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Nathalie Le Floc'H, Florence Gondret, J. Jacques Matte, H el ene Quesnel. Towards amino acid recommendations for specific physiological and patho-physiological states in pigs. Proceedings of the Nutrition Society, 2012, 71 (3), pp.425 - 432. 10.1017/S0029665112000560 . hal-01210472

**HAL Id: hal-01210472**

**<https://hal.science/hal-01210472>**

Submitted on 29 May 2020

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## Symposium on ‘Metabolic flexibility in animal and human nutrition’ Session IV: Nutritional compounds for optimised healthspan and life performance

### Towards amino acid recommendations for specific physiological and patho-physiological states in pigs

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The objective of this review is to provide an overview of the implication of amino acids (AA) in important physiological functions. This is done in the context of pig production where the competition for AA utilisation is exacerbated by constraints to maximise productive responses and the necessity to reduce dietary protein input for environmental, economic and sanitary issues. Therefore, there is an opportunity to refine the nutritional recommendations by exploring the physiological roles of AA. For example, methionine and cysteine, either in selenised or sulfur forms, are directly involved in the regulation of the glutathione antioxidative system. In sows, glutathione antioxidative system may contribute to improving ovulation conditions through control of oxidative pressure. Supplementation of sow diets with L-arginine, a precursor of NO and polyamines, may stimulate placental growth, promoting conceptus survival, growth and tissue development. The beneficial effect of arginine supplementation has been also suggested to improve lactation performance. Feed intake is usually the first response that is impacted by an inadequate AA supply. Valine and tryptophan imbalances may act as signals for decreasing feed intake. AA are also important nutrients for maintaining the animal’s defence systems. Threonine, one of the main constituents of mucin protein, is important for gut development during the postnatal period. It may exert a protective effect that reduces the impact of weaning on gut morphology and associated disturbances. Finally, tryptophan is involved in the regulation of the defence system through its action as a precursor of antioxidants and its effect on the inflammatory response.

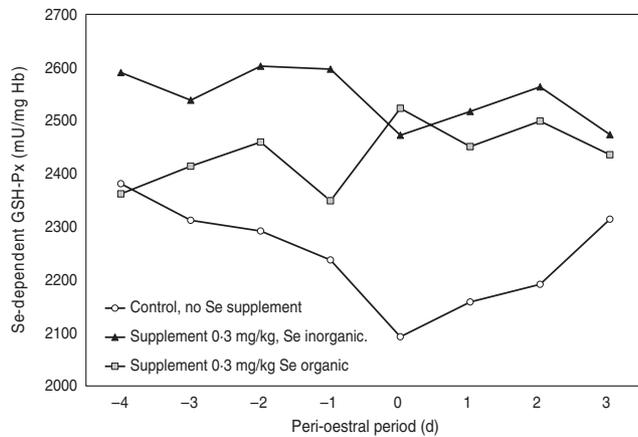
#### Amino acid: Pig: Body defence: Reproduction: Development: Growth

There is a growing interest in considering the non-proteinogenic functions of some amino acids (AA) in physiological roles such as health, stress response and tissue development<sup>(1)</sup>, and livestock species are concerned by these questions<sup>(2)</sup>. The pig is an interesting model species to refine our knowledge on the roles of AA, because decades of selection on productive traits such as prolificacy, body fatness and growth rate may have modified nutrient partitioning and exacerbated the competition between

physiological functions for AA utilisation. At present, nutritional recommendations for pigs are mainly established to maximise performance traits. These traits are mainly associated with maximising protein deposition during pre- and post-natal growth or exportation of protein in milk<sup>(3)</sup>. This is due to the fact that AA are used in large quantities for the synthesis of peptides and proteins, and that large amounts of AA are implicated in body protein turnover<sup>(4)</sup>. However, this is true only for the twenty AA

**Abbreviations:** AA, amino acid; GSH-Px, glutathione peroxidase; IDO, indoleamine 2,3-dioxygenase.

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**Fig. 1.** Blood Se-dependent glutathione peroxidase (GSH-Px) activity during the peri-estrus period in gilts according to the level and source of dietary Se. Gilts were offered a control diet containing a basal level of 0.2 mg/kg Se, or a diet supplemented with 0.3 mg/kg of either inorganic selenite (MSe) or organic Se (OSe). Day 0 = third estrus. One unit (U) of GSH-Px activity equals 1  $\mu$ mol of NADPH oxidised per minute. Values are Least Squares means and maxima SE were 206.5, 123.9, 147.5, 146.8, 129.7, 155.8, 165.1 and 195.6 for days -4, -3, -2, -1, 0, 1, 2 and 3 (adapted from<sup>(13)</sup>).

