



Balance and form of amino acid intake in the diet : physiological and metabolic consequences in pigs

Francis Amann Eugenio

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L'ENVIRONNEMENT**

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Par

Francis Amann EUGENIO

**Équilibre et forme de l'apport en acides aminés dans l'alimentation :
Conséquences physiologiques et métaboliques chez le porc**

**English title: Balance and form of amino acid intake in the diet: physiological and metabolic
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SYNTHÈSE EN FRANÇAIS

1. Introduction

Ce projet de thèse CIFRE (convention n° 2018/1771) a été réalisé dans le cadre d'un partenariat entre l'Institut National de Recherche pour l'Agriculture, l'Alimentation, et l'Environnement (INRAE) et une entreprise, BCF Life Sciences (Pleucadeuc, France). Cette entreprise est spécialisée dans la valorisation des plumes de volailles par une hydrolyse intensive principalement pour la production de L-cystine et de L-tyrosine ainsi qu'un hydrolysate riche en acides aminés (AA) qui est utilisé comme source de protéines pour l'alimentation animale, notamment pour animaux de compagnie et pour poissons.

Pour les animaux, les protéines sont essentielles à la vie car ces macronutriments constituent de nombreuses biomolécules qui ont une variété de fonctions importantes et cruciales. La synthèse de toutes les protéines dont l'animal peut avoir besoin se produit tant qu'il dispose des substrats pour le faire. Les plus élémentaires de ces substrats sont les vingt AA protéinogéniques. Tant que l'animal dispose d'une quantité suffisante de ces AA par rapport à ses besoins, la synthèse normale des protéines peut avoir lieu. Comme les animaux n'ont pas la capacité de synthétiser certains des AA protéinogéniques, ils doivent les obtenir via leur alimentation (Wu 2014). Cependant, les AA sont fournis par une variété de sources de protéines qui apportent des AA sous différentes formes (i.e., protéine intacte ou AA libre) et des profils variables (i.e., équilibré ou déséquilibré). Ces deux propriétés des protéines alimentaires peuvent avoir un impact significatif sur la biodisponibilité des AA pour les pores et peuvent affecter leur statut métabolique et physiologique. Un profil équilibré en AA alimentaire peut avoir des effets positifs sur l'utilisation de l'AA pour la synthèse des protéines et réduire l'excrétion d'azote (Ten Have et al. 2007; Kampman-van de Hoek et al. 2016). Cependant, la forme des AA alimentaires peut influencer l'absorption par l'animal (Liu et al. 2013; Chrystal et al. 2020a), ce qui peut affecter sa physiologie intestinale (Zhang et al. 2013; Morales et al. 2017) ; ainsi que

l'utilisation par les tissus périphériques (Gwin et al. 2020; Weijzen et al. 2022). Ces deux aspects, l'équilibre et la forme des AA alimentaires, sont le sujet principal de cette thèse.

Pour poursuivre notre étude de l'équilibre et de la forme de l'apport, nous avons choisi d'utiliser une source de protéines modèle qui est constituée de plumes traitées par hydrolyse acide extensive (EHF) fabriqué par BCF Life Sciences. Cela nous a permis d'utiliser un produit dont le profil d'AA est intrinsèquement déséquilibré, avec de faibles niveaux d'AA comme lysine, méthionine, histidine, et tyrosine. D'autre part cette méthode de traitement transforme toutes les protéines sous une forme qui peut être absorbée immédiatement sans besoin de digestion (Kersanté et al. 2021). En effet, la fraction protéique des EHF est constituée à 82% d'AA libres tandis que le reste est sous forme de petits peptides. En raison de la forme des AA dans les EHF, nous pouvons facilement l'équilibrer à l'aide d'AA libres sans avoir l'effet confondant de donner des AA qui sont absorbés à des taux différents (Batterham and Bayley 1989). Actuellement, les plumes sont principalement utilisées dans l'alimentation animale sous la forme de farine, après un traitement à la vapeur et sous pression. Par rapport aux EHF, la farine de plumes a une digestibilité plus faible car sa fraction protéique doit encore être digérée avant d'être absorbée (Grazziotin et al. 2006). En utilisant l'EHF et la farine de plumes, nous pouvons donc étudier l'effet de la forme des AA alimentaires en utilisant des produits provenant de la même source et ayant un profil d'AA comparable. En utilisant l'EHF comme source de protéines modèle, l'objectif de la thèse était d'étudier les conséquences métaboliques et physiologiques de l'alimentation des animaux (1.) avec un régime dont le profil en AA est déséquilibré et (2.) un régime dont les AA se présentent sous la forme d'AA libres et de petits peptides au lieu de protéines intactes.

2. Procédures expérimentales

Comme la réponse métabolique et physiologique des animaux est très liée à leur biologie, les expériences *in vitro* ne seraient pas en mesure de reproduire avec précision certains mécanismes que nous voulions mesurer dans la thèse. Nous avons donc décidé que la meilleure approche était une approche *in vivo*, avec des expériences sur les animaux. Nous avons choisi le porc comme animal modèle car il est suffisamment grand pour effectuer des prélèvements sanguins en série. Les porcs utilisent généralement les AA ingérés pendant un repas pour leur croissance et leur entretien. Les concentrations plasmatiques d'AA sont affectées par l'absorption des AA alimentaires, la dégradation des protéines, la synthèse *de novo*, ainsi que l'absorption d'AA et la synthèse protéique des tissus périphériques. Le métabolisme animal étant souvent compris comme un taux métabolique, notre approche a consisté à mesurer les changements dans les concentrations plasmatiques des métabolites, y compris les AA, pour représenter la réponse de l'animal au régime alimentaire (Kimball and Jefferson 2002; Lobley and Lapierre 2003; Bos et al. 2003). En effectuant les mesures postprandiales, nous avons pu attribuer les changements des concentrations plasmatiques à la réponse au régime alimentaire.

L'intestin des porcs est très adaptable à divers facteurs, notamment à la disponibilité des nutriments dans le régime alimentaire (Boudry et al. 2002). On peut donc s'attendre à ce que l'alimentation en AA ayant des cinétiques de digestion et d'absorption différentes, comme des protéines et des AA libres, induise des changements dans la physiologie de l'intestin grêle. Cependant, l'intestin est un organe long et tubulaire, souvent divisé en trois sections distinctes (i.e., duodénum, jéjunum et l'iléon). L'effet de la cinétique d'absorption des AA de chaque source (farine ou hydrolysate) sur la physiologie intestinale peut donc différer selon la section d'intestin étudiée.

Nous avons réalisé trois expériences dans cette thèse, chacune ayant ses propres objectifs scientifiques indépendants. Les expériences ont été approuvées par le Ministère de la Recherche

(première expérience : n° 2019041610073508; deuxième et troisième expérience : n° 27196-2020091509061764_v2) conformément à la directive sur l'expérimentation animale (2010/63).

Tous les porcs dans les expériences qui ont été équipé de cathéters jugulaires ont été placés afin d'effectuer des prélèvements sanguins fréquents sans douleur ni stress.

Dans la première expérience, l'objectif était de mesurer l'évolution de la concentration plasmatiques des métabolites et des hormones (AA, glucose, insuline, acides gras libres, lactate et urée) après l'ingestion d'un repas test. Cette expérience a été réalisée avec 4 Yucatan mini-porcs adultes (38.7 ± 2.9 kg) et 4 porcs en croissance (Piétrain x [Large white x Landrace]; 48.9 ± 0.8 kg). L'utilisation de ces deux types de porcs nous permet d'étudier la réponse métabolique d'animaux ayant une utilisation contrastée des AA. Les porcs adultes ont un faible dépôt de protéines car ils mangent principalement pour se maintenir, tandis que les porcs en croissance ont un dépôt élevé de protéines car ils grandissent. Le repas test est une technique expérimentale dans laquelle un porc est nourri avec un régime après un état de jeûne (>12 h) et la réponse postprandiale est suivie par des prélèvements sanguins en série (pendant 6 h). Dans le régime BAL, L-Lys, L-Met, L-His, L-Ile et L-Tyr ont été ajoutés pour équilibrer le profil AA des EHF en fournissant tous les AA essentiels limitants. Pour s'assurer que la quantité d'AA totaux était la même pour tous les régimes (pour les porcs adultes et en croissance), des AA purifiés non essentiels L-Ala, L-Asp, L-Glu et L-Gly ont été ajoutés dans tous les régimes. Enfin, L-Trp a été ajouté à tous les régimes dans la même quantité, car l'EHF ne contient pas cet AA qui est essentiel chez les porcs. Pour les repas tests, chaque régime a été donné à chaque porc trois fois (6 repas tests par porc au total), permettant à chaque porc d'être son propre témoin. Les jours en dehors des repas tests, les porcs ont été nourris avec le régime BAL.

Dans la deuxième expérience, l'objectif était d'observer la réponse métabolique postprandiale des porcs en croissance après l'alimentation de différentes formes d'AA. Nous avons donc préparé trois régimes alimentaires expérimentaux avec différentes formes de l'apport d'AA

alimentaires : des protéines intactes (**INT**), ou de l'AA libre et des petits peptides provenant de EHF (**HYD**), ou sous forme d'AA libre utilisant des AA purifiés (**FAA**). Ces trois régimes présentaient des profils AA similaires. Nous avons donné ces régimes alimentaires à 30 porcs en croissance (Piétrain x [Large white x Landrace] ; 33.7 ± 4.1 kg). Comme l'expérience 1, nous avons utilisé la technique du repas test dans cette expérience. Mais, cette procédure n'a été effectuée qu'une seule fois pour chaque porc.

Enfin, pour la troisième expérience, nous avons étudié les effets de la forme des AA alimentaires sur la physiologie digestive des porcs en utilisant les mêmes porcs et les mêmes régimes expérimentaux que ceux de la deuxième expérience. Après 14 jours d'alimentation, nous avons abattu les porcs 3 h après leur avoir donné un régime avec des marqueurs indigestibles. Nous avons ensuite prélevé des tissus du duodénum, du jéjunum et de l'iléon afin de les utiliser pour des mesures la morphologie intestinale et l'expression de différents transporteurs d'AA. Nous avons également prélevé des échantillons de digesta pour mesurer la digestibilité des AA.

3. Expérience 1 : Réponse du métabolisme postprandial de porcs adultes et en croissance après l'alimentation avec un profil d'acides aminés équilibré et déséquilibré

Les concentrations plasmatiques des métabolites que nous avons mesurées se situaient dans la fourchette des concentrations mesurées dans d'autres expériences (Mohamed et al. 2019 ; Campos et al. 2019). Mais les concentrations plasmatiques ne représentent pas les flux de nutriments, qui sont directement contrôlés par le métabolisme des porcs (Cynober 2002; Liao et al. 2018). Ainsi, pour mieux caractériser la réponse métabolique globale des porcs, nous avons développé un modèle conceptuel qui simplifie la cinétique postprandiale des porcs en trois ou quatre paramètres avec une signification biologique (van Milgen et al. 2022).

Conformément à notre hypothèse, l'alimentation des porcs avec un régime au profil en AA équilibré entraîne une utilisation plus rapide des AA alimentaires (i.e., pour la synthèse des protéines) par rapport à l'alimentation avec un profil en AA déséquilibré (Tuitoek et al. 1997; Moehn et al. 2004a; Kampman-van de Hoek et al. 2016). Nous avons observé cela en estimant l'aire sous la courbe (*AUC*, $\mu\text{M}\cdot\text{min}$) à l'aide de notre modèle, qui s'est trouvé être généralement plus faible pour le régime BAL par rapport au régime UNB. L'*AUC* est représentatif de l'exposition métabolique des porcs aux AA alimentaires après l'alimentation, c'est le résultat de l'influx (i.e., absorption alimentaire après le métabolisme de l'intestin et foie, mobilisation à partir d'autres tissus) et de l'efflux (i.e., synthèse des protéines, conversion en d'autres molécules, catabolisme pour excrétion) d'AA dans le plasma après l'alimentation (van Milgen et al. 2022). Il y a deux explications au fait qu'elle puisse être plus basse. La première est qu'elle peut signifier une plus rapide disparition d'AA dans le plasma, ce qui suggère une utilisation postprandiale plus rapide. D'autre part, cela pourrait signifier que la quantité d'AA dans l'aliment est faible. Cette deuxième hypothèse pourrait expliquer les *AUC* de Lys, Met, et His particulièrement faibles lors de l'alimentation avec le régime UNB, car ces AA sont limitant dans ce régime. En outre, leur concentration finale (lors du dernier prélèvement sanguin) était inférieure à leur concentration initiale (dans l'expérience, celle-ci était mesurée par le paramètre

C_{δ} , μM). Nous pensons que cela est dû à leur demande continue après l'alimentation, même si leur apport dans le régime est limité.

Nous avons également confirmé les résultats d'une autre expérience qui a montré que les porcs en croissance ont des besoins en protéines et en AA plus élevés que les porcs adultes. En effet, leur dépôt de protéines est encore élevé alors que les porcs adultes mangent surtout pour l'entretien, avec des taux de synthèse de protéines plus faibles (Moehn et al. 2004b). Cela se reflète dans l'*AUC* des porcs en croissance qui est généralement plus faible que l'*AUC* des porcs adultes. De plus, les porcs en croissance semblent être plus affectés par le déséquilibre de l'AA alimentaire puisque leur *AUC* pour le régime UNB est beaucoup plus grande que l'*AUC* des porcs adultes nourris avec UNB. Cela peut être dû au fait que nous avons comparé l'*AUC*, qui comprend à la fois l'influx et l'efflux des AA dans le plasma. Étant donné que les porcs en croissance ont un taux de synthèse des protéines plus élevé (donc un efflux plus important) que les porcs adultes, il est logique qu'ils soient plus affectés par un profil en AA déséquilibré. Cela explique également pourquoi les porcs en croissance semblent être plus sensibles que les porcs adultes à un régime alimentaire dont le profil en AA est déséquilibré. Lorsque l'alimentation contient des quantités limitées d'AA, les porcs en croissance ont plus de difficultés que les porcs adultes à assimiler les AA libres absorbés du plasma dans les tissus (Kerr et al. 2003; Moehn et al. 2004b).

