recently, in Large White pigs, polymorphism was identified in the *KMO*
407 gene coding for kynurenine 3-monooxygenase that hydroxylates kynurenine to 3-hydroxy-kynurenine; the
408 polymorphism for the genotype for *KMO* affected the extent to which the plasma levels of kynurenine and
409 kynurenic acid were elevated in response to Trp supplementation (Trevisi et al., 2015b). Furthermore, the 3-
410 hydroxykynurenine/kynurenic acid ratio in plasma, representing the different actions of *KMO* and kynurenic
411 acid transaminase enzymes, differed among the different genetic variants for *KMO*. This implied that the
412 response of pigs to dietary Trp levels could be influenced by the genetic background as recently suggested
413 (Le Floc'h et al. 2017) and by their ability to produce different kynurenine metabolites during inflammatory
414 states.

415

416 **8. Functional amino acids and feeding strategies**

417 Environmental, social and economic reasons justify the demand for higher feed efficiency and more
418 specifically nitrogen-utilization efficiency in animal production (Wu et al., 2014c). A strategy to improve
419 protein utilization in pigs and to prevent gut disorders is the reduction of dietary crude protein (CP)
420 concomitant with adequate supplementation of free AAs. Gloaguen et al. (2014) confirmed the efficacy of
421 this strategy and the possibility to formulate very low-CP (13.5%) diets that maintain the growth of 10 to 20
422 kg pigs through the inclusion of free AAs. Furthermore, moderate dietary protein restriction (CP 13-15.3%)
423 was demonstrated to be beneficial for a healthy balance in the gut microbiota and metabolic activity in the
424 large intestine of pigs, and improved ileal mucosal barrier function (Fan et al., 2017; Peng et al., 2017). The
425 reduction in dietary CP content allows for reduction in nitrogen intake and may avoid AA excesses, thereby
426 preventing excessive metabolic loads. Besides reductions in the use of feed antibiotics, low CP diets may
427 provide an opportunity to supply specific functional AAs that would also contribute to limiting metabolic
428 disturbances associated with inflammatory states. Functional AAs are defined as those AAs that regulate key
429 metabolic pathways to improve health, survival, growth, development, lactation, and reproduction of

430 organisms (Wu, 2010) or which form biologically active peptides or proteins. These AAs include those that
431 can be synthesized and those that cannot be synthesized *de novo* in animal cells.

432 Dietary free AAs appear in the peripheral plasma more quickly than AAs arising from intact proteins (Yen
433 et al., 2004). Yen et al. (2004) reported maximal portal and arterial plasma Lys and Thr concentrations in pigs
434 1 h postprandial with the provision of free AAs, while the peak level for protein-bound AAs occurred at 2.5 h
435 postprandial. The difference in the time of appearance of AAs in the peripheral blood, which results from
436 their provision in different forms, may be a physiological basis for preventive or therapeutic nutritional
437 intervention via addition of single AAs or AA blends to drinking water or the feeding system. Besides,
438 supplemental AAs, such as free Arg, Gln, Glu, Gly, and Trp, enter the lumen of the small intestine and are
439 taken up rapidly by the gut, where they regulate gene expression, cell signaling, antioxidative responses, and
440 immunity (Wu, 2009). Temporary, targeted provision with functional AAs as powder on top of the commercial
441 diet might be beneficial to overcome intestinal dysfunction during critical periods of production, such as
442 weaning stress or pathogen exposure. For example, Le Floc'h et al. (2008) reported that inflammation
443 increased Trp catabolism and thus decreased Trp availability for growth (see section 7). Consequently, it may
444 be assumed that the targeted provision of free Trp beyond the requirements for growth may contribute to a
445 steady level of plasma Trp and therefore an increased availability for immune response and muscle growth.

446 Much attention has recently been directed to studying Leu signaling in animal nutrition. For example,
447 pulsatile delivery of Leu to neonatal pigs fed a milk replacer orogastrically increases lean growth by 25%
448 (Boutry et al., 2016), likely due to insulin-stimulated translation initiation (Davis et al., 2015). Wilson et al.
449 (2009) demonstrated that ingestion of a meal providing one-sixth of the daily dietary Leu requirements
450 provoked the most rapid stimulation of muscle protein synthesis with highly efficient peak activation within
451 30 min. Consequently, with pulsatile supplementation with a functional AA, frequent or *ad libitum* feeding
452 to the healthy pig should be the preferred feeding strategy in order to guarantee a balanced supply of AAs,
453 leading to similar rates of oxidation of excess essential AAs from diets containing either free or protein-bound
454 AAs.