constituting body protein. At present, the revision of recommendations is necessary because of increasing constraints on pig production. The necessity to reduce the dietary crude protein content to cope with environmental issues may increase the risk that several AA are supplied in inadequate or sub-limiting amounts for physiological functions, some of which may not directly be associated with productive traits. There is also an increasing demand to reduce the utilisation of medication, mainly antibiotics, while preserving health and welfare. In fact, along with some micronutrients as cofactors, some AA act as precursors for neurotransmitters, signal molecules and as modulators of the immune system. Nutrition and recommendations should therefore include knowledge of the involvement of AA in metabolic pathways, AA metabolism and animal physiology. The objectives of this review are to identify AA involved in important physiological functions and to provide experimental evidence showing that the dietary AA supply may influence the response of the animal. In some cases, the AA profile can be modulated within the range of current nutritional recommendations. Other AA may need to be supplied in much larger amounts to induce a substantial effect. In this review, we will focus on the critical periods of pig production during which the opportunities to modulate the response of the pig by nutrition are clearly questioned.

### Effects of amino acids on reproductive performance of the sows

#### *Amino acids, antioxidative status and reproduction: methionine and cysteine*

In reproducing animals, the hormonal regulation of the ovarian metabolism generates reactive oxygen species and

free radicals<sup>(5)</sup> that must be neutralised locally by antioxidants such as vitamin E, vitamin C and by the Se-dependent glutathione peroxidase (GSH-Px) system. A constant and adequate antioxidant status is critical for optimal ovulation both in terms of quantity and quality. This is particularly important in hyperprolific lines of sows, because of the poor quality of oocytes shed by the supplemental follicles<sup>(6)</sup>.

The metabolism of oxidised and reduced glutathione, as well as the enzymes GSH-Px and glutathione reductase, are closely related to cysteine and methionine via the key metabolite homocysteine. Homocysteine is an intermediate AA that can be metabolised by transmethylation (towards methionine) or transsulfuration (towards cysteine and glutathione, the precursor of GSH-Px). In fact, reactive oxygen species and peroxides up-regulate transsulfuration and down-regulate transmethylation<sup>(7)</sup>. Therefore, the presence (or the production) of oxidative metabolites stimulates the transsulfuration pathway, which will in turn neutralise these metabolites.

The animal metabolism does not distinguish between sulfur forms of methionine and cysteine and their Se analogues<sup>(8)</sup>. Under oxidative pressure, both seleno-cysteine and seleno-homocysteine are thus key factors just like their sulfur forms. Seleno-cysteine is either supplied directly by the diet or is derived from seleno-methionine after transsulfuration, a metabolic reaction that is up-regulated by redox changes<sup>(7)</sup>. Then, it is mineralised to selenide and Se phosphate<sup>(9-11)</sup> and resynthesised into a seleno-cysteinyl moiety that modulates gene expression and activates Se-dependent enzyme, GSH-Px<sup>(9,12)</sup>. This metabolic control was observed recently in pigs<sup>(13)</sup> by studying homeostasis of Se-dependent GSH-Px during the peri-estral period under oxidative pressure (Fig. 1). The Se-dependent GSH-Px activity dropped shortly after ovulation in control gilts, while the synthesis of GSH-Px was maintained and enhanced by dietary supplementation of respectively inorganic and organic Se. This drop in GSH-Px activity of the control gilts is in agreement with the earlier-mentioned transient and local demand for antioxidants at ovulation. In contrast, there was an up-regulation of GSH-Px in gilts supplemented with organic Se in synchrony with the oxidative conditions brought by ovulation. The inorganic source of Se (selenite, the dietary form routinely used in animal nutrition) is rapidly transformed to selenide and Se-phosphate<sup>(9-11)</sup> and short-circuits the transsulfuration and the regulation of GSH-Px by redox changes. Therefore, it appears that inorganic Se produces a readily and persistent response to GSH-Px, independent of the need for this enzyme, while the response to seleno-AA directs the Se flow through a succession of metabolic steps in response to redox changes.