Nous avons également trouvé que le métabolisme de premier passage dans l'intestin et le foie joue un rôle relativement important dans l'homéostasie de l'AA chez les porcs adultes. En effet, les différences d'apport en AA alimentaire entre BAL et UNB entraînent des différences plus faibles dans les concentrations plasmatiques. Nous avons mesuré cela dans la différence de paramètre Q (μM) entre deux régimes. Le paramètre Q représente la quantité apparente d'AA alimentaire apparaissant dans le plasma, il a été calculé en utilisant le modèle. En utilisant cette méthode, nous avons trouvé qu'une quantité moindre d'AA alimentaire atteint le plasma des

porcs adultes par rapport aux porcs en croissance. Nous attribuons cette différence à la différence au sort de l'AA alimentaire entre les porcs adultes et les porcs en croissance. Chez les adultes, les AA alimentaire sont principalement utilisés pour le renouvellement des protéines corporelles, y compris les sécrétions endogènes, et le catabolisme. Ces processus sont principalement contrôlés par le foie (Munro 1974). Alors que les 'AA sont principalement utilisés pour la synthèse et l'accrétion des protéines musculaires chez les porcs en croissance (Whittemore et al. 2001; Moehn et al. 2004a) ; cela peut signifier que les porcs en croissance apportent le plus d'AA possible dans le plasma pour supporter les besoins des tissus périphériques.

4. Expérience 2 : Réponse métabolique des porcs après l'alimentation d'un régime avec des formes différentes d'acides aminés

L'expérience 2 a été réalisée avec la technique de repas test comme celle de la première expérience. Nous avons donc également modélisé les concentrations plasmatiques postprandiales des AA. Les résultats ont clairement montré que les protéines intactes et les AA libres sont absorbés à des taux très différents. Ce que nous avons trouvé était conforme à notre hypothèse et aux résultats d'autres expériences (Liu et al. 2013; Weijzen et al. 2022). Le régime INT a donné lieu à une cinétique très plate par rapport à HYD et FAA, cela était évident simplement en regardant leur cinétique plasmatique postprandiale mais plus encore dans les paramètres estimés du modèle. Ces différences étaient très probablement dues aux différences de dynamique digestive entre les régimes expérimentales, d'autres expériences ayant abouti à des résultats similaires (Weijzen et al. 2022).

Cependant, nous avons trouvé que l'alimentation FAA entraîne une apparition plus rapide et des concentrations plus élevées d'AA alimentaire dans le plasma par rapport à HYD. Comme mentionné précédemment, bien que le HYD soit principalement composé d'AA libres, de petits peptides sont toujours présents. Ceci n'est pas en accord avec d'autres expériences qui ont trouvé que l'alimentation en peptides entraîne une absorption plus rapide des AA alimentaires (Hara et al. 1984; Hegarty et al. 2015). Mais c'est peut-être parce que les peptides ne représentent qu'une petite partie de des AA totaux dans HYD. Cependant, une autre explication pourrait être que par rapport aux FAA, il pourrait y avoir plus d'AA retenus dans l'intestin grêle et le foie lorsque les porcs sont nourris avec le régime HYD. Nous l'avons vu lorsque nous avons calculé le Q des régimes et l'avons mis en relation avec la quantité d'AA et avons constaté qu'il était plus faible pour HYD par rapport aux FAA. Il se peut que la rétention des petits peptides dans l'intestin grêle est plus élevée que celle des AA libres. Il se pourrait donc que le HYD ait été absorbé plus rapidement, mais que moins d'AA soient apparus dans le plasma.

Cependant, on ne sait toujours pas si le taux d'apparition rapide des AA alimentaires dans le plasma (i.e., HYD et FAA) est meilleur qu'un taux d'apparition lent (i.e., INT). Même si ces résultats entraînent une différence très marquée dans la forme de la cinétique postprandiale, nous ne savons pas vraiment s'ils induisent une différence dans l'utilisation ou la synthèse protéique des animaux. c'est notamment vu dans la concentration d'urée postprandiale et ce que l'on constate dans une étude précédente (Chrystal et al. 2020b). L'alimentation de INT entraîne une concentration en urée plasmatique plus élevée à l'état de jeûne par rapport à l'alimentation HYD et FAA. Mais après l'alimentation, nous voyons clairement que la concentration d'urée plasmatique diminuent avec le régime INT alors qu'elle augmente avec HYD et FAA. Comme l'urée est souvent utilisée comme un proxy pour estimer le catabolisme de l'AA (Chen et al. 1995; Bos et al. 2003), nous pouvons dire qu'il y avait relativement plus d'AA catabolisé dans la période postprandiale lorsque le régime est composé d'AA libre et de petits peptides au lieu des protéines intactes. Néanmoins, les concentrations d'urée étaient toujours plus élevées pour l'INT à la fois dans la concentration à jeun et tout au long de la période postprandiale.

5. Expérience 3 : Réponse physiologique des porcs après l'alimentation avec des formes différentes d'acides aminés

Dans la troisième expérience, comme prévu, nous avons vu que la digestibilité des AA de INT était beaucoup plus faible que celle de HYD et FAA. Ce que nous avons mesuré pour INT est proche de ce qui est rapporté par d'autres expériences (Sulabo et al. 2013; Park et al. 2020a). Bien qu'une observation très notable ait été que la digestibilité de HYD était généralement plus élevée que celle de FAA. Les formes d'AA présentes dans ces deux régimes sont facilement absorbables sans qu'il soit nécessaire de les hydrolyser. Nous nous attendions donc à ce que leur digestibilité soit assez similaire. Une explication possible de ce observation est que l'absorption des petits peptides est plus rapide que celle des AA libres individuels, ce qui est confirmé par d'autres études (Hara et al. 1984; Ten Have et al. 2007)

Les données morphologiques mesurée sur les trois sections de l'intestin grêle du porc dans cette expérience sont conformes à celles rapportées par d'autres expériences (Guay et al., 2006; S. Park et al., 2020). Une plus grande surface de l'intestin (i.e., la longueur des villosités par rapport à la profondeur de la crypte) permet une plus grande absorption et la libération d'une plus grande quantité d'enzymes digestives (Dibner and Richards 2004; schulze 2006). De plus, l'alimentation des porcs avec des régimes hautement digestibles entraîne une augmentation de la hauteur des villosités et du ratio villosités/crypte (Yun et al. 2005; Park et al. 2020b), ce que nos résultats ont pu confirmer mais uniquement dans le jéjunum.

Nous avons vu que l'expression de PepT1 était plus élevée dans le duodénum des porcs nourris avec INT. Ceci était totalement inattendu car qu'il ne devrait pas y avoir beaucoup de peptides facilement absorbables pour INT dans cette section de l'intestin. Le temps de transit de la partie soluble du digesta est très rapide dans la partie proximale de l'intestin grêle, cela n'a peut-être pas donné assez de temps pour que la plupart des peptides de l'HYD soient absorbés dans le duodénum (Schop et al. 2019; Selle and Liu 2019). Cela peut également être la raison pour laquelle l'expression de PepT1 dans le jéjunum était plus élevée pour HYD par rapport au

duodénum. Par ailleurs, l'expression de ASCT2 et CAT-1 dans le jéjunum était la plus élevée chez les porcs nourris au HYD ; ces deux transporteurs peuvent transporter la plupart des AA (Bröer and Fairweather 2018). Cela peut contribuer à expliquer la digestibilité plus élevée de HYD par rapport à FAA.

6. Conclusion

Chez les porcs nourris avec un régime présentant un profil d'AA déséquilibré et des quantités limitées de certains AA essentiels, comme les EHF, les cinétiques plasmatiques montre que les concentrations en AA essentiels limitant sont inférieures aux concentrations à jeun. Cependant, le fait d'équilibrer le profil en AA des régimes alimentaires en fournissant des AA limitants avec des AA purifiés entraîne généralement des concentrations plasmatiques d'AA plus faibles, ce qui suggère une utilisation globale plus élevée des AA au cours de la période postprandiale. Il convient toutefois de faire preuve de prudence, lorsqu'il s'agit d'équilibrer le profil AA des protéines intactes avec les AA libres. Contrairement aux EHF, les AA des sources protéiques sont absorbés moins rapidement par rapport aux AA libres, ce qui peut également entraîner un déséquilibre transitoire des AA lorsque des AA libres sont ajoutés en grandes quantités, en raison d'un différentiel de temps d'arriver des AA alimentaires dans le plasma. Cela signifie également qu'il existe une différence de biodisponibilité entre les AA provenant de protéines intactes et de les AA libres, ce qui peut influencer le statut métabolique global des porcs. Enfin, la forme de l'AA alimentaire peut entraîner des changements dans la physiologie des différentes sections de l'intestin grêle des porcs, comme la morphologie de la surface de la lumen et l'expression du gène de différents transporteurs d'AA et de peptide

INTRODUCTION

The goal of feeding animals is to provide nutrients that are crucial for their life. Among these nutrients are amino acids (**AA**), which are used by animals to synthesize its own proteins, as an energy source, and as precursors for other biomolecules (Wu 2014). In animal feeds, AA are usually supplied by different protein sources with varying AA profiles and digestibility. The nutritional values of protein sources for animal diets are usually evaluated on their AA content and profile, particularly for the content of essential AA, as well as their digestibility. High quality protein sources for animal feeds are usually in competition with those used in human diets. To make animal feed more sustainable, limiting this competition by using protein sources in animal diets that are not used for human nutrition can be a solution (Gasco et al. 2020). Nowadays, there are multiple technological methods to valorise different protein sources that can also improve their nutritive value and digestibility. However, these protein sources need to be evaluated for their nutritional properties to determine their effects on animal performance, metabolism, physiology, and health.

One of the human-inedible protein sources is feathers, a typical by-product of poultry raising operations that is also produced in bulk. Using feathers in animal feed greatly contributes to a more circular economy as this means that we are reusing animal by-products that would otherwise just be waste as an input for animal feed. Currently, feathers are being used in animal diets as feather meal after processing by steam and pressure. However, a limitation of its use in feed is that it has disproportionately low amounts certain essential AA like His, Lys, and Met coupled with a relatively low digestibility (Grazziotin et al. 2006; Kerr et al. 2019)

The thesis is a CIFRE thesis in partnership with BCF Life Sciences (Pleucadeuc, France) that specializes in the valorisation of feathers by extensive acid hydrolysis to produce purified L-Tyr and L-Cys, which leaves a product with a mix of the rest of the AA of feathers (**EHF**). Currently, EHF is being used in pet food diets as a hypoallergenic and highly digestible protein source and in aquaculture feed as a palatability booster. Due to the method of processing, the

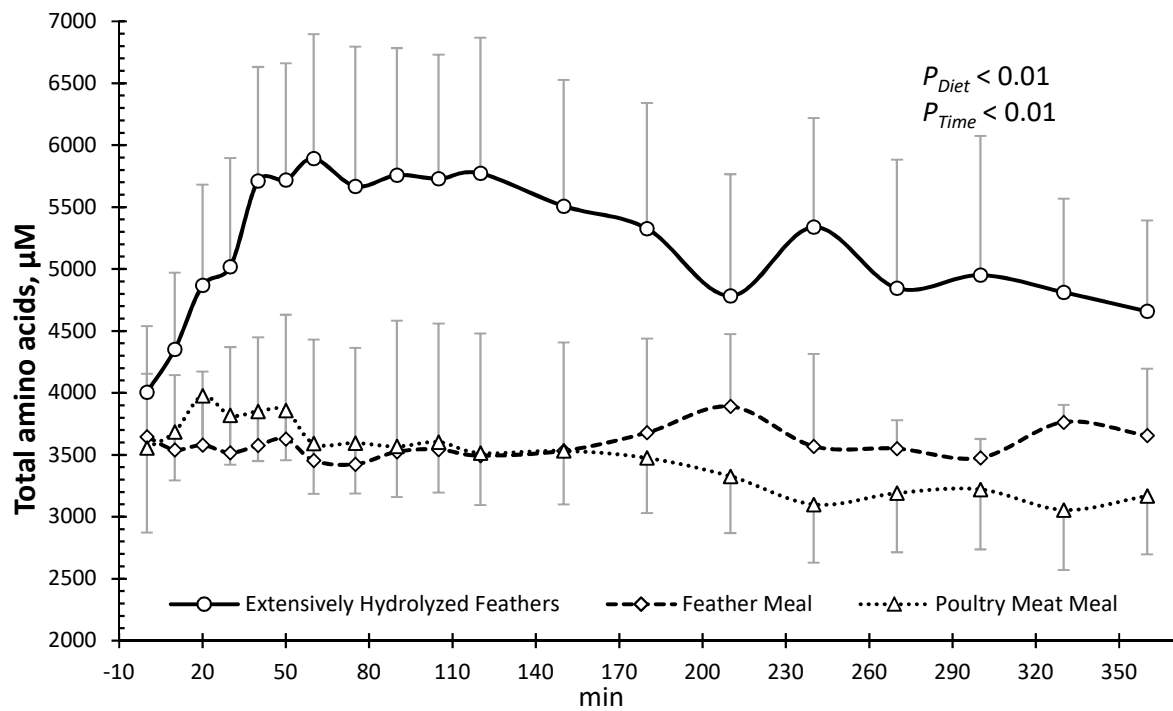


Figure 1. Postprandial plasma kinetics (μM) of total amino acids of adult mini-pigs (n = 6) after a meal test with three different experimental diets providing the same amount of total amino acids with either an extremely hydrolyzed feather meal, feather meal, or poultry meal as the sole protein source

protein fraction of this product is transformed to free AA (82% of all AA) and small peptides (di- and tri-peptides with ≤ 800 Da molecular weight). Although, the process also makes its AA profile more unbalanced than feather meal as, in addition to the very low amounts of His, Lys, and Met, it is also deficient in other essential AA like Tyr and Cys as they are taken out to be sold as purified AA. Furthermore, it is completely devoid of Trp that is inevitably destroyed with acid hydrolysis. Even though it might not be interesting in a nutritional point of view when used alone, its unique properties of being made up of free AA and small peptides makes it very different from the most commonly used protein sources in animal nutrition and makes it a good candidate to be used as a model protein source for studies involving dietary AA balance and form.

