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

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1	e 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, H_2S , 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

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

30

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

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

37

38 1. Introduction

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

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 required for DNA and protein synthesis (Wu et al., 2013a). Furthermore, Gln is a precursor for the synthesis 166 167 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 tricarboxylic acid cycle enzymes (Pi et al., 2014) or ileal diamine oxidases (Wang et al., 2016). 183 184 Supplementation of GIn 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

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

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

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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.

The most abundant AAs in some mucins, immunoglobulins, and other intestinal defense proteins are summarized in Table 1. These values were calculated from their AA composition obtained from the gene sequence reported in the Ensemble database for pigs (or for human when the sequence was not available). In general, the amount of Thr in endogenous protein losses, particularly in young pigs (Adeola et al., 2016), agrees with the abundance of this AA, representing 16% of total AAs in mucins (Lien et al., 1997). Data in Table 1 also help to understand some conflicting results about the requirement for Thr during digestive disorders in pigs. In neonate pigs, adequate Thr is critical to maintain the necessary mucin production (Law et al., 2007). Experimentally induced ileitis increased the utilization of arterial-blood Thr by the portal-drained
viscera, and ileal mucin synthesis in mini pigs (Rémond et al., 2009). However, when the dietary ratio of
Thr:Lys was increased from 65% to 70%, ETEC-susceptible weaned pigs orally challenged with ETEC did not
exhibit a change in the total mucin content in jejunal mucosal scrapings (Trevisi et al., 2015a). The lack of an
effect of supplementary Thr can be explained, in part, by the fact that mucin-13, which is mainly expressed
in the jejunum of pigs, is not rich in Thr, in contrast to other mucins that are dominant in other segments of
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

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

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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

(2-hydroxy-4-(methylthio)butanoate), total sulfur AAs (Met + Cys), or N-acetyl-cysteine to diets also help
 maintain the intracellular GSH pool, as well as intestinal redox status and integrity in weaned pigs (Bauchart 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.

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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 GIn, 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.

As noted previously in sections 3 and 5, Arg has many roles: protecting against oxidative stress and inflammation, activating mTOR in intestinal tissue, modulating the intestinal inflammatory response, and attenuating villus atrophy. As a functional AA, Arg has a wide range of applications in swine production (Hou et al., 2016b). For example, in neonatal pigs, dietary supplementation with 0.2% and 0.4% Arg to 7- to 21-dold milk-fed pigs, artificially reared on a liquid-milk feeding system, dose-dependently enhanced plasma Arg 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 concentrations of Arg, ornithine, and Pro in plasma by 77%, 53%, and 30%, respectively, as well as the number 342 343 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).

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7. Tryptophan and the kynurenine pathway

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

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

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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%) was demonstrated to be beneficial for a healthy balance in the gut microbiota and metabolic activity in the 423 424 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 their provision in different forms, may be a physiological basis for preventive or therapeutic nutritional 436 437 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.

As described previously for Trp, the response of animals to dietary Leu and Gln in terms of whole 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 GIn, 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 GIn 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).

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

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Proteins	The most represented AA ¹	Percentage
		of total AA number ¹
Mucin 1 (more expressed in stomach)	Thr	17.4
	Ser	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
- Immunoglobulins		
IgA constant chain (Human)	Thr	9.6
IgM constant chain (Human)	Thr	9.6
Joining chain of Multimeric IgA And IgM	Thr	9.1
- Defense and antibacterial, lectins		
Regenerating Family Member 3 Gamma	Ser	13.8
LY6/PLAUR Domain Containing 8	Thr	11.1
	Ser	9.9
Lysozyme	Leu	10.1
		o ٦
Haptoglobin	Val	0.2

909 **Table 1** Calculation of the most abundant amino acids in some relevant defense proteins secreted by the gut

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

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



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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

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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 release L-arginine (Arg), L-glutamine (Gln), L-glutamate (Glu), L-proline and other amino acids. Almost all 934 935 Glu (95-97%) and Asp (95%), most Gln (70%), 40% Pro, and 40% Arg in the lumen are metabolized by the small intestine, primarily in enterocytes and by bacteria) (Hou et al., 2016b). Within enterocytes, L-citrulline 936 (Cit) is synthesized from Gln, Glu and Pro. These cells convert a small percentage of the Cit (10%) into Arg 937 938 and release 90% of the Cit, while hydrolyzing Arg into L-ornithine (Orn) through the action of arginase (Wu 1997). Arg and Gln inhibit the expression of toll-like receptor-4 (TLR-4) and nuclear factor kappa-light-939 chain-enhancer of activated B cells (NFkB) in response to inflammation (Hou et al. 2015a). The liver 940 receives little Glu and Asp and a relatively small amount of Gln from the portal vein, and does not take up 941 942 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.