Several reactions of the transsulfuration pathway, which is involved in the fate of organic Se for the control of the GSH-Px system, are pyridoxine (vitamin B<sub>6</sub>)-dependent<sup>(11)</sup>. In a recent experiment, the importance of pyridoxine for an adequate flow of organic Se (Se-cysteine) towards the GSH-Px system was demonstrated in response to oxidative pressure induced by the peri-estrus period in sows<sup>(14)</sup>. Indeed, gene expressions of GSH-Px and Se-cysteine oxidase (control of the flow of Se-cysteine to the GSH-Px system) in the

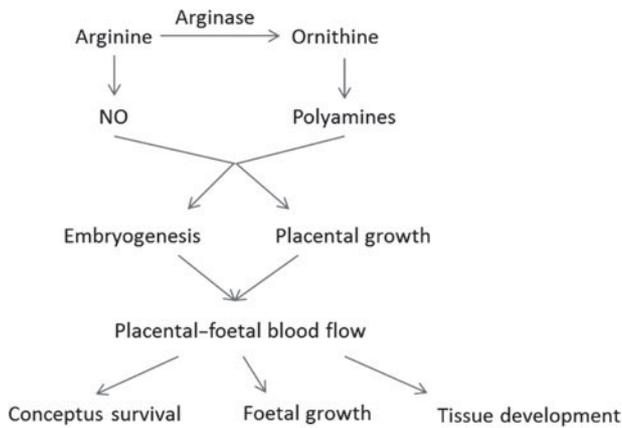


Fig. 2. Schematic involvement of arginine, NO and polyamines in fetal development (adapted from<sup>(15)</sup>).

cytosol of both liver and kidney cells were 50–70% greater in gilts supplemented with an organic source of Se and vitamin B<sub>6</sub> as compared with the other groups, either unsupplemented, supplemented with inorganic Se (with or without vitamin B<sub>6</sub>), or with organic Se without vitamin B<sub>6</sub>. In terms of reproductive performance, ovulation rate was approximately 23% greater in gilts supplemented with organic Se and vitamin B<sub>6</sub><sup>(14)</sup>. These last two effects highlight the crucial role of micronutrients modulating the metabolic pathways of AA, such as seleno-analogues of methionine and cysteine, towards regulation of antioxidation and eventually optimal ovulation conditions.

#### Fetus development and lactation: L-arginine

A moderate to extensive dietary supplementation with specific AA can have important physiological effects on reproduction. L-Arginine is the common precursor for NO and polyamines, which are key regulators of angiogenesis, embryogenesis, and placental and fetal growth. Arginine is particularly abundant in porcine allantoic fluid, and associated with high rates of synthesis of NO and polyamines in the placenta during the first half of pregnancy<sup>(15)</sup>. This led to the hypothesis that dietary supplementation with L-arginine to sows may stimulate placental growth to promote conceptus survival, growth and development of some tissues (Fig. 2). Studies have consistently shown an effect of L-arginine supplementation during gestation on reproductive performance; however, the effects vary according to treatment period and dose. When given during early gestation (0–25 d of gestation)<sup>(16)</sup>, L-arginine improved placental vascularity regardless of the dose. However, it had no effect on survival and weight of the embryos at a dose of 8 g/d supplemented arginine but had an adverse effect on these traits at a dose of 16 g/d. When supplementation started during embryonic implantation and lasted for 2 weeks (from 14 to 28 d of gestation) at a dose of 25 g/d supplemented arginine, a positive effect was observed on placental vascularity<sup>(17)</sup>. Additionally, it resulted in an increased number of fetuses (+1 to +3 fetuses)<sup>(17,18)</sup> or in an increased number of piglets born alive (+1 piglet)<sup>(19)</sup>. Importantly, this treatment did not

reduce within-litter birth weight<sup>(19)</sup>. When given from 30 d of gestation to term at the dose of 20 g/d supplemented arginine, a positive effect on litter size was still observed (+2 piglets born alive) without detrimental effects on within-litter birth weight and variation in birth weight<sup>(20)</sup>.