In a previous study performed by INRAE PEGASE (Saint-Gilles, France), it was found that feeding adult pigs with EHF results in elevated concentrations of total AA in the plasma up to 6 hours after feeding compared to other protein sources (i.e., feather meal and poultry meat meal) providing the same amount of TAA whose profiles were deliberately left unbalanced (Figure 1 adapted from Meniga et al. 2017). Elevated plasma AA concentrations for extended periods (i.e., 6 hours in this case) can be an indication of low AA bioavailability and utilization (i.e., for protein synthesis, bioconversion to other molecules, etc.) by peripheral tissues (Lobley and Lapierre 2003). Thus, feeding pigs with EHF can induce a unique metabolic and physiological response compared to typical protein sources used in animal diets. It was hypothesized that the unexpected response of pigs fed EHF was the consequence of its unbalanced AA profile and the fact that its AA fraction is made up of free AA and small peptides. Hence, the scientific questions of the thesis were formulated. Using pigs as an animal model and EHF as a model protein source, the thesis aims to study the postprandial AA metabolism and digestive physiology of feeding pigs (1.) with a diet with an unbalanced AA

profile and (2.) a diet who's AA comes in the form of free AA and small peptides instead of intact proteins.

The dissertation is divided into four main sections. In the first section, a review of the literature was done to better understand how AA metabolism can be affected by dietary AA balance and form and how EHF can be used as a model to study these concepts. The subsection dealing with feeding monogastric animals with different dietary AA forms was published as a literature review (Eugenio et al. 2022). For the second section, we present the rationale of our experimental strategies and briefly describe the techniques that we used in the animal experiments of the thesis. The first experiment (Exp. 1) deals with the metabolic consequences of feeding adult and growing pigs with a diet with either a balanced or unbalanced AA profile. For the second experiment, we studied the postprandial AA metabolism (Exp. 2A) and digestive physiology (Exp. 2B) of pigs in response to feeding diets that supplies AA in different forms. We present and discuss the results of these experiments in the third section of the dissertation. The fourth and final section includes the general discussion that synthesizes the results of the experiments and examines what we learned and the potential limitations and gaps in our research. It also includes future perspectives and research ideas based on our findings.

LITERATURE REVIEW

The justification of the use of EHF as an experimental model to study the effects of the balance and form of dietary AA on the metabolic and physiological responses of animals requires a thorough discussion on each of these topics. We begin with a brief overview of protein and AA metabolism with an emphasis on the postprandial period (subsection 1). We then focus on the effects of the balance and form of dietary AA and their effects on AA metabolism and physiology of animals to contextualize the themes that will be discussed in the thesis. One of the outputs of the thesis was a review paper focusing on feeding AA supplied as proteins, peptides, or free form to monogastric animals. The review paper is used here as part of the bibliography (subsection 2) and is followed by a subsection dealing with the metabolic effects of dietary AA balance. Lastly, we will discuss the technical and nutritional properties of EHF and other keratinous products used in animal nutrition.

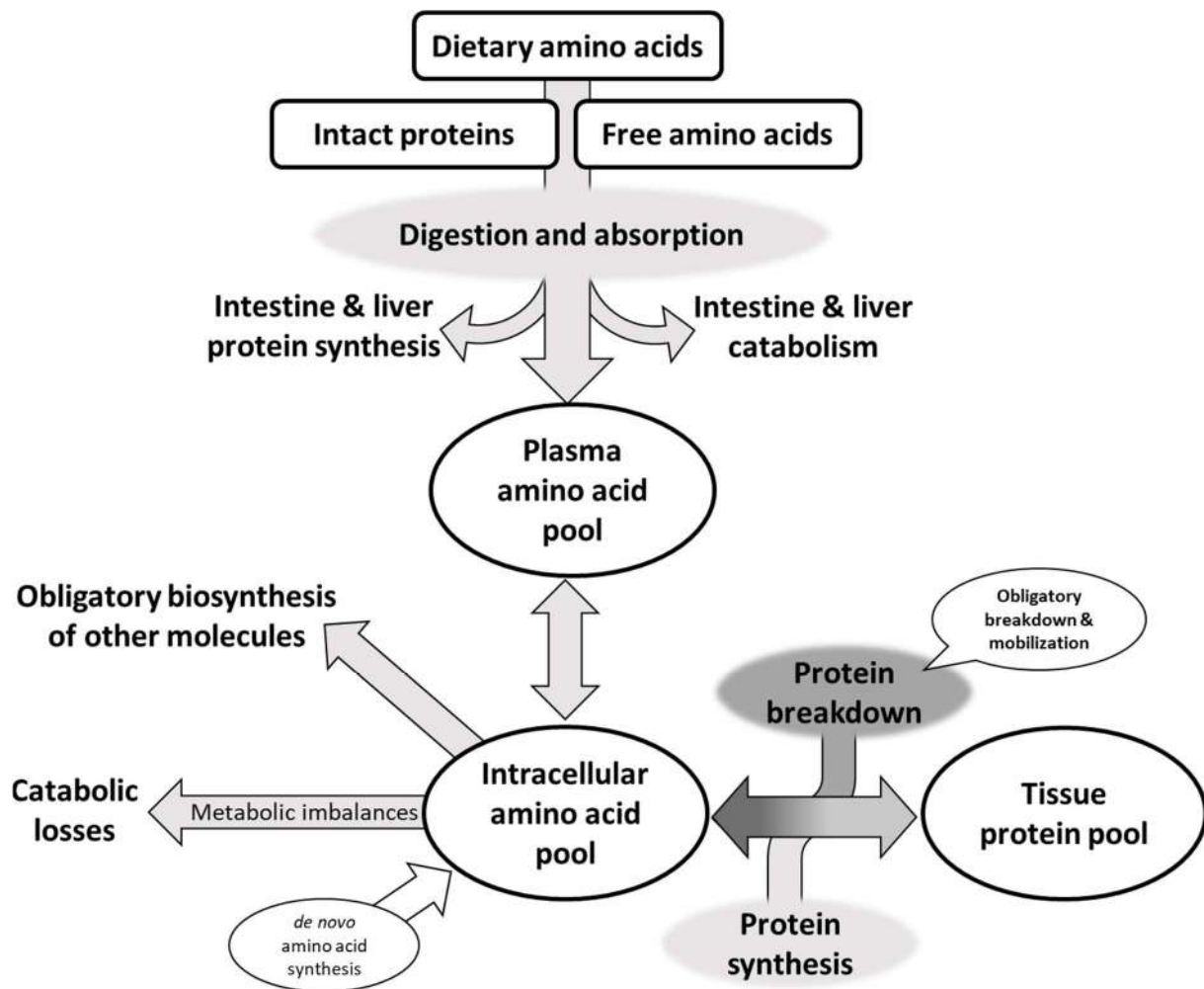


Figure 2. Postprandial amino acid metabolism involving the constant exchange of amino acids between the plasma and tissues of animals

1. Amino acid metabolism during the postprandial period

The immediate period after feeding is typically called the postprandial period. For AA, the postprandial period includes the digestion of dietary proteins, absorption of free AA and small peptides by the enterocytes, the metabolism of AA by the intestine and liver, the appearance of AA in the plasma, and the subsequent uptake and metabolism by different tissues. All these processes happen within a couple of hours after ingestion (Mariotti et al. 2000; Bos et al. 2003). After absorption, dietary AA will be used first by enterocytes and hepatocytes (i.e., for their protein synthesis or for bioconversion to other molecules) before delivering and making them available in the systemic circulation for the uptake by peripheral tissues (Pratt et al. 2002). Hence, the metabolic use of AA by the intestine and the liver can affect the amount and profile of AA appearing in the plasma. All plasma AA beyond the collective need of the peripheral tissues will ultimately be catabolised and lost (Brosnan 2003; Bröer and Bröer 2017).

The use of dietary AA by the intestine and liver is straightforward as they retain what they need before delivering AA to the peripheral. The use of AA by peripheral tissues depends on many factors like the metabolic requirement and status of animal. The rate of utilization of AA, or at least cellular uptake, is increased after feeding (Pratt et al. 2002; Liao et al. 2018). In Figure 2, we see how the arrival of dietary AA in the plasma can influence the constant and obligatory protein synthesis and breakdown (also known as protein turnover) and the interchange of AA between three AA pools of the body, namely the plasma free AA pool, intracellular free AA pool, and the tissue protein pool (Lobley 2003; Bröer and Bröer 2017).

Protein turnover is a biological fact of life for all animals as it enables the obligatory exchange of AA between the different pools. This exchange of AA is an important mechanism for the replacement of any damaged protein, the supply of nitrogen for the *de novo* synthesis of AA, and to accommodate for the changing demands of the animal (Pratt et al. 2002; Moughan 2003). Without a stimulus (i.e., feeding), this turnover is constant and the reason why the

concentrations of plasma AA is never zero even after prolonged fasting. The period when the turnover is constant is often called a steady state, where the AA inflow and AA outflow is equal (Munro 1974; Bertolo et al. 2000). But after feeding and as dietary AA arrive in the plasma, the plasma AA pool temporarily gets bigger, which drives the uptake of AA by tissues and protein synthesis (Cynober 2002; Bos et al. 2003; Lobley 2003). The increase in metabolic rate is temporary, eventually slowing down once the plasma AA pool gets smaller once plasma AA gets taken up by tissues and either utilized (i.e., for protein synthesis or catabolism for bioconversion to other molecules) or to be lost after excreted. At any given time, the amount of AA in the three pools (or the size of the pools) is both maintained and regulated by the constant exchange of AA with each other (Wheatley et al. 1986; Brosnan 2003)

2. Feeding intact proteins, peptides, or free amino acids to monogastric farm animals

Review paper published in the journal *Amino Acids*

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See the published material in Annex 1

To become more sustainable, the negative environmental impact of farm animal production has to be reduced. This has led to global adjustments in animal feeding, like the reduction of the crude protein (CP) content in the diet and the better adjustment of dietary supplies to the nutritional requirements of the animal for maintenance and other physiological needs (e.g., growth, reproduction). Soybean meal is the most widely used protein source in diets of monogastric farm animals such as pigs and poultry, due to its favourable AA profile and high digestibility (Cromwell 2000). However, its use around the globe has been questioned because of the carbon and nitrogen footprint related to its production, which is mainly concentrated in the United States and Brazil. These aspects have contributed to a reduced use of soybean meal in non-producing countries and the need to use alternative, locally-sourced ingredients (Florou-Paneri et al. 2014; Garcia-Launay et al. 2014). Also, a considerable part of the protein supply of monogastric farm animals originates from cereals (e.g., corn, wheat, barley). However, the AA composition of cereals and other plant protein sources such as rapeseed meal is not as good as the AA composition of soybean meal to fulfil the AA requirement. This, in combination with the reduction of CP, has led to the use of free AA (e.g., Lys, Met, Thr, Trp, and Val) to ensure a proper supply of AA while making the animal diet cheaper and more convenient to formulate.

The production of animal-derived products (e.g., meat, milk, and eggs) also results in the production of by-products that are not used for human consumption (e.g., feathers, blood, and offals). These by-products have been used as protein sources in animal feed but the risk of pathogen transmission has reduced their use, especially in Europe. To reduce this risk, animal proteins can be hydrolysed into peptides, a shorter chain of AA, which also improves the

digestibility of proteins that are resistant “by nature” such as feathers. Hydrolysis of protein sources provides an opportunity to valorise these by-products and turn them into viable alternative AA sources in animal diets (Dieterich et al. 2014; Bah et al. 2016).

In monogastric farm animals, dietary AA can be supplied as intact proteins, peptides, or as free AA. Free AA and small peptides can be absorbed directly by the gut while intact proteins and larger peptides have to be hydrolysed through digestion in the gastrointestinal tract (Wu 1998; Krehbiel and Matthews 2003). Consequently, different dietary forms of AA are absorbed at different rates, which can have consequences on the postprandial metabolism of AA. This literature review explores the interests and perspectives of supplying dietary AA in free form, as peptides, or as intact protein, focussing on their use in the diets of monogastric farm animals like pigs and poultry. It may help to understand AA metabolism in a context of using alternative forms and sources of AA in farm animal diets.

2.1. Digestion of dietary protein and absorption of amino acids and small peptides

The provision of dietary AA is required for the production of animal-derived products as they are the building blocks of proteins constituting meat, milk, and eggs. Proteins play diverse functions such as serving as structural components (e.g., collagen, myosin, actin), as metabolic substrates, and as catalysts to various chemical reactions like enzymes (Cox and Nelson 1942; Wu 2013). Although there are about 700 naturally occurring AA, the focus of this review is on the standard proteinogenic AA for animals, which are the 20 AA used for protein synthesis that are encoded for in the standard genetic code (Cynober 2002; Wu 2013). Selenocysteine, a rare AA found in some animal proteins (Clark and Pazdernik 2013), is not considered in this review. Peptides can be categorized by the number of AA residues they have, which ranges from di- and tri-peptides to longer oligopeptides with up to 20 AA residues. Those with even more AA residues (but less than 50 residues, corresponding to <10,000 Da molecular weight) are simply

Table 1. Protein and amino acid digestibility (%) of different protein sources fed to monogastric farm animals^{a,b}

Protein source	CP	LYS	MET	TAA	Reference/s
Soybean meal	70.0	79.9	80.1	74.4	Cervantes-Pahm and Stein (2010) ^d
Fermented soy	70.1	72.7	83.7	76.5	Cervantes-Pahm and Stein. (2010) ^d
Wheat gluten	88.6	77.6	83.2	82.7	Chae et al. (1999) ^c
Fish meal	70.8	82.9	86.0	75.2	Cervantes-Pahm and Stein. (2010) ^d
Whey protein		93.3	89.9		Gottlob et al. (2006) ^c
Meat & bone meal	67.8	56.5	65.6	67.8	Wang et al. (2018) ^d
Spray-dried plasma	81.8	81.2	82.4	82.3	Jeong et al. (2016) ^c
Skim milk	81.7	83.8	85.8	83.6	Chae et al. (1999) ^c
Casein	81.3	92.6	95.1	84.7	Cervantes-Pahm and Stein. (2010) ^d
Feather meal	57.8	62.0	66.5		Kerr et al. (2019) and Grazziotin et al. (2006) ^d
Faba beans	89.0	81.2	55.0		van der Peet-Schwering et al. (2006) ^d
Peas	91.9	90.9	66.8		van der Peet-Schwering et al. (2006) ^d
Canola meal	72.3	66.4	70.3		van der Peet-Schwering et al. (2006) ^d

^aSummary of the ileal digestibility of some dietary protein sources. Free amino acids are not included in the table as they are considered to be completely digestible

^bValues are presented as percentage for crude protein (CP), lysine (Lys), methionine (Met), and total amino acids (TAA).

^cApparent ileal digestible values – measured by the proportion of ingested AA left in the distal ileum

^dStandardized ileal digestible values - Apparent ileal digestibility corrected for endogenous losses

called polypeptides, while a common protein has more than 50 AA residues and has a more complex three-dimensional structure (Ten Have et al. 2007; Munro 2012).