455 As described previously for Trp, the response of animals to dietary Leu and Gln in terms of whole
456 body growth depends on the health status and production level of the pig. Frost and Lang (2011) estimated

457 a threshold for dietary Leu that was higher during inflammation than under healthy conditions. Inflammation
458 has been reported to reduce the sensitivity of skeletal muscle to Leu (Lang and Frost, 2005), thereby impairing
459 muscle protein synthesis via the mTOR signaling pathway. Furthermore, Leu could act as an N donor for
460 synthesis of Gln, which is considered to be a conditionally essential AA during weaning (Wu et al., 1996b) or
461 disease (Karinch et al., 2001) but promotes Leu uptake by the muscle as well. Therefore, targeted additional
462 administration of Leu and Gln might alleviate weight loss during disease by maintaining muscle protein
463 synthesis. However, whether these findings could have practical applications for swine production remains
464 to be determined. Based on the current literature, the provision of particular AAs may be useful to target
465 specific AA functions with a flexibility to adjust age- or health status-specific requirements of the animals.

466

467 **9. Future perspectives**

468 Besides being the building blocks of proteins, AAs are also precursors for the synthesis of bioactive
469 peptides and low-molecular-weight metabolites with major physiological and regulatory functions in animals.
470 Because the small intestine is the terminal site for nutrient digestion and absorption and yet is highly
471 susceptible to infection, inflammation, and injury, there has been growing interest in the use of specific AAs
472 to improve intestinal health, integrity, and function in swine at the various stages of physiological
473 development and during various phases of pork production (Wu, 2018). While it is well established that
474 swine diets must contain essential AAs, adequate provision of traditionally classified non-essential AAs (e.g.,
475 Arg, Glu, Gln, and Gly) is also critical to ensure optimum intestinal and whole-body health, growth rate and
476 feed efficiency in pigs. The term “nutritionally non-essential AA” has now been recognized as a misnomer in
477 nutritional sciences, and animals (including growing, gestating and lactating pigs, as well as gilts and boars)
478 have dietary requirements for those AAs (Hou and Wu, 2017). The availability of feed-grade crystalline AAs,
479 particularly functional AAs, for supplementation to low-protein diets is expected to play an important role in
480 the sustainability of pig production worldwide to limit the negative impact of pig production on the
481 environment (Garcia-Launay et al., 2014), while meeting the increasing demand for human consumption of
482 high-quality animal protein (Wu et al., 2014b and 2014c).

483 The recent progress on the “omic” sciences has provided insights into AA metabolism in animals,
484 including swine. These high-throughput studies, including targeted gene association studies and
485 metabolomic approaches, have resulted in a better understanding of the gut microbiota and in the
486 identification of gene markers for important transmissible diseases. The advanced methodologies will further
487 stimulate research to better define dietary AA requirements for pigs with different phenotypes and
488 genotypes.

489

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495

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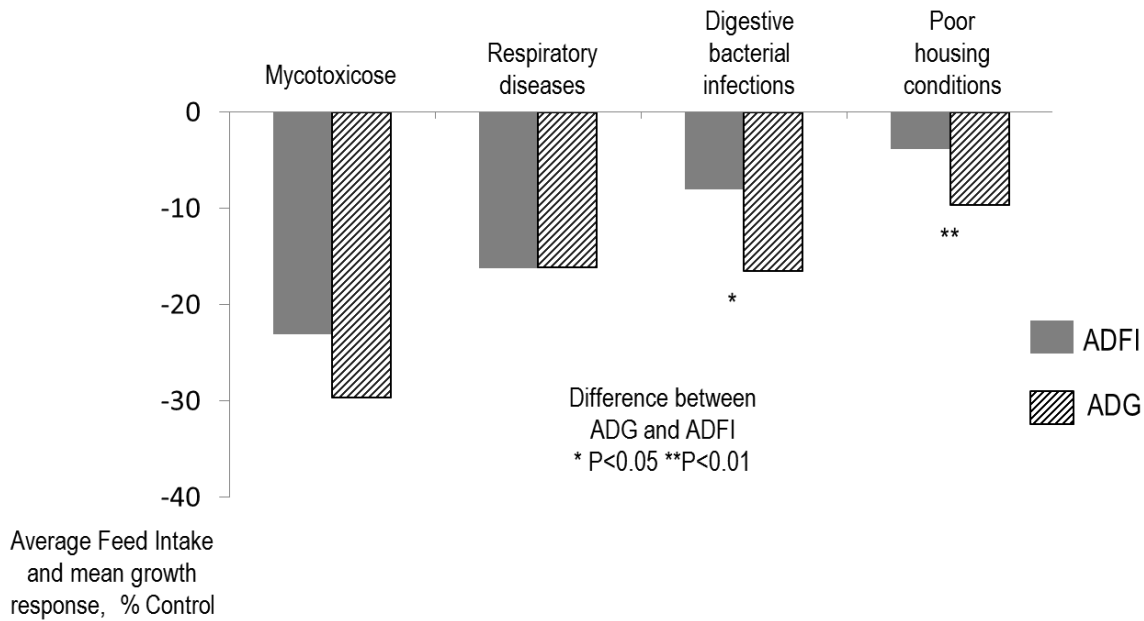
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908