Dietary supplementation with L-arginine may also benefit piglets, irrespective of the L-arginine given to the lactation sow<sup>(21)</sup> or directly to the piglet<sup>(22,23)</sup>. Supplementing the lactation diet with 1% L-arginine has been shown to increase the concentration of most AA in milk compared with the control group<sup>(21)</sup>. This greater transfer of AA in the milk could be related to the positive effects of L-arginine on vascularity and blood flow to the mammary gland<sup>(24)</sup>. In support of this hypothesis, a short-term infusion of a NO donor has been shown to increase the blood flow to the mammary gland in goats<sup>(25)</sup>. Supplementing L-arginine to the diets of primiparous sows may also increase milk production<sup>(21)</sup>. Moreover, piglets receiving milk-based diet supplemented with 0.4% L-arginine had greater plasma concentrations of insulin and growth hormone<sup>(22,23)</sup>. All these studies reported an increase in weight gain of the piglets during the treatment period that may be due, at least partly, to an increase in protein synthesis and the signalling activity of the mammalian target of rapamycin in skeletal muscle<sup>(24)</sup>. Direct effects of L-arginine on tissue development have been reported *in vivo* and *in vitro*. In growing-finishing pigs, dietary L-arginine supplementation increased muscle gain by enhancing muscle protein, glycogen and fat content, while decreasing carcass fat content<sup>(26)</sup>. This is consistent with the finding that L-arginine supplementation favoured lipogenesis in muscle and lipolysis in adipose tissue<sup>(27)</sup>. Less consistent effects of arginine and NO have been reported *in vitro*. Arginine-induced pre-adipocyte differentiation in rats<sup>(28)</sup>, whereas NO suppressed some markers of differentiation in 3T3-L1 murine cell line through suppressing the transcriptional activity of the key regulator PPAR $\gamma$ <sup>(29)</sup>. These results suggest that the L-arginine supply may affect the balance between fat and muscle development in pigs, but specific studies are required to identify the most critical periods.

#### Amino acids are involved in the control of body protein deposition

In growing pigs, formulation of low protein diets is associated with a reduction of nitrogen output and energy losses. Moreover, it is one of the best strategies to reduce digestive disorders<sup>(30)</sup>. However, the supply of several AA could then be limiting for protein deposition and growth. These effects can be attributed to a direct effect on protein synthesis and deposition because one or several AA will be supplied in insufficient amounts to fulfill the potential for protein synthesis. Moreover, some AA are now known to act as signal molecules in the control of feed intake, appetite, or protein synthesis. Therefore, a better knowledge of the roles of AA in the control of feed intake and protein synthesis is needed to formulate diets for optimal feed efficiency and growth rate.

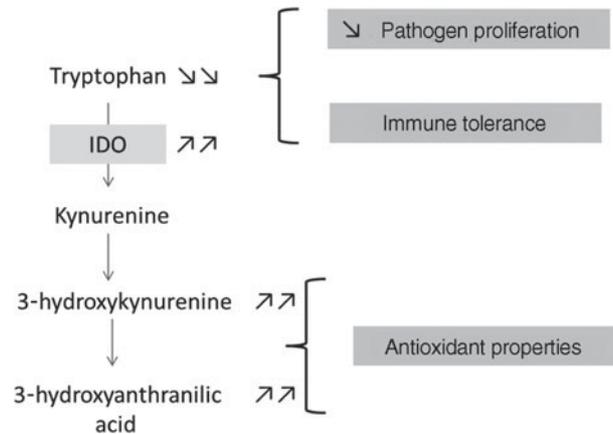
### Control of feed intake by amino acids: tryptophan and valine

An inadequate or imbalanced AA supply implies that one (or more) AA is provided in disproportionate amount compared with the others. Many species are able to detect a dietary AA imbalance<sup>(31)</sup> and react by reducing feed intake. This is particularly the case for two essential AA, tryptophan and valine. In pigs, the effect of tryptophan and valine deficiencies on growth are mainly associated with a reduction in appetite and feed intake<sup>(32–35)</sup>. In growing pigs, the depressive effect of a tryptophan deficiency on feed intake was enhanced by increasing the level of protein and thus the level of large neutral AA (valine, isoleucine, leucine, tyrosine, phenylalanine and methionine). These AA compete with tryptophan for transport across the blood–brain barrier<sup>(34,36)</sup> because they share the same transport system. The depressive effect of valine deficiency on feed intake was enhanced by an excess supply of leucine known to stimulate the activity of the branched-chain keto acid dehydrogenase complex, thereby increasing the catabolism of valine in rats and pigs<sup>(35,37)</sup>. Recent data showed that pigs rejected a valine-imbalanced diet within 2 d of exposure to the diet by reducing the daily number of meals<sup>(38)</sup>. This behavioural response suggests that the response to a valine imbalance results from post-absorptive feedback signals.