Digestion is the breakdown of large macromolecules into absorbable nutrients (Kong and Singh 2008). Digestibility is the coefficient of disappearance of a nutrient from the gastrointestinal tract after ingestion (Sauer and Ozimek 1986). For monogastric farm animals, protein digestion involves the breakdown of dietary proteins and large peptides through hydrolysis in the stomach and small intestine. The resulting small peptides and free AA are the forms that can then be absorbed by the small intestine. Digestibility of protein in animal diets involves the measurement of what is left of its constituent AA after passing the whole, or a section, of the gastrointestinal tract. Since there is virtually no absorption of AA in the large intestine, experimental methods for estimating “true” protein digestibility until the distal ileum, the last section of the small intestine, have been put in place and the ileal digestibility for many available ingredients used in monogastric animal diets has been determined. The protein and AA digestibility of different intact protein sources varies (Table 1), which may be attributed to the complexity of their protein structure or even their AA profile (Cox and Nelson 1942; Bhattarai et al. 2017). Pre-hydrolysis of proteins increases their digestibility compared to the native (intact) form (Heimbürger et al. 1997).

Absorption of free AA and small peptides from the small intestinal lumen into the systemic circulation involves nutrient transporters located in the enterocyte membrane. Some studies show that the distribution of AA and peptide transporters in the gut is influenced by the form by which the AA are provided (Morales et al. 2017). In pigs, the expression of genes coding for AA transporters is upregulated when AA are provided in free form as opposed to their intact protein counterparts (Zhang et al. 2013; Morales et al. 2017). Likewise, the expression of intestinal PepT1, the main dietary peptide transporter in the small intestine, is upregulated in

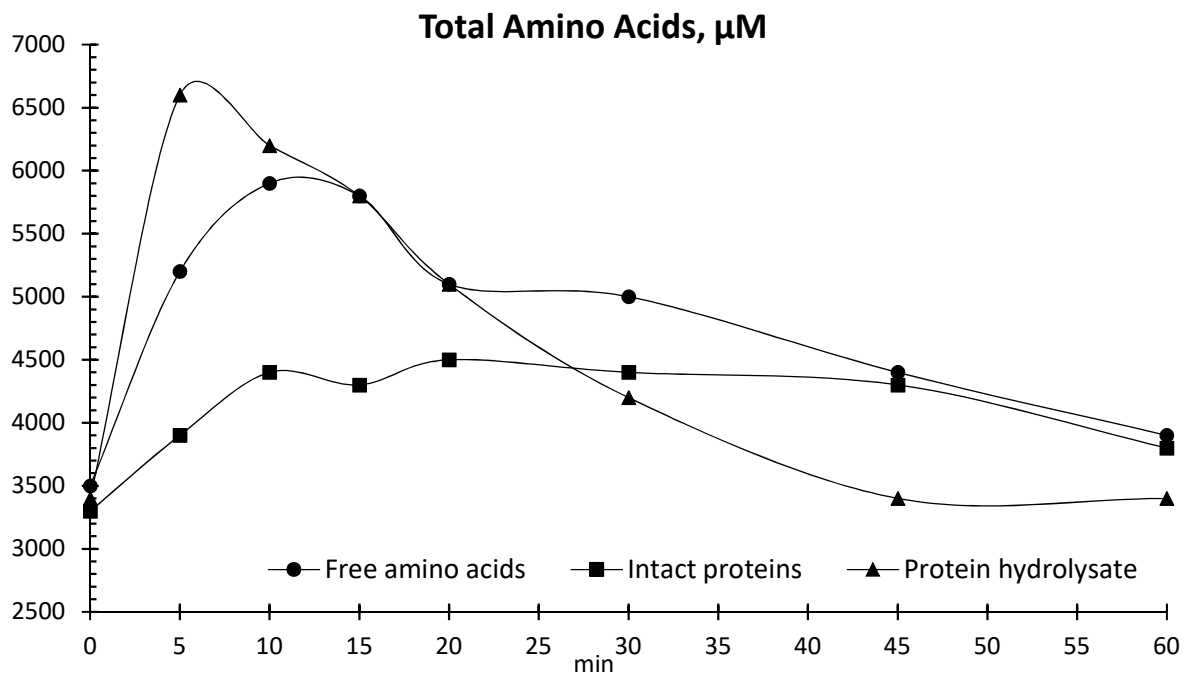


Figure 3. Plasma concentrations of total proteinogenic amino acids (μM) in the portal vein of rats after gastric infusion of intact protein (■), hydrolysate (▲), or free amino acids (●) with the same amino acid profile (Kodera et al. 2006)

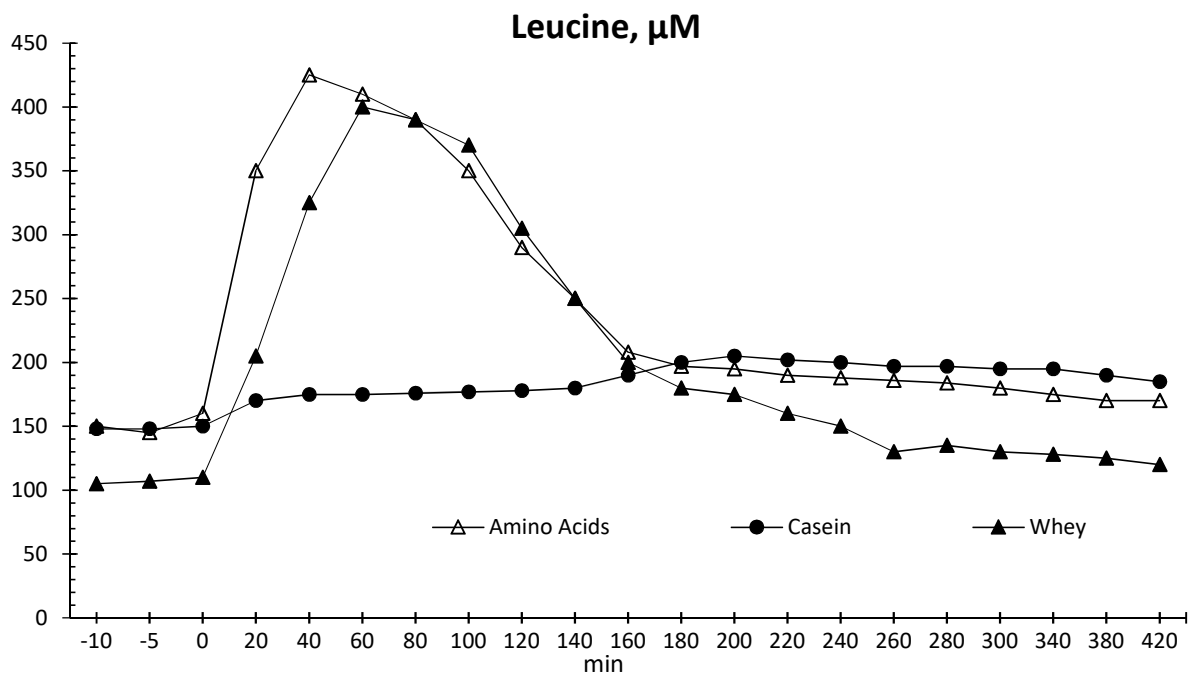


Figure 4. Postprandial leucine plasma concentration (μM) of healthy young men given free amino acids (Δ), whey protein (\blacktriangle), and casein (\bullet) protein sources with identical amino acid profile (Dangin et al. 2001)

pigs fed a diet with a high protein content, especially if the protein is highly digestible (Gilbert et al. 2008a).

Protein sources with similar digestibility values do not necessarily have the same kinetics of digestion and absorption. The kinetics of digestion and absorption is difficult to study *in vitro* as it involves different and complex mechanisms and, *in vivo*, it requires multiple cannulation along the gut, which can be complicated and raises ethical concerns that limit its use. The appearance of AA in the systemic circulation after feeding may also be used to study the dynamics of digestion and absorption. However, the postprandial plasma concentration of AA reflects not only the combined dynamics of digestion and absorption, but also the metabolism by the splanchnic tissues and the protein turnover and AA metabolism of the whole organism (Liao et al. 2018). After feeding, the concentrations of AA in the peripheral blood steadily increase followed by a gradual decline (Figures 3 and 4). The magnitude of this increase and decrease is more important when feeding rapidly absorbed AA like free AA and hydrolysed proteins. In rats (Figure 3), gastric administration of intact proteins, hydrolysates, or free AA with the same AA profile results in a quicker appearance of AA in the plasma for free AA and hydrolysates as opposed to intact proteins. Thus, the plasma AA kinetics of free AA and hydrolysates results in an asymmetric bell-shaped curve while it was rather flat for intact proteins (Kodera et al. 2006), but this is not the case for all intact protein sources. In young men, the postprandial plasma Leu concentration reaches its maximum within one hour after ingestion of whey protein, with a kinetic curve looking similar to that of feeding free AA and hydrolysates (Figure 4). However, feeding casein results in a kinetic curve that remained flat throughout the experiment (Dangin et al. 2001), like for intact soy proteins. Though feeding free AA and hydrolysates results in a faster appearance of AA in the plasma, the rate of appearance of AA after the ingestion of different intact proteins may be variable, which allows to classify intact proteins as so-called fast- and slow-proteins (Dangin et al. 2001). Fast-proteins

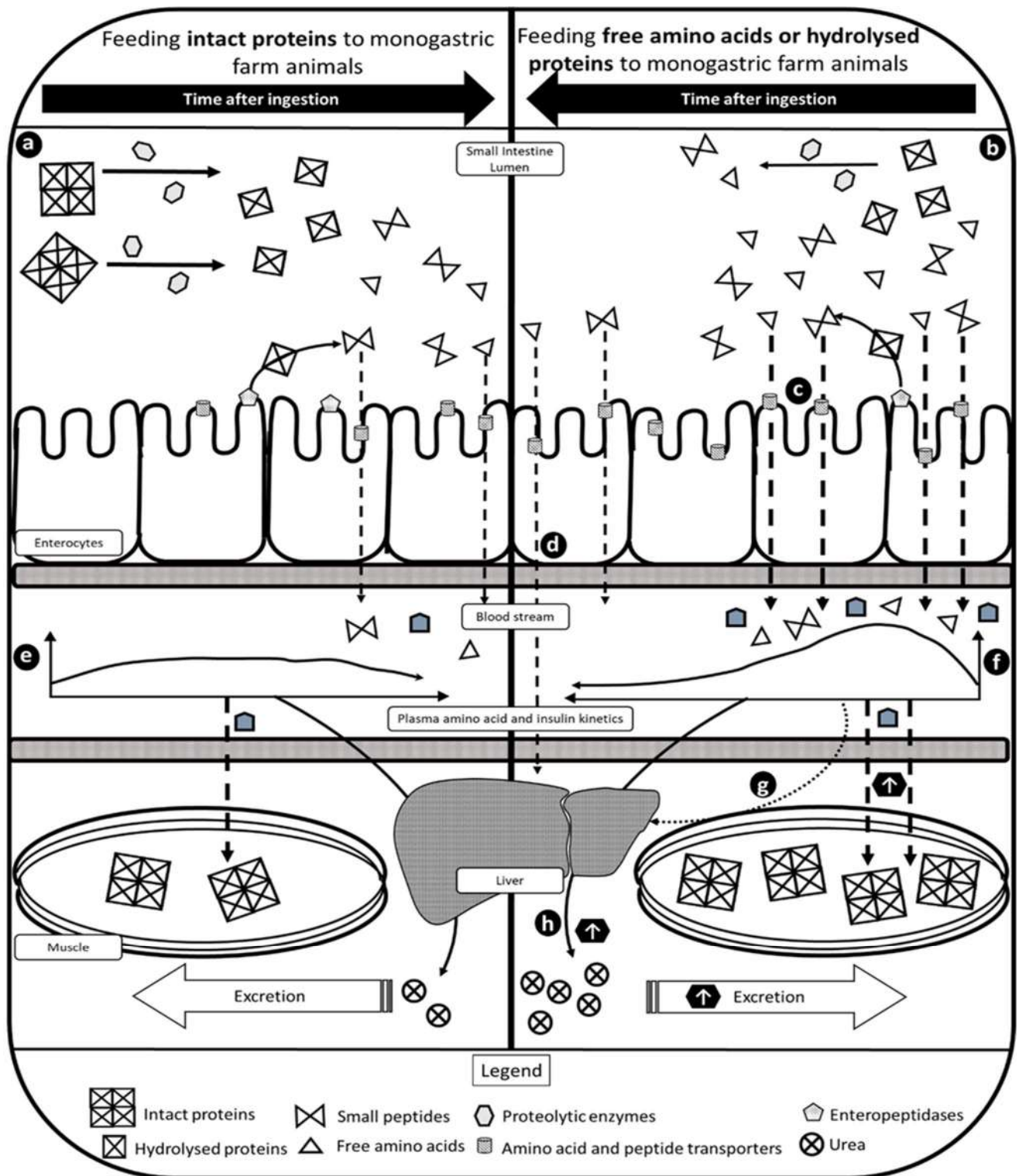


Figure 5. Model of the different metabolic and physiological effects of feeding monogastric farm animals with different forms of dietary amino acid that is given as intact protein or as hydrolysed protein or free amino acids. In the intestinal level, because intact proteins still needs to be digested, more proteolytic enzymes are released after its ingestion contributing to more endogenous protein losses (a) while feeding hydrolysates or free amino acids lessens the need for these enzymes as hydrolysates are more easily digested and free amino acids are immediately available for absorption (b). Furthermore, due to the greater availability of hydrolysates and free amino acids, there are more amino acid and peptide transporters found in the proximal part of the small intestine (c) although with this increased substrate availability, amino acids may be directed to the liver for deamination/catabolism (d). Since protein digestion takes time, the appearance of its products in the plasma is more constant resulting in a characteristically flat kinetics of amino acids after feeding intact proteins (e). Feeding hydrolysates and free amino acids induces a quicker appearance of plasma amino acids and an asymmetric distribution of postprandial plasma amino acids kinetics that is skewed towards the beginning (f). Plasma amino acids induces the release of insulin that increases the uptake of amino acids by the muscle and increase protein synthesis. However, in this postprandial state, due to the rapid appearance of amino acids in the plasma after feeding hydrolysates or free amino acids, there is a period of temporary imbalance of amino acid uptake of sites of protein synthesis, like the muscle, that causes more amino acids to be catabolized compared to feeding intact proteins (g). As a consequence of this increased rate of amino acid catabolism, more urea is produced by the liver which can also be interpreted as a lower efficiency of use of dietary nitrogen (h)

(e.g., whey) are proteins that are quickly digested and absorbed, and the released AA appear quickly in the blood. Conversely, slow-proteins (e.g., casein, soybean meal) are digested slower and their constituent AA appear with a more delayed increase in plasma postprandial concentrations (Koopman et al. 2009). Therefore, the postprandial AA kinetics of slow-proteins often look flat compared to fast-proteins.