Proteins	The most represented AA ¹	Percentage of total AA number ¹
<i>- Mucins</i>		
Mucin 1 (more expressed in stomach)	Thr	17.4
	<i>Ser</i>	15.5
Mucin2 (Human)	Thr	17.5
Mucin 13 (more expressed in jejunum)	Asn	8.5
Mucin 20 (more expressed in colon)	Thr	15.5
<i>- Immunoglobulins</i>		
IgA constant chain (Human)	Thr	9.6
IgM constant chain (Human)	Thr	9.6
Joining chain of Multimeric IgA And IgM	Thr	9.1
<i>- Defense and antibacterial, lectins</i>		
Regenerating Family Member 3 Gamma	Ser	13.8
LY6/PLAUR Domain Containing 8	Thr	11.1
	<i>Ser</i>	9.9
Lysozyme	Leu	10.1
Haptoglobin	Val	8.2
Alkaline phosphatase, intestinal	Arg / Leu	9.9 each

910 ¹ The relative abundance of amino acids in a protein was calculated from the counting of each amino acid
 911 and of the total of amino acids encoded for by the gene's DNA sequence reported in Ensemble data base
 912 for pigs (or for human, when the sequence was not available). The second most abundant amino acid is in
 913 italics. Thr = threonine; Ser = serine; Asn = asparagine; Leu = leucine; Val = valine; Arg = arginine.

914

915 **Fig. 1.** Figure 1. Impacts of health disturbances on average daily feed intake (ADFI) and average daily
916 growth (ADG) in % of the value for control healthy pigs (adapted from Pastorelli et al. 2012). The symbols
917 *and ** indicate that the impacts of health disturbances differed for ADG and ADFI at $P < 0.05$ and $P < 0.01$,
918 respectively.