The physiological mechanisms involved in the rejection of an AA imbalanced diet are probably different among AA and have not been clearly identified in pigs. In rats, an AA imbalance is detected by the brain in the cortex piriform anterior<sup>(39)</sup>. An AA deficiency results in an accumulation of its uncharged tRNA, which activates the general control of non-depressible-2 kinase in the cortex piriform anterior. A valine deficiency increased the expression of somatostatin mRNA in the hypothalamus in the anorectic mice, and intracerebroventricular administration of somatostatin reduced the feed intake of mice<sup>(40)</sup>. Moreover, because tryptophan is a precursor of serotonin, a brain neuromediator involved in the control of feed intake in pigs, this pathway might be involved in the rejection effect of a tryptophan-deficient diet. Serotonin manipulation is known to affect meal size<sup>(34,41)</sup>. Tryptophan could be also involved in the peripheral control of appetite through a modulation of the rate of gastric emptying<sup>(42)</sup>, insulin secretion and sensitivity<sup>(42,43)</sup>. Tryptophan effect on appetite could be mediated by ghrelin<sup>(44)</sup>, a gut hormone involved in the regulation of feed intake. Indeed, tryptophan supplementation to low-tryptophan diets induced ghrelin secretion, expression of ghrelin mRNA in the stomach and duodenum of piglets, and increased feed intake. Tryptophan also stimulates the release of cholecystokinin, a hormone that decreases meal size<sup>(45)</sup>.

### Control of protein synthesis by amino acids: leucine

The role of leucine, one of the three branched-chain AA, has been known for a long time for its ability to stimulate muscle protein synthesis and inhibit protein degradation in rodents<sup>(46,47)</sup> and young piglets<sup>(48–50)</sup>. The role of leucine as a signal molecule for the intracellular pathway leading



**Fig. 3.** Roles of tryptophan catabolism by the enzyme indoleamine 2,3-dioxygenase (IDO) on the animal's defence system. ↗, ↘, increase and decrease respectively.

to activation of the first step of protein synthesis has been widely explored. Leucine is known to activate mammalian target of rapamycin leading to activation of the translation initiation factor and then to the aggregation of ribosome and mRNA translation<sup>(51)</sup>. Recently, this effect has been reported in young growing pigs fed a low-protein diet supplemented with leucine up to 30% higher than the recommendations for maximum growth rate<sup>(52)</sup>. After 2 weeks of treatment, growth rate and tissue protein synthesis were increased in pigs fed the leucine-supplemented diet and this effect was independent of feed intake. This result opens new ways to improve feed efficiency and growth rate through nutrition.

### Amino acids are involved in health preservation

#### Preservation of gut homeostasis: threonine

In young piglets, threonine has been shown to be indispensable for gut development and physiology. Threonine is extracted from the small intestine in greater proportion than the other essential AA<sup>(53)</sup>. In orally fed piglets, the threonine requirement, evaluated by the indicator AA oxidation technique, is twice that of parenterally fed piglets<sup>(54)</sup>. The high rate of intestinal threonine extraction is not explained by threonine catabolism<sup>(53,55)</sup> but is associated with intestinal protein synthesis, especially for the synthesis of mucins<sup>(56,57)</sup>. The threonine content in mucin ranges from 13 to 26% of total constituting AA<sup>(58,59)</sup>. Mucins are excreted in the lumen, and their constituting AA are poorly re-absorbed. Therefore threonine is found in high concentrations in ileal endogenous protein losses<sup>(60)</sup>. This questions the impact of dietary threonine supply on gut homeostasis. In piglets, a threonine deficiency impaired total protein and mucin synthesis<sup>(61,62)</sup> and also had an impact on other functions of the small intestine. In piglets, a low threonine supply (70% of recommendations) for 2 weeks induced atrophy of the villi associated with a reduction in aminopeptidase N activity in the ileum<sup>(63)</sup>. It also increased paracellular permeability and glucose absorption capacity<sup>(64)</sup>, and modified ileal gene expression

profiles. Notably, the increase in the expression of genes involved in immune and defence functions associated with the increased paracellular permeability suggests that threonine is essential to preserve intestinal integrity. These morphological and functional disturbances are also observed during starvation or malnutrition (e.g. after weaning) and are frequently associated with an increased risk of gut disorders<sup>(65–67)</sup>. The impact of dietary threonine on weaning diet as a strategy to minimise gut disorders deserves further attention<sup>(68)</sup>.