2.2. Consequences of feeding intact proteins, hydrolysates, or free amino acids on animal metabolism

The postprandial metabolic responses of animals after feeding intact proteins and protein hydrolysates or free AA are summarized in Figure 5. The kinetics of digestion and absorption determine the metabolic fate of AA by non-digestive tissues (Wang et al. 2021a). More specifically, this fate is mainly due to the rate of AA appearance in the plasma. A rapid increase and high concentrations of AA in the peripheral circulation induced by feeding free AA or hydrolysates result in contrasting outcomes. It increases the rate of AA deamination (catabolism) by the liver (De Feo et al. 1992; Davis et al. 2002) while also enhancing protein synthesis of tissues like the muscle (Dangin et al. 2001). The increase in muscle protein synthesis is also induced by the insulin response after a meal by favouring the utilisation of AA for protein synthesis while decreasing protein breakdown (O'Connor et al. 2003; Paddon-Jones et al. 2004). Although insulin and AA act independently on protein synthesis (O'Connor et al. 2003), they synergistically stimulate muscle protein synthesis after meal ingestion. Feeding free AA or hydrolysates induces a higher postprandial peak of insulin compared to feeding intact proteins (Calbet and Holst 2004) with certain AA like Arg, Leu, Phe, and Gln being known to stimulate insulin secretion (van Loon et al. 2000; O'Connor et al. 2003). Particularly, Leu has been shown to increase the sensitivity of muscle to insulin and stimulates muscle protein synthesis (Anthony et al. 2002). Compared to feeding hydrolysates or free AA, the postprandial insulin secretion and plasma AA concentrations are lower for intact proteins (van Loon et al. 2000), which can lead to lower protein synthesis and retention (Koopman et al. 2009).

Feeding animals diets with free AA and peptides also increases AA catabolism and therefore results in an inefficient use of AA (Davis et al. 2002; Hou et al. 2017). Some studies found that feeding free AA instead of intact proteins resulted in an inefficient use of AA for protein retention (Batterham and Murison 1981; Batterham and Bayley 1989). With a sudden and quick appearance of AA in the plasma after ingestion of free AA, the sites of protein synthesis are assumed to be saturated, which can reduce the uptake and use of AA by tissues (Batterham and Bayley 1989). Since AA are not stored in the body, free AA remain in the plasma and are subject to catabolism (Boirie et al. 1997a; Guillet et al. 2004). Conversely, the delayed plasma appearance of AA released by the digestion of intact proteins results in lower catabolism of AA and, even though it results in a lower rate of protein synthesis, may result in a better nitrogen balance after the meal (Boirie et al. 1997a).

In practical conditions, farm animals are fed with a mixture of the different forms of AA (i.e., protein-bound and free AA). In pigs, the absorption of total Lys and Thr was lower when pigs were fed a low CP diet supplemented with free Lys and Thr than when these AA were provided in the same amount as intact proteins (Yen et al. 2004). This occurs despite a faster absorption of these two AA when supplied as free form as indicated by the maximum plasma concentrations of Lys and Thr in the portal vein that were observed two hours earlier than for pigs fed intact protein. A more gradual appearance of dietary AA in the systemic circulation as observed after ingestion of intact proteins may lead to a greater protein utilization and muscle protein accretion (Reidy et al. 2013; Wang et al. 2021a).

In the lumen of the gut, proteins of non-dietary origin are commonly referred to as “endogenous proteins”. Feeding intact proteins results in greater endogenous protein production than with feeding free AA (Nyachoti et al. 1997). The endogenous proteins are nitrogen-containing compounds like digestive enzymes, products of the mucosal membrane (e.g., mucins), and sloughed off epithelial cells (Awati et al. 2009). A significant part of these endogenous proteins

(e.g., gastric and pancreatic secretions and enzymes) is produced in response to digesta present in the gastro-intestinal tract (Lobley 2003). Feeding free AA instead of intact proteins, diminishes the secretion of proteolytic enzymes and could therefore affect the “apparent” protein digestion (Butts et al. 1993; Adeola et al. 2016). In addition, endogenous secretions can be partially re-absorbed and are not necessarily lost for the animal (Souffrant et al. 1986). Endogenous secretions thus have both a direct (i.e., providing the digestive enzymes) and an indirect effect (i.e., by contributing to protein secretion and absorption in the gut) on digestion and, eventually on plasma AA appearance.

The gut itself also uses dietary AA, thereby affecting the overall bioavailability of these AA. The portal-drained viscera, which include the stomach, intestines, pancreas, and spleen, are estimated to use 30 to 60% of AA like Thr, Lys, and Phe (Stoll and Burrin 2006), while they only account for less than 10% of the body weight in monogastric animals. Some of these dietary AA are metabolized by the gut to produce other molecules. For example, the gut tissue has been shown convert dietary Met to Cys that is one of the three AA constituting glutathione, a major antioxidant in the gut (Riedijk et al. 2007; Li et al. 2014). Apart from the gut tissue, the microbiota found in the small intestinal lumen also use dietary AA (Apajalahti and Vienola 2016). In broilers, the AA requirement of lactic acid producing bacteria (i.e., *Lactobacillus* spp., *Streptococcus* spp., and *Enterococcus* spp.), which is the dominant microbiota population in the small intestine, is very close to the AA requirement of birds (Dai et al. 2013; Apajalahti and Vienola 2016). This may mean that the gut microbiota can potentially compete with the animal for dietary AA, which might be more critical for essential AA. Proteins that are not digested in the small intestine are fermented in the large intestine, affecting the microbial ecosystem and favour the proliferation of harmful bacteria (Apajalahti and Vienola 2016). Conversely, feeding free AA or hydrolysed proteins that are absorbed very quickly in the small intestine prevents

Table 2. Nutritional classification of proteinogenic amino acids in the diets of monogastric farm animals.^a

Mammals ^b			Poultry ^c		
EAA	CEAA	NEAA	EAA	CEAA	NEAA
Cys	Gln	Ala	Arg	Gln	Ala
His	Glu	Asn	Cys	Glu	Asn
Ile	Gly	Asp	Gly	Cys ^e	Asp
Leu	Pro	Ser	His	Tyr ^f	Ser
Lys	Arg ^d		Ile		
Met	Cys ^e		Leu		
Phe	Tyr ^f		Lys		
Thr			Met		
Trp			Phe		
Tyr			Pro		
Val			Thr		
			Trp		
			Tyr		
			Val		

^aTable derived from Wu et al. (2014); Amino acids are listed in their three-letter IUPAC abbreviation; EAA – Essential amino acids; CEAA – Conditionally essential amino acids; NEAA – Non-essential amino acids

^bIncludes pigs and ruminants, also applicable for rodents

^cIncludes chickens, ducks, quails, and geese

^dArginine is classified as a conditionally essential amino acid for pig by NRC (2012)

^eCystine; can be synthesized in the liver by conversion of Met and Ser, but Met needs to be in sufficient amount

^fCan be synthesized in the liver by conversion of Phe as long as it is in sufficient amounts

the proliferation of harmful bacteria (Wang et al. 2016b; Zhang and Piao 2021) and may contribute to maintain the absorptive and digestive capacities of the small intestine.

2.3. Feeding free amino acids and hydrolysed proteins to complement intact proteins in animal diets

In terrestrial farm animal nutrition, dietary AA are mostly provided as intact proteins from soybean meal and cereal grains. Other protein sources include cereal by-products, legumes, oil seed meals, and animal-by products (Florou-Paneri et al. 2014) and the use of these sources differs between countries and geographic areas. During the last decades, there has been an increased use of free AA, which are often added to supplement and complement low-protein diets to ensure the proper supply of AA and allows formulating diets with a composition close to the requirements of the animal. Dietary AA are commonly classified as nutritionally essential or non-essential AA. The essential AA are those that should be provided by the diet because of the inability of the animal to synthesize the carbon backbone and those that are insufficiently synthesized. Non-essential AA can be synthesized *de novo* using metabolic intermediates (D'Mello 2003). However, there is a difference between the biochemical and the biological capacity to synthesize non-essential AA. In young pigs, reducing the dietary supply of NEAA has been shown to limit protein synthesis and growth (Deng et al. 2009). Thus, even though animals may have the capacity to synthesize non-essential AA, they may have a dietary requirement for these AA (Wu et al. 2013) at certain production stages. For example, during pregnancy, lactation, and weaning Arg, Glu, Gln, Gly, and Pro may become limiting for pigs and require a dietary supply (Wu et al. 2014). Therefore, it is difficult to define the “essentiality” of AA because all AA are conditionally essential (Table 2).

Ideal protein is a concept used in animal nutrition to express the AA requirements of the animal. It refers to the balance of dietary AA that is needed to exactly cover the productive potential of farm animals. It concerns all the essential AA and some conditionally essential AA. The capacity to synthesize the other non-essential AA is assumed to be sufficient to cover the

requirements (Emmert and Baker 1997). The AA profile of ideal protein is expressed as a ratio relative to of Lys, which is the first limiting AA in typical diets. However, it ignores possible interactions among AA (e.g., among the branched-chain AA) (Kim et al. 2001a; van Milgen and Dourmad 2015). Also, the ideal protein requirement depends on the physiological state of the animal (e.g., weaned piglets, growing pigs, gestating and lactating sows, laying hens) and on the capacity to synthesize certain AA (e.g., Pro and Gly in poultry), and therefore, different ideal protein ratios have been proposed. The concept of ideal protein is simple and has been widely used in formulating diets for monogastric animals.

2.3.1. Use of free amino acids in farm animal nutrition

The concept of ideal protein largely contributed to the use of free AA in animal diets. Furthermore, it is believed that the CP of the diet can be reduced without affecting performance as long as the supply of essential AA and non-essential AA is ensured (Heo et al. 2009; Gloaguen et al. 2014). Thus, the provision of synthetic essential AA to complement intact proteins in the diet allows that farm animals can perform according to their genetic potential, as dictated by the ideal protein concept. The highest efficiency of nitrogen utilization is achieved when the supply of all AA exactly matches the requirement. This means that more nitrogen will be used for protein retention and production of animal derived-products and less nitrogen is excreted (Garcia-Launay et al. 2014). Providing protein sources that are not easily digested results in a lower efficiency of nitrogen utilization. Likewise, provision of AA in excess lowers nitrogen efficiency.

Free AA in pure, crystalline form like L-Lys, DL-Met, L-Thr, L-Trp, and L-Val are commonly added to commercial animal diets. The L-enantiomers are produced by fermentation and subsequent purification, whereas DL-Met is produced through chemical synthesis. Other commercially available AA include L-Met and hydroxy analogues of Met. The reduction of the CP content of the diet accompanied by supplementation of free AA has been called the “protein-

sparing effect of free AA” (Han et al. 1995). However, there are practical limitations to the extent of this effect. Providing a very low CP diet to broilers (4.5% lower CP than the control diet) reduced their growth performance even though the diet was supplemented by all limiting EAA according to the ideal protein concept (Wang et al. 2021b). Similarly, finishing pigs fed with diets with very low levels of CP (11 vs 14%) supplemented with EAA had lower growth performance than those fed a control diet (Tuitoek et al. 1997). Recent studies suggest that the synthesis of NEAA in pigs depends on the availability of EAA and glucose (Hou et al. 2017), both of which are primarily sourced from the diet. Also, direct supplementation of NEAA in free form may alleviate the need for their *de novo* synthesis when AA nitrogen is limiting (Gloaguen et al. 2014).

The provision of Lys, Met, and Thr in synthetic form in broiler diets resulted in higher plasma concentrations of these AA than when they were provided as intact proteins (Chrystal et al. 2020a). In pigs, the Lys and Thr plasma concentrations remained higher for a longer period when these AA were provided in free form instead of intact proteins (Morales et al. 2020). High plasma concentrations after ingestion of free AA for an extended period may indicate that these AA are not used for protein synthesis, which may be a signal of inefficiency in AA utilization. This may be caused by an AA imbalance in plasma due to the rapid appearance of AA (Yen et al. 2004). Batterham and Bayley (1989) suggested that this imbalance may also result in increased AA catabolism and therefore in a lower AA efficiency of feeding diets with free AA. To summarize, the bioavailability of AA provided in free form by the diet may not be the same as that coming from intact proteins. It remains unknown if there is a ratio between AA coming from intact proteins and free AA that optimizes AA utilisation for protein retention.

There is a growing interest in the targeted utilisation of free AA for roles other than being constituents of body proteins and peptides. These AA are usually provided as free AA instead of intact proteins as their dietary amounts can only be precisely adjusted using free AA. Certain

AA (e.g., Asn, Thr, Ser, and Val) have effects on the modulation of metabolic pathways (Wu et al. 2010; Wu 2013), the regulation of protein synthesis and turnover, carbohydrate, and AA metabolism (Wu et al. 2010; Dai et al. 2013), and feed intake (Trevisi et al. 2018), while others (e.g., Arg, Gln, Glu, Gly, and Trp) are involved in signalling, immunity, and antioxidative responses (Wu et al. 2009, 2010). The supplementation of Gln in the diet of young broiler chickens has been shown to improve intestinal health with longer villi length, greater intestinal IgG and IgA concentrations, and improved growth performance compared to birds fed a control diet (Bartell and Batal 2007; Chalvon-Demersay et al. 2021). Stress hormones and stress-related behaviour were also reduced in young chicks when given diets that were supplemented with Ala, Ser, and Pro (Kurauchi et al. 2009). Otherwise, AA like Trp, Gln, Leu, and Ala can support health and can be adjusted based on age and the health status of animals (Le Floc'h et al. 2018).