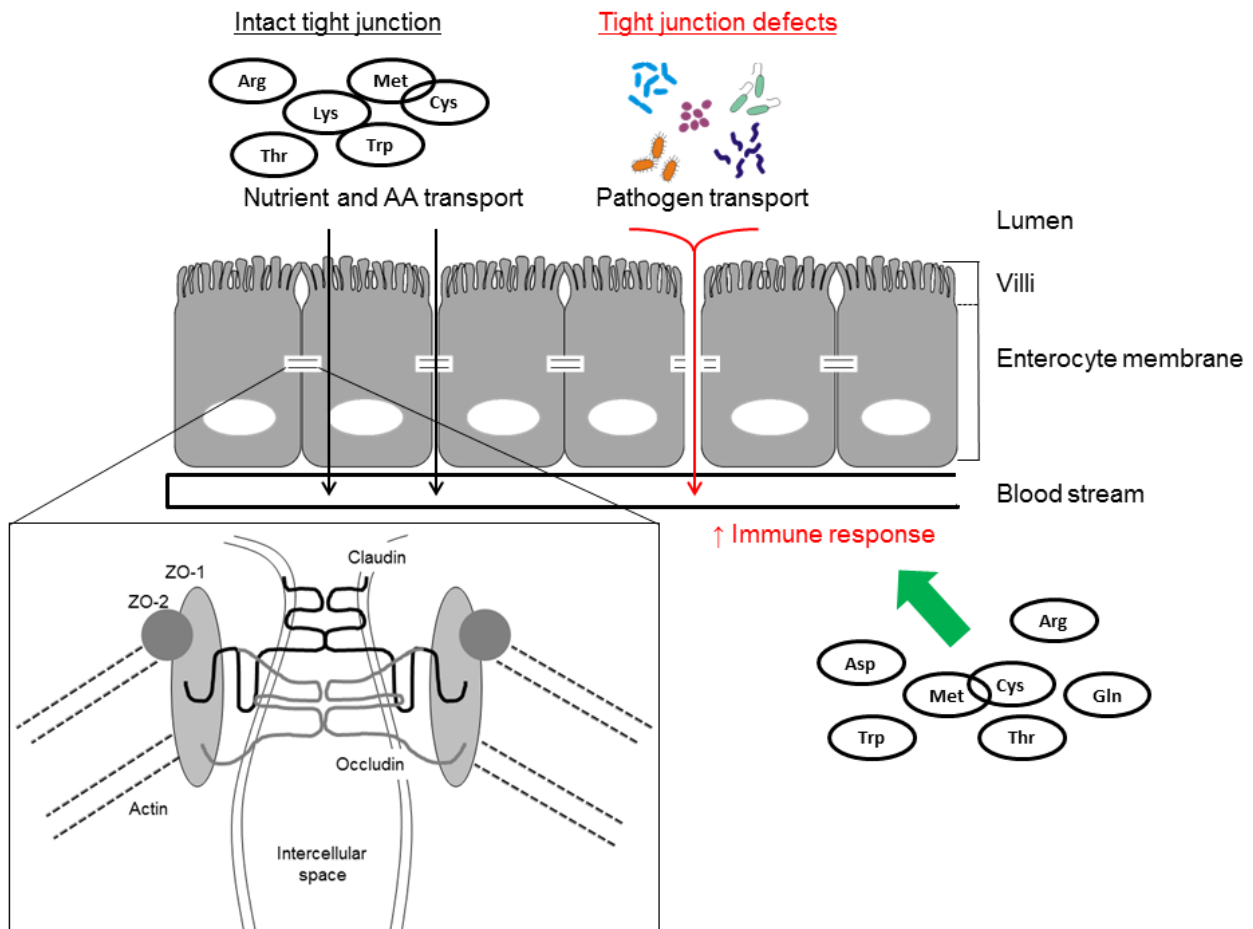


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920

921 **Figure 2.** Mucosal barrier function and the structure of intestinal tight junctions (TJ). The intestinal
922 epithelium provides a physical barrier to luminal bacteria, toxins, and antigens. The mucosal barrier is
923 structured by different barrier components, including the TJ. The TJ structure is formed by membrane
924 proteins such as occludin, members of the claudin family, and *zonula occludens* proteins ZO-1 and ZO-2. TJs
925 regulate the paracellular passages of nutrients (e.g., amino acids) between adjacent cells, and their uptake
926 into the blood stream. Any TJ barrier impairment allows for the passage of noxious molecules, which can
927 induce the excessive activation of mucosal immune cells and inflammation. Therefore, intestinal mucosal
928 barrier defects are associated with the initiation and development of various intestinal and systemic
929 diseases. Dietary and circulating amino acids contribute to anti-inflammatory response in the body.

930



931

932 **Figure 3.** Metabolism of the arginine-family of amino acids in the small intestine and liver of post-weaning,
 933 growing pigs under fed conditions. Dietary protein is hydrolyzed in the lumen of the small intestine to
 934 release L-arginine (Arg), L-glutamine (Gln), L-glutamate (Glu), L-proline and other amino acids. Almost all
 935 Glu (95-97%) and Asp (95%), most Gln (70%), 40% Pro, and 40% Arg in the lumen are metabolized by the
 936 small intestine, primarily in enterocytes and by bacteria) (Hou et al., 2016b). Within enterocytes, L-citrulline
 937 (Cit) is synthesized from Gln, Glu and Pro. These cells convert a small percentage of the Cit (10%) into Arg
 938 and release 90% of the Cit, while hydrolyzing Arg into L-ornithine (Orn) through the action of arginase (Wu
 939 1997). Arg and Gln inhibit the expression of toll-like receptor-4 (TLR-4) and nuclear factor kappa-light-
 940 chain-enhancer of activated B cells (NFkB) in response to inflammation (Hou et al. 2015a). The liver
 941 receives little Glu and Asp and a relatively small amount of Gln from the portal vein, and does not take up
 942 Cit. In multiple tissues of pigs, Cit is effectively converted into Arg, which is metabolized to ornithine,
 943 proline, glutamate and glutamine. The sign (-) denotes inhibition of gene expression in response to
 944 inflammation.