#### *Control of inflammatory response: tryptophan*

In pig farms, permanent exposure to antigens, unfavourable sanitary conditions and moderate infectious diseases induce an inflammatory status that limits growth by reducing feed intake<sup>(69)</sup> and affecting nutrient metabolism<sup>(70,71)</sup>. Indeed, AA are partitioned from body protein deposition towards tissues and functions involved in the animal's defence system<sup>(72)</sup>. If an AA is supplied in limited amount, this partition is not necessarily fully compensated by endogenous supplies. The consequence could be a reduction of AA available for protein deposition, the defence system, or for both. The impact of an inflammatory challenge on AA metabolism has been demonstrated in several species for dispensable AA such as cysteine involved in glutathione synthesis<sup>(73)</sup>, glutamine used as a fuel for rapidly dividing cells such as enterocytes and lymphocytes<sup>(74)</sup>, and tryptophan and its metabolites implied in the inflammatory response.

Tryptophan is one of the indispensable AA that is found in low concentration in body proteins and plasma. This relatively low amount of tryptophan is singular considering that this AA is involved in several physiological functions such as the regulation of growth, mood, behaviour and immune responses<sup>(75)</sup>. Depletion of plasma tryptophan has been reported during experimentally induced inflammation in pigs<sup>(76,77)</sup>, mice<sup>(78)</sup> and human subjects<sup>(79,80)</sup>. This decrease can be attributed to the synthesis of acute phase proteins, which have a high tryptophan content<sup>(81)</sup>, and/or to an increase in tryptophan catabolism. Indoleamine 2,3-dioxygenase (IDO), a rate-limiting enzyme for the catabolism of tryptophan to kynurenine, is induced by interferon- $\gamma$ <sup>(80,82)</sup> and by inflammation in pigs<sup>(83,84)</sup>.

Increased tryptophan catabolism and IDO activation during inflammation are considered as mechanisms involved in the defence system. Three hypotheses have been proposed (Fig. 3). The first one is that local tryptophan depletion resulting from IDO activation in macrophages would be a mechanism controlling proliferation of bacteria, virus and parasite<sup>(80,82,85)</sup>. The second one is that IDO regulates T-cell activity. Such a mechanism has been shown during gestation where IDO exerts a protective role by preventing the fetal allograft rejection by maternal T-lymphocytes<sup>(86)</sup>. Finally, IDO induction during immune activation may protect cells from oxidative damage<sup>(87)</sup>. Indeed, 3-hydroxyanthranilic acid and 3-hydroxykynurenine, produced from tryptophan through the IDO–kynurenine pathway, have antioxidant properties<sup>(88)</sup>. This suggests that dietary tryptophan could influence the inflammatory response and the animal health. In support of

this hypothesis, pigs suffering from an experimentally induced lung inflammation and offered a diet with tryptophan concentration slightly higher than the recommendations had lower acute phase protein concentration and were healthier compared with pigs offered a diet moderately deficient in tryptophan<sup>(84)</sup>. In a porcine model of induced colitis, tryptophan supplementation loading down-regulated inflammation, restored local immune response and reduced colitis symptoms<sup>(89)</sup>.

The consequences of inflammation on tryptophan metabolism probably impaired the availability of tryptophan for body protein deposition and growth<sup>(90)</sup>. However, additional crystalline tryptophan did not totally prevent growth retardation caused by inflammation. Nevertheless, an improvement in growth rate by additional tryptophan was observed in pigs suffering from a moderate inflammation compared with control pigs<sup>(91)</sup>. Additional tryptophan allowed pigs to partially compensate for the effects of an *Escherichia coli* infection by increasing feed intake and maintaining growth<sup>(92)</sup>. These results suggest that dietary tryptophan should be maintained at an adequate level to preserve health during critical phases of pig rearing.

#### **Conclusion**

Further investigations are required to improve our knowledge on AA metabolism and their implications in various physiological functions. One of the challenges in the future will be to identify novel traits and indicators that are modulated by the AA supply and that could be used to revise nutritional requirements and recommendations. Moreover, ranges for safety and efficiency of AA utilisation should be clearly established, especially when AA are supplied in large amounts. The interactions between AA and other nutrients also merit further consideration. Despite the increasing availability of synthetic AA or AA precursors, their utilisation could be still limited by their cost and specific regulation.

#### **Acknowledgements**

The authors are grateful to the Oskar Kellner Symposium committee. We acknowledge our colleagues from INRA PEGASE, France and Dairy and Swine R & D Centre, Agriculture and Agri-Food Canada, Canada for their contributions. The authors have no conflict of interest. All the authors contributed significantly to the preparation of this manuscript.

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