2.3.2. Hydrolysed proteins or peptides in farm animal nutrition

The hydrolysis of intact protein sources opens up the possibility of using inedible, waste, or by-products in animal diets and therefore reduce competition with the food supply for humans, while also increasing the nutrient value of common ingredients by making their protein fragment more digestible (Hou et al. 2017). These ingredients are collectively called hydrolysed proteins or hydrolysates, which essentially means that they provide AA as peptides instead of intact proteins. Hydrolysis of intact protein sources improves their nutritional value and also reduces anti-nutritional factors that may be present (Pasupuleti and Braun 2008; Martínez-Alvarez et al. 2015). For soybean meal and other highly digestible materials, the improvement may be marginal, but pre-hydrolysis of materials that are not easily digested improves their nutritional value substantially. Hydrolysis of non-edible materials like feathers (with a protein digestibility of less than 10%) results in a five-fold increase of its protein digestibility while a more intensive hydrolysis process renders the feathers almost completely digestible (Grazziotin et al. 2006). Hydrolysis techniques by chemical (i.e., acidic or basic) or physical means (i.e.,

steam and pressure) are used for both animal-derived proteins (e.g., casein, whey, blood, meat, mucosa) and plant-based proteins (e.g., soybean, wheat, barley) (Pasupuleti et al. 2008; Dieterich et al. 2014). Keratin-based materials (e.g., feathers, wool, and hair) can be processed through these means and can also be treated by controlled bacterial breakdown (Pasupuleti and Braun 2008). Other protein sources can be processed through microbial fermentation techniques that not only improves digestibility but also modifies the AA profile of the material (Jones et al. 2010; Cervantes-Pahm and Stein 2010). Extensive hydrolysis results in a greater proportion of small peptides and free AA compared to those that were only partially or briefly hydrolysed (Bouhamed and Kechaou 2017), but it may also result in the degradation of the AA (Papadopoulos et al. 1986; Bellagamba et al. 2015). Certain hydrolysis conditions induce crosslinking of AA or racemization with a negative impact on digestibility (Friedman 1999; Bouhamed and Kechaou 2017).

Like certain free AA, peptide-rich ingredients can have properties other than being a highly digestible AA source. Peptides with less than 20 AA residues and abundant in Arg, Lys, and Pro residues induce beneficial effects like immune modulation and antimicrobial activities (López-Barrios et al. 2014). Hydrolysed protein products have been tested for their immunomodulation and anti-oxidant properties (Nørgaard et al. 2012; Hou et al. 2017) and as alternatives to antimicrobial to support health and growth performance of animals (Hou et al. 2017). However, the benefits on growth performance of hydrolysed proteins in animal feeds is inconsistent and their functional properties are not fully understood (Nørgaard et al. 2012; Martínez-Alvarez et al. 2015). These functional properties have been attributed to bioactive peptides such as opioid peptides (4 to 8 AA residues) that alter feeding behaviour by binding to certain receptors in the brain (Hou et al. 2017). In addition, some bioactive peptides exert antimicrobial effects by damaging the membrane of harmful bacteria or directly affecting their metabolism (López-Barrios et al. 2014; Hou et al. 2017). Protein hydrolysates are usually fed

to young animals with high AA requirements and with a less mature immune and digestive system like weaned piglets and chicks (Min et al. 2004; Hou et al. 2017). Young broiler chickens fed a diet supplemented with hydrolysed pig mucosa had greater performance compared to those fed the control diet (Frikha et al. 2014), but no effect was observed in older chickens. This might be because younger animals are more susceptible to the functional properties of hydrolysates as they are still developing their immune and digestive systems. Poudel et al. (2020) reported changes in the faecal microbiota of young pigs fed a peptide-based feed additive and hypothesised that such changes may favour the maturity of the digestive tract. Chemical and physical hydrolysis techniques involve processes that indiscriminately cut the protein into smaller fragments. As a result, the resulting product may have different peptides in terms of size and AA composition, which may explain the inconsistencies on the observed effects on the animal. Thus, animal performance may vary substantially and enhanced performance cannot be pinpointed to a particular peptide in the hydrolysate. There is a general understanding regarding the positive effects of hydrolysis of protein sources, but further characterisation of the resulting peptides is still needed. This information is important because it may provide “additional value” to these ingredients over and above to the value of a highly digestible source of AA.

2.4. Conclusion

Although farm animal diets contain intact proteins, there has been an increasing trend to substitute and complete the diet with free AA or hydrolysates. Though feeding these new forms has advantages, there is still much to be learned. A better characterization of the peptides from currently available sources is needed to identify the nutritional and functional properties of peptides. Future research should identify and quantify how the dietary form of AA affects metabolism. For example, it is unknown if the animal can metabolically differentiate between free and protein-bound dietary AA. Most of the recent studies regarding dietary form of AA

deal with their short-term effects, but there is limited information on the long-term effects on metabolism and physiology. For nutritionists, the challenge will be to determine the ideal proportion of AA provided as intact proteins or in free form for optimizing animal performance and health in a sustainable way.

3. Dietary amino acid balance and unbalance: biological consequences

We know that feeding animals with diets whose AA content is either in excess or deficient for at least one AA (relative to their AA requirement) can cause feed refusal and impair protein synthesis (Gietzen 1993; Kampman-van de Hoek et al. 2016). We also know that there are specific interactions between dietary AA especially those among AA whose chemical structures are similar, where these interactions can hinder each other's availability and utilization for protein synthesis (Yun et al. 1991; Cemin et al. 2019). Numerous studies have been made to define the AA requirements of animals, and diets are now formulated to ensure that they can be balanced to be close as possible to the animal's requirements. We will first define and characterize the different types of imbalances of AA caused by feeding diets with unbalanced AA and then discuss their biological consequences.

3.1. Defining “unbalance” and “imbalance”

First, we need to define the terms “unbalance/d” and “imbalance/d” as these are continuously used throughout the thesis. The semantics of the two terms may be similar, as they can both be adjectives. We mainly used the term “unbalance/d” as a verb that pertains to the AA profile of the diet. Large deviations of the AA profile of the diet relative to the animal's requirement means that the dietary AA profile is “unbalanced”. While the term “imbalance/d” is mainly used as a noun describing the state of a biological system and in this thesis, we mainly use it to talk about the plasma or a specific tissue. The disproportion in terms of the AA supply at the sites of protein synthesis induces a state of AA “imbalance”. Throughout the discussion, the logical flow of things is that an “unbalanced” diet can cause an “imbalanced” metabolic state.

3.2. Unbalanced dietary amino acids: deficiency, excess, and antagonism

A main goal of protein and AA nutrition is not only to ensure that the diet provides AA, but also to ensure that the supply is in the correct proportion relative to the animal's requirement. A diet with unbalanced AA can refer to either excess or deficiency of the amount of AA. As

previously said (see subsection 2.3.1 in this literature review), unbalanced dietary AA can cause negative metabolic and physiological responses especially because the process of both protein breakdown and synthesis can be impaired (Kumta and Harper 1961; Harper 1964).

Our focus in this section is on how the dietary AA profile can cause AA imbalances. An unbalanced dietary AA profile can be just as detrimental as any negative factor like inflammation, injury, and stress in inducing a disruption of AA metabolism (Eggum et al. 1981; Cynober 2002; Cervantes et al. 2017). The pioneering works of Alfred Harper and colleagues (1961; 1964; 1965; 1970; 1970) provided strict definitions as to what constitutes an AA imbalance within the context of the dietary AA profile and feeding. According to them, an AA imbalance is the negative state observed when one or more AA become limiting because they are either lacking in the diet or they have a relative deficiency due to the addition of excess amounts of one or more AA. These negative effects can sometimes be attenuated by the supplementation of the most limiting AA or structurally similar AA (Harper et al. 1970; Salter et al. 1990). Hence, there are three main categories of AA imbalances: deficiency, excess, and antagonism.

The AA imbalance caused by the deficiency of one or more AA can limit protein synthesis as all proteinogenic AA should be available at the sites of protein synthesis (Munro 2012). If not, protein synthesis would be hindered (Kim et al. 2001b). This is mostly the case for essential AA as they cannot be synthesized by the animals and are required to be supplied by the diet. The first tissues to be crossed by absorbed essential AA are the intestine and liver who both have high metabolic requirements for AA and can thus modify the AA profile of the plasma (Gietzen 1993; Bauchart-Thevret et al. 2011). A limited supply of essential AA for peripheral tissues limits their protein synthesis which means that all the other AA will be in relative excess and ultimately catabolised (Brosnan 2003; Liao et al. 2015). This type of AA imbalance can be

corrected by adding the deficient AA in the diet (Brown and Cline 1974; Kampman-van de Hoek et al. 2016).

Another type of AA imbalance is AA antagonism. The antagonism of AA is the inhibitory effect of one AA to other structurally similar AA (Harper 1964; Roura et al. 2008). This type of AA imbalance involves the interactions between AA in that excessive intake of one AA increases the dietary requirement of another AA (Sauberlich 1961). Antagonisms among AA can exist at two levels: antagonism between AA sharing the same enzyme for catabolism and antagonism between AA sharing the same transporters in the gut during absorption and in peripheral tissues (Calvert et al. 1982; Block et al. 1985; Krehbiel and Matthews 2003). Some of the most well-known AA antagonisms are those between branched-chain AA, who share the same enzyme for transamination, and between Lys and Arg, who share the same transporters (Sauberlich 1961; Brosnan 2003; Cemin et al. 2019). Like for the case of AA deficiency, AA antagonism can be fixed by the addition of the other, structurally similar AA.

Harper (1964) initially defined AA toxicity as the negative condition caused by having excessive quantities of any or all dietary AA. It is often accompanied by an immediate reduction of feed intake and increased AA catabolism (Benevenga et al. 1976; Liao et al. 2015). Unlike AA deficiency and antagonism, AA toxicity cannot be alleviated by supplementation of other AA. Presently, AA toxicity is often used as a general term to describe the adverse effects of excessive AA intake even though the toxic levels of individual AA may be different (Muramatsu et al. 1976; Benevenga et al. 1976). For example, rats have more tolerance for excess dietary Thr than for excess Tyr (Schweizer 1947; Sauberlich 1961; Tedesco et al. 2018). Currently, a dose-response for each AA is needed to define the point when each AA becomes toxic before commercially available free AA can be put on the market (Garlick 2004). However, the most adverse effects of AA toxicity were those observed and induced in very specific

laboratory conditions (i.e., force feeding, very large supply, intravenous injections) that may not be observed in normal circumstances (Benevenga and Steele 1984; Gupta et al. 2013).

3.3. Metabolic and physiological consequences

Most of the AA imbalances result in negative responses like feed refusal, growth retardation, reduced nitrogen and AA utilization, and development of pathological lesions in extreme situations (Harper 1964; Leung et al. 1968; Muramatsu et al. 1976; Toohey 2014). We highlight some of these responses in the next subsections.

3.3.1. Consequences on feed intake

The very first sign of an AA imbalance is typically the depression of feed intake and even complete feed refusal (Harper 1964; Li et al. 2007; Cemin et al. 2019). Reduced feed intake may be a measure to protect animals from any metabolic harm or damage as has been seen in situations of forced-feeding (Peng and Harper 1970; Clement and Harper 1974). This reduction in feed intake is almost immediate for most of the imbalances and a complete refusal can even happen for a few days when the diet is completely devoid of at least one essential AA (Gietzen 1993). Furthermore, pigs also seem to have the capacity to choose a more AA balanced diet over an unbalanced one when offered a choice (Edmonds et al. 1987).

The underlying biochemical explanation to feed intake depression has been hypothesized in older studies (Harper and Rogers 1965; Peng and Harper 1970). The authors suppose that upon feeding a diet with unbalanced AA, the liver (and possibly the intestine) will retain the most limiting AA effectively further reducing even more its supply to peripheral tissues. As the AA supply to the plasma becomes more unbalanced over time, cells are triggered to release protein kinase GCN2 which can be detected by the anterior piriform cortex of the brain which ultimately results in feed refusal (Maurin et al. 2005; Shuzhen et al. 2005). This sequence of the postprandial response of animals tells us some important concepts that are used repeatedly in this thesis. First is that unbalanced dietary AA can limit protein synthesis of peripheral tissues

and secondly, the AA imbalance is detected by the brain and induces negative physiological effects.

3.3.2. Consequences on protein deposition

As feeding animals with a diet with unbalanced AA limits protein synthesis, it can be associated by a reduction in protein deposition (Kumta and Harper 1961; Edmonds et al. 1987). Due to impaired protein synthesis, tissue proteins would need to be broken down to mobilize AA in an effort to supply the missing essential AA in the systemic circulation (Kimball and Jefferson 2002). Feeding diets with unbalanced AA can thus have a negative effect on overall protein balance and synthesis. A diet with just one missing essential AA can result in the same detrimental effect on protein deposition as feeding a protein-free diet (Leung et al. 1968; Verney and Sidransky 1975). As compromised protein deposition can easily be seen through the growth performance of animals, growth retardation is the most convenient way to verify the reduction in protein deposition.

We also see this reduction of performance in instances when AA are in excess in the diet. In an early study of Sauberlich (1961), he studied the toxicity of 19 proteinogenic AA (except Gln) by feeding rats diets where AA were individually added at 5% higher than normal levels and found that there was a systematic depression of their growth. The depression varied for each AA with the most drastic effect found for excess Met. Meanwhile, there was only a very minimal depression in growth when Glu, Pro, and Ser were provided in excess, and excess Ala did not even negatively affect growth (Sauberlich 1961). Although, feeding AA is relatively safe and animals, with the exception of certain AA (i.e., Met), typically have a certain tolerance for the different imbalances previously described. In fact, the animal can adapt to these situations and after an initial feed intake reduction, animals will adapt and return to their normal feed intake (Harper et al. 1970; Dilger et al. 2007). Still, these types of information becomes increasingly important because of the increasing supplementation of free AA in animal diets.

3.3.3. Consequences on metabolism

Whether AA are lacking or in excess in the diet, protein synthesis will be hindered causing high plasma AA concentrations and a higher proportion of catabolized AA will need to be excreted (Cynober 2002; Kimball and Jefferson 2002; Lobley 2003). Several studies have found that feeding animals with diets with unbalanced dietary AA induces an imbalance in the plasma level compared to feeding a diet with a balanced AA profile (Morrison et al. 1961; Zimmerman and Scott 1965; Gustafson et al. 1986). However, plasma AA concentrations can return to normal, relative to that of animals fed a balanced diet, once the dietary AA profile is corrected. For example, a diet with excess Leu resulted in depression in feed intake and growth in chickens, and can be prevented by “balancing” it with the other branched-chain AA, Ile and Val (Calvert et al. 1982; Cemin et al. 2019).

Among all proteinogenic AA, excesses of the sulphur-containing AA is one of the most harmful (Sauberlich 1961; Yang and Liao 2019). Pigs preferred to eat diets with extra (4%) Arg, Lys, or Thr compared to diets with extra Met (Edmonds et al. 1987). Longer feeding (at least 6 days) of excess Met can result to infertility, cardiovascular disease, and liver damage (Benevenga et al. 1976; Matsueda and Niiyama 1982; Fau et al. 1988). Excess free Cys has neurotoxic effects in developing mammals and can even cause some deaths in rats (Sauberlich 1961). The authors attributed this to the reaction of Cys with pyridoxal 5-phosphate, the active form of vitamin B6 involved in all transamination reactions, which caused the depletion of pyridoxal 5-phosphate (Osman et al. 1997; Dilger et al. 2007). Kumta and Harper (1961) found that feeding unbalanced dietary AA with excess Met and Phe to rats hindered their ability to synthesize Trp pyrrolase, an enzyme that catalyses Trp oxidation to *N*-formyl-L-kynurenine, which is an important pathway for the production of the coenzyme nicotinamide adenine dinucleotide (Pilotte et al. 2012). Ribosomal polysome aggregation in liver cells was reduced upon feeding AA unbalanced diets, ultimately compromising protein synthesis (Clement and Harper 1974).

3.3.4. What about using amino acids as functional molecules?

The concept of defining AA requirements in relation to the pathophysiological state of the animal is not new (Grimble 1992; Egerland et al. 1996; Grimble and Grimble 1998). It has been recently replaced by the concept of functional AA, which corresponds to the utilisation of AA for purposes other than their nutritive functions (Le Floch et al. 2018). A very broad definition of functional AA was previously presented that indicated that these AA are those that can have roles in improving health, growth, development, reproduction, and lactation of animals by regulating certain key metabolic pathways when provided in amounts higher than the requirement (Wu et al. 2010). Hence, there seems to be a thin line between excess dietary AA being functional and when it starts being in excess and potentially toxic for healthy animals. Although, with this definition, as AA demand is reduced during periods of stress and disease, it means that all AA maybe considered as functional AA (Le Floch et al. 2004; Cervantes et al. 2017). Still, this feeding strategy is currently employed by supplementing certain AA like Arg, Gln, Pro, and Trp among others to animal diets on top of the requirement with positive results to growth and performance (Beaumont et al. 2021; Chalvon-Demersay et al. 2021; Rodrigues et al. 2021).

3.4. Conclusion

Unbalanced dietary AA can impair animal metabolism. It can greatly reduce plasma concentrations of limiting AA and can have a profound negative effect on protein synthesis. For the case of AA deficiency or antagonism, the AA imbalances can be corrected by the dietary supplementation of certain AA while for the case of excess AA, certain AA can be more detrimental to AA metabolism than others. Amino acid catabolism is also increased in response to these imbalances. Therefore, care is needed when formulating animal diets and knowledge on AA biochemistry and animal physiology is needed to be able to properly provide animals with balanced dietary AA.

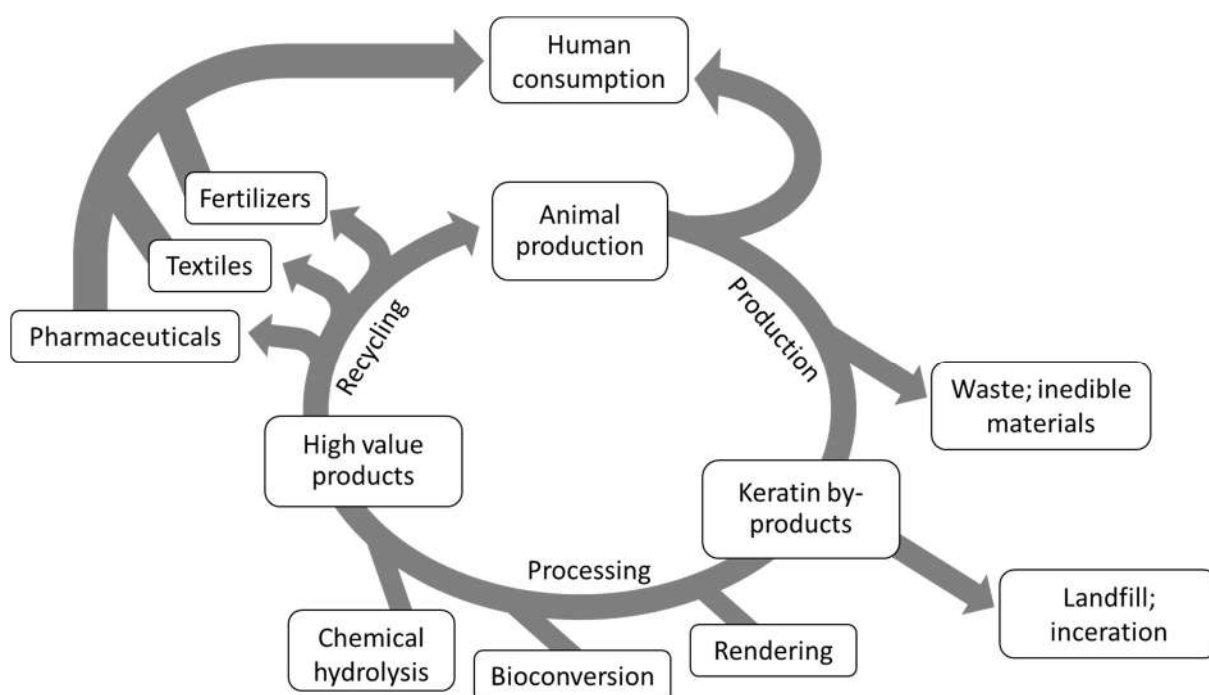


Figure 6. Circular production system of keratin by-products in the animal production cycle.

Table 3. Major poultry production by-products and some of their potential uses.^a

By-product	% BW ^b	Protein content, %	Potential use
Blood	5	≤ 30	Production of hydrolysed bioactive peptides Animal feed ingredient (blood meal)
Residual meat, bone, and offals	6	10-30	Animal feed ingredient (meat meal) Mineral source Enzymes Lipids Growth media and emulsifier Flavouring materials
Feathers	8	≥ 85	Animal feed ingredient (feather meal) Biomedical uses Leather production Textiles and clothes (insulation) Fertilizers Production of activated carbon Source of single amino acids

^aData on table is adapted from Sharma & Gupta (2016), Shestakova *et al.* (2021), Seidavi *et al.* (2019) and Lasekan *et al.* (2013)

^bPercent body weight of poultry species

4. Feather proteins

Around 20 million tons of animal by-products, those not included in the dressed carcass, are processed annually and reused in agriculture (Marti et al. 2011; National Research Council 2012). Reusing by-products of the animal production industry reduces wastage and improves the sustainability of animal production (Gooding 2012; Kerr et al. 2019). For poultry production, by-products include blood, meat trimmings, bones, offals, and feathers. Among these, feathers make up the majority of the by-product material produced (Fagbemi et al. 2020). In this thesis, our focus is on feathers, a keratinous material, and its transformation into an ingredient that can be used for animal diets. A closed-loop system of animal production, particularly for reusing keratinous by-products is shown in Figure 6 while a summary of potential uses of some major poultry by-products can be found in Table 3.

Presently in Europe, poultry by-product such as poultry meat meal, bone meal, and feather meal are classified as processed animal protein (**PAP**). Among these, the use of feather meal might be the most sustainable as it prevents the unsustainable disposal of feathers by incineration or in landfills and feathers are not very biodegradable (Shestakova et al. 2021). The difficulty to naturally degrade feathers is because they are principally composed of keratin, a very rigid protein whose physiological functions require it to be tough. The durability of this protein is one of the main reasons (along with food safety) why feathers needs to first be hydrolysed (at least partially) to release its otherwise non-bioavailable AA (Lee et al. 2016; Machado et al. 2018). The most common technique to process feathers is a simple hydrolysis technique by steam and pressure. After such processing, feathers are converted into feather meal, the form of feathers that is commonly used in animal feeds as PAP and as organic fertilizers. This section of the bibliography will deal with the use of feathers as an AA source for animal feed and highlighting EHF and how it can be used as a model AA source to answer the scientific questions of the thesis.

Table 4. Amino acid composition (g·kg⁻¹) of different keratinous materials.^a

Amino Acid	α -keratin		β -keratin
	Human hair	Wool	Feathers
Ala	39.9	50.4	55.6
Arg	117	95.5	66.6
Asx ^b	54.2	62.5	60.4
Cys ^c	245	130	70.7
Glx ^d	166	134	95.3
Gly	67.9	92.0	101
His	16.4	8.42	5.73
Ile	29.9	33.9	44.8
Leu	113	104	89.4
Lys	72.2	52.9	20.5
Met	8.06	5.41	4.67
Phe	25.3	31.7	44.5
Pro	37.5	61.9	81.1
Ser	102	116	128
Thr	66.5	60.4	46.4
Trp			2.98
Tyr	26.6	46.0	23.4
Val	53.3	55.6	76.8

^aValues are average of values from Wang *et al.* (2016a), Esparza *et al.* (2018), Robbins and Kelly (1970), Ward *et al.* (1955) and internal analyses.

^bAspartic acid and asparagine

^cCystine

^dGlutamic acid and glutamine

4.1. Composition and chemical structure of feathers

Feathers are the external covering uniquely found in avian species. They are attached to their epidermis and acts as a barrier to the external environment that provides insulation and waterproofing for the birds (Wang et al. 2016a). Feathers are hard but flexible, have low molecular density, and are hydrophobic (Reddy and Yang 2007). These properties are the result of their chemical structure as they mainly consist of small, uniformly sized low molecular weight proteins arranged in a fibrous-like filament structure (Ullah et al. 2011) with each filament connected with strong hydrogen and sulphide bonds (Schor and Krimm 1961; Esparza et al. 2018). This type of protein structure is a characteristic of those belonging to the keratin family of fibrous proteins which can exist as α - and β -keratin (Schor and Krimm 1961). The two types of keratins have different properties as they differ in their chemical structure, with β -keratins having shorter polypeptide chains which allows the protein to be arranged in a pleated conformation called β -sheets, making it more compact and rigid compared to the more flexible α -keratin whose protein is arranged in α -helix structure (Bragulla and Homberger 2009; Esparza et al. 2018). This difference in their protein structure stems from only slight differences in the AA composition (Table 4). Feathers are almost entirely made up of β -keratin, while human hair and sheep wool are made up of α -keratin (Wang et al. 2016a). Higher Gly and Ser concentration in β -keratins allows for more hydrogen bonds between the protein filaments (Calvaresi et al. 2016). The concentrations of these two AA are increasing from human hair, sheep wool, to poultry feathers, which is why human hair can be expected to be softest and feathers to be the hardest among the three.

4.2. Production, utilization, and nutritive value of feather meal

According to the Global Feather Meal Market Research Report for 2021 (360 research reports), feather meal is an economically important material for animal feed with a global market value of 360 million USD as of 2021. The United Nations' Food and Agriculture Organization

Table 5. Typical nutrient composition of feather meal^a

Nutrient	Unit	Average	Min	Max	n
Dry matter	%	93.7	92.0	95.0	7
Crude protein	%	86.5	85.1	88.5	6
Crude fibre	%	1.40	1.00	1.40	3
Ether extract	%	8.25	6.35	9.20	3
Ash	%	5.63	3.20	5.90	5
Minerals					
Calcium	%	0.41	0.33	0.48	2
Phosphorus	%	0.48	0.45	0.50	2
Potassium	%	0.22	0.10	0.33	2
Sodium	%	0.34	0.32	0.35	2
Chloride	%	0.23	0.20	0.26	2
Amino Acids					
Alanine	g·kg ⁻¹	44.8	38.5	59.2	7
Arginine	g·kg ⁻¹	64.0	56.3	80.9	7
Aspartic Acid ^b	g·kg ⁻¹	60.2	49.5	67.0	7
Cyst(e)ine	g·kg ⁻¹	43.7	28.7	59.1	7
Glutamic Acid ^c	g·kg ⁻¹	96.0	84.0	109	7
Glycine	g·kg ⁻¹	77.1	63.3	127	7
Histidine	g·kg ⁻¹	8.11	5.46	13.0	7
Isoleucine	g·kg ⁻¹	39.7	3.64	54.7	7
Leucine	g·kg ⁻¹	66.4	6.6	97.7	7
Lysine	g·kg ⁻¹	18.2	2.01	30.2	7
Methionine	g·kg ⁻¹	6.72	5.00	9.30	7
Phenylalanine	g·kg ⁻¹	44.9	39.5	56.4	7
Proline	g·kg ⁻¹	93.7	83.3	167	7
Serine	g·kg ⁻¹	102	82.0	125	7
Threonine	g·kg ⁻¹	45.5	37.2	55.8	7
Tryptophan	g·kg ⁻¹	6.42	4.90	9.30	4
Tyrosine	g·kg ⁻¹	25.8	21.2	28.8	7
Valine	g·kg ⁻¹	68.3	57.5	84.9	7

^aValues are average of values from Kerr *et al.* (2019), Hicks and Verbeek (2016), Shestakova *et al.* (2021), Moritz *et al.* (2001), NRC (2012), and 2 internal analyses.

^bIncludes aspartate

^cIncludes glutamate

estimated that 135 million MT of poultry livestock was produced in 2020 (FAO 2021). Assuming that feathers account for 8% of the total body weight of birds (see Table 3), there should be around 10.8 million MT of feather waste globally for 2020. Current market data from Global Market Insight reported that for this year, global feather meal production is only around 1.90 to 2.10 million MT. This means that feather transformation into feather meal is underutilized with only around 20% of global feather by-products being valorised into feather meal. Nevertheless, market reports show that more than 95% of produced feather meal goes to animal feeds. Around 60% of which are used in poultry feeds, 25% in cattle diets, and 10% in fish and aquaculture diets with the rest being used for pets and pig diets. In Europe, feather meal and other PAP were only authorized to be fed to fish and pets, but they were recently granted authorization to be fed to pigs under regulation (EC) number 2021/1372 of the European parliament and of the council of as of 17 August 2021. According to the regulations, feather meal should be derived from category 3 feathers as those from healthy birds killed in an accredited slaughterhouse or poultry rendering plant. With the new European regulations and its extended use in pig diets, we could expect an increase in the utilization and global demand for feather meal to be used in animal diets.

Feathers contain 85-90% protein (Kim and Patterson 2000; Kumar et al. 2012) and 3-5% minerals, as much as half of which can be sulphur as feathers is rich in Cys (Ward et al. 1955; Esparza et al. 2018). The composition of feather meal is summarized in Table 5. Compared to typical protein sources used in animal diets, the AA composition shows very high levels of Cys, Ser, Pro, and Glu while having very low levels of Lys and Met. Furthermore, protein digestibility usually only ranges from 70 to 80% (Kim and Patterson 2000; Grazziotin et al. 2006; Kerr et al. 2019). Valorising feathers by rendering (steam and pressure) does not alter its AA profile but cuts up the keratin protein into a less rigid protein accessible to digestive enzymes. Without this step, raw feathers are only around 10% digestible (Grazziotin et al.

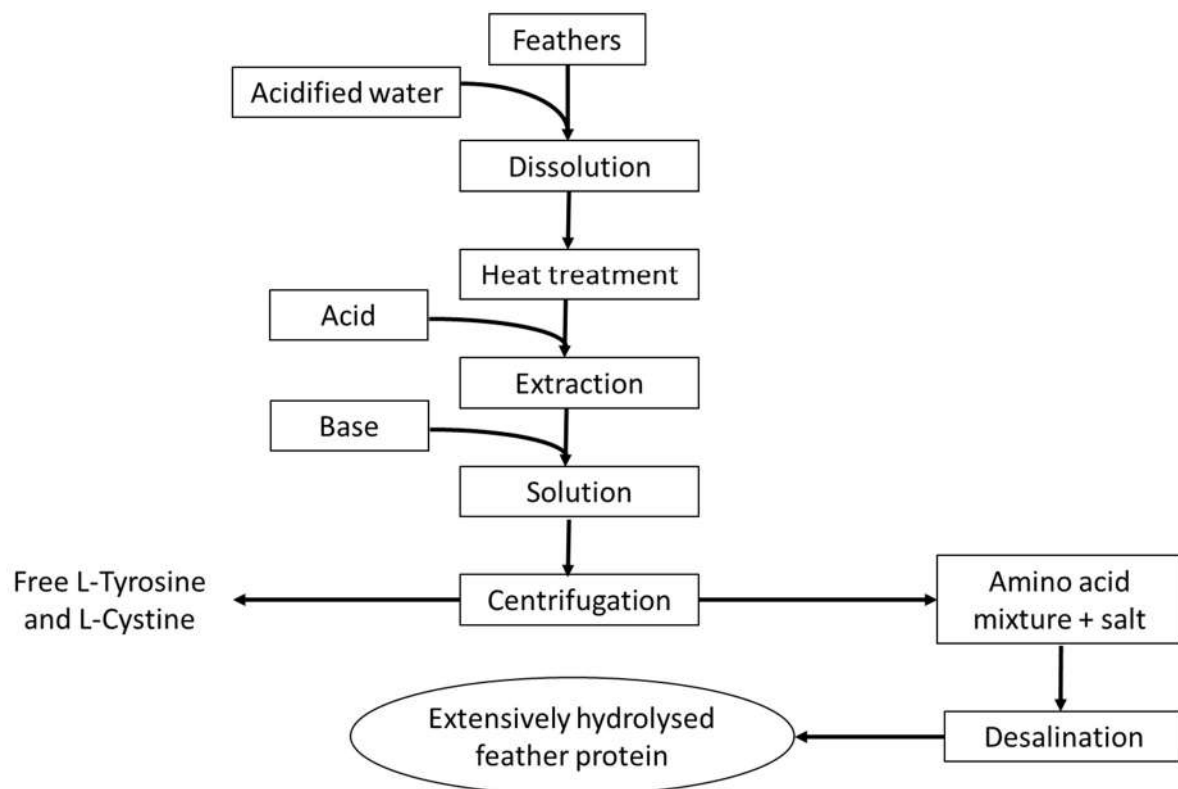


Figure 7. Production flowchart of the extensively hydrolysed feather used as the model protein source in the thesis

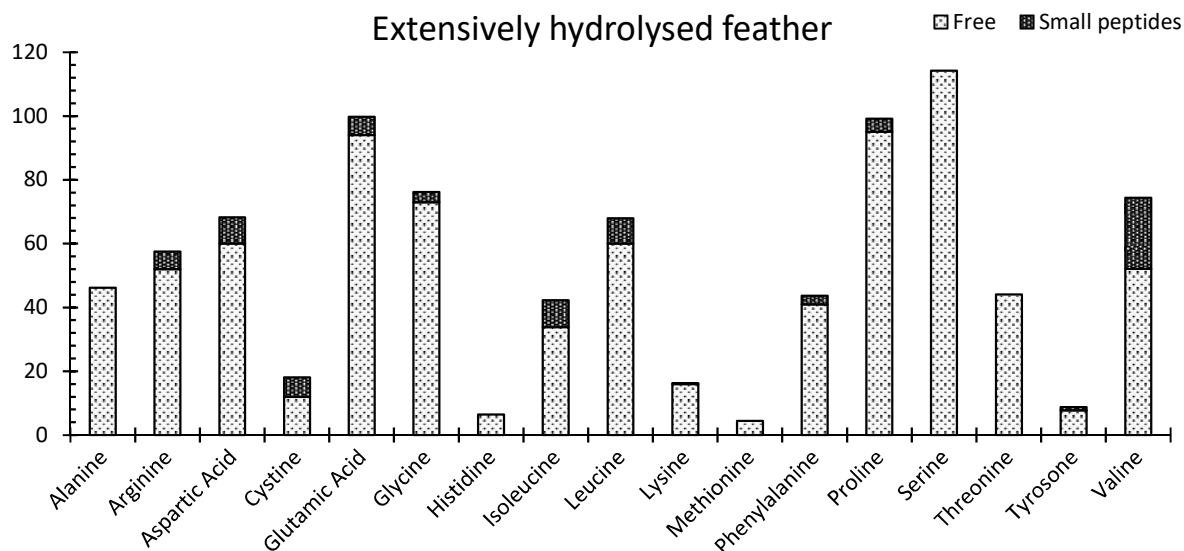


Figure 8. Amino acid (g·kg⁻¹) profile of the extensively hydrolysed feather which is exclusively made up of free amino acids and small peptides (≤ 800 Da)

2006). Some other emerging techniques to process feather is through enzymatic hydrolysis with keratinase and other proteases (Clemente 2000; Kumar et al. 2012) while another is through the introduction of microorganisms that degrade keratin (Grazziotin et al. 2006; Karaveli and Deniz 2021). With the former, the AA profile of raw feathers will not be changed but due to the microbial fermentation of the protein, the AA profile is altered. The degree of alteration will depend on the microorganism used and the time of incubation (Kim and Patterson 2000). Newer chemical methods of feather processing is through chemical hydrolysis. This can be done with either an acidic or basic medium with the goal of further increasing AA availability of hydrolysed feathers (Kim et al. 2002; Lee et al. 2016; Bouhamed and Kechaou 2017). The digestibility and AA profile of feather meal is variable among samples as this largely depends on processing methods that are not well standardized between manufacturers (Papadopoulos et al. 1986; Park et al. 2019). Generally, the nutritional value of feather meal is lower than that of other poultry PAP (Park et al. 2019) mainly because of the unbalanced AA profile (i.e., low Lys, Met, and His) and relatively low digestibility. Thus, the inclusion of feather meal in animal diets has been limited to around 10% as it can depress growth and lower feed intake when given in higher amounts due to its poor nutritional value (Kim and Patterson 2000; van Heugten and van Kempen 2002). It is improbable that increasing digestibility will increase feather meal use in the diet because of its AA profile. So, a possible solution to increase rendered feather utilization is not through the valorisation of feather meal for animal feeding, but from other industries that can have uses for feathers and its constituting AA (see Table 3 and Figure 6)

4.3. Extensively hydrolysed feather protein

4.3.1. Process and utilization

An innovative processing method of a French company (BCF Life Sciences, Inc.) allows to hydrolyse feathers and take out some crystalline AA like Cys and Tyr (Figure 7) to be used as supplements for human nutrition. This also adds more value to the protein fraction of feathers

Table 6. Nutrient composition of feather meal compared to the extensively hydrolysed feather

Nutrient	Unit	Feather Meal	EHF
Dry matter	%	93.7	98.3
Crude protein	%	86.5	88.0
Ash	%	5.63	7.23
Sodium	%	0.34	2.60
Chloride	%	0.23	2.42
Total amino acids	%	91.2	88.7
Free amino acids	%		82.3
Amino Acids			
Ala	g·kg ⁻¹	44.8	44.5
Arg	g·kg ⁻¹	64.0	55.8
Asp	g·kg ⁻¹	60.2	62.3
Cys	g·kg ⁻¹	43.7	15.6
Glu	g·kg ⁻¹	96.0	95.7
Gly	g·kg ⁻¹	77.1	72.4
His	g·kg ⁻¹	8.11	6.00
Ile	g·kg ⁻¹	39.7	38.8
Leu	g·kg ⁻¹	66.4	67.0
Lys	g·kg ⁻¹	18.2	16.7
Met	g·kg ⁻¹	6.72	5.00
Phe	g·kg ⁻¹	44.9	43.1
Pro	g·kg ⁻¹	93.7	95.1
Ser	g·kg ⁻¹	102	115
Thr	g·kg ⁻¹	45.5	43.8
Trp	g·kg ⁻¹	6.42	
Tyr	g·kg ⁻¹	25.8	7.10
Val	g·kg ⁻¹	68.3	62.9

as individual purified AA can be sold in a more premium price point. Furthermore, this process also leaves a product (EHF) composed of a mix of the remaining AA fraction of feathers. The commercial EHF (Protamine, BCF Life Sciences, Inc., Pleucadeuc, France) is produced by extensive acid hydrolysis of feathers and is composed of a mix of AA with a very stable product composition. Like feather meal, it has a very high nitrogen and AA content (88.7% total). Due to the extensive hydrolysis process, the AA of EHF come in the form of free AA and small peptides (82.0% of total AA are in free form, the rest are small peptides) which makes it a highly digestible product (Figure 8). The EHF is used in diets of fish and aquatic species (i.e. shrimps) to promote feed intake and as a growth promoter (Kersanté et al. 2021). Furthermore, since all of its protein fraction comes in free AA or in low molecular weight peptides (<800 Da), it is also used in specialized diets for carnivorous pets with allergic diseases (Vanderhoof et al. 2016).

4.3.2. Nutritive values and lack of data in species of interest

The nutritive profile of EHF compared to feather meal is given in Table 6. The nutrient content and AA profile of both products is similar, hence, the AA profile of EHF is also unbalanced. As the production of EHF involves the extraction of Cys and Tyr, these AA are present in lower amount in EHF compared to feather meal. Unlike for the rendering process of feather meal production, Trp is destroyed during acid hydrolysis so EHF is devoid of this AA. Total AA *in vivo* digestibility of EHF measured in cecectomized cockerels, was around 97% with each AA, except Tyr and Trp, having at least 90% digestibility (unpublished).

One of the biggest commercial use of EHF is as an AA source for pet food. Hence, the main interests of the company is to characterize the nutritive value of this product for pets. However, due to the difficulties associated with animal experiments involving pets, an alternative animal model was used. Therefore, an adult pig model can be used as they mostly eat for maintenance, much like the majority of pets. For that purpose, the adult mini-pig model is practical to use

because of its adult size and metabolism. A previous experiment performed by the team (Meniga et al. 2017) showed that feeding adult mini-pigs with EHF results in prolonged elevated plasma AA concentrations, which may signal inefficient use of AA (Liao et al. 2018; Weijzen et al. 2022). The initial hypothesis is that this might have been caused by the intrinsic properties of EHF, whose AA profile is strongly unbalanced and/or supplies AA as free and small peptides (Kumta and Harper 1962; Batterham et al. 1986; Bos et al. 2003).

4.3.3. Extensively hydrolysed feather as an experimental model

Even with the limitations of EHF in feeding to pigs (i.e., unbalanced AA profile and palatability issue), this material has an opportunity as a model AA source to study the consequences of both the form and balance of AA in diets on pig metabolism and physiology. In AA balance studies, the protein sources used are usually intact proteins and balancing the AA profile is achieved by adding free AA (Ten Have et al. 2007; Spring et al. 2020). While this design is generally acceptable, providing AA in different forms in the diet can cause a transient AA imbalance in the sites of protein synthesis caused by the differences in the arrival times in the blood of AA coming from the digestion of intact protein sources and from readily absorbable free AA (Batterham 1974; Yen et al. 1991). With using EHF to study dietary AA profile balance, it allows us to overcome these confounding effects.

EXPERIMENTAL METHODS

Table 7. Summary of the animal experiments of the thesis.

Exp.	Objective	Animal model	n	Weight, kg	Experimental diets	Measured responses
1	Metabolic response of adult and growing pigs after feeding a balanced or unbalanced diet	Adult Yucatan mini-pig	6	38.7 ± 2.9	BAL – diet with a balanced amino acid profile	Postprandial plasma amino acid, glucose, insulin and urea concentrations
		Piétrain x (Large-White x Landrace) growing pigs	6	48.9 ± 0.8	UNB – diet with an unbalanced amino acid profile	
2	Metabolic and digestive response of growing pigs after feeding amino acids as intact proteins or as free amino acids and small peptides	Piétrain x (Large-White x Landrace) growing pigs	30	33.7 ± 4.10	INT – intact protein (feather meal)	Metabolic response: postprandial plasma amino acid, glucose, insulin, and urea concentrations Digestive response: amino acid digestibility, intestinal morphology, and gene expression of amino acid transporters
					HYD – free amino acids and small peptides (EHF)	
					FAA – free amino acids	

1. Scientific questions and animal experiments

The objectives of the thesis are to determine whether the dietary AA balance and form can have an effect on the AA metabolism and intestinal physiology of pigs. Since both responses result from complex interactions between digestion and metabolism, the best approach is through *in vivo* methods with animal experiments. We performed two animal experiments, each with their own objectives that are in line with answering the thesis' scientific questions. An overview of these experiments is presented in Table 7. In the first experiment (Exp. 1), we used two pig models of different physiological stages that have contrasting uses of dietary AA to study the metabolic effect of feeding a diet with a balanced AA profile compared to diet with an unbalanced AA profile. The second experiment studied the metabolic (Exp. 2A) and digestive (Exp. 2B) responses of growing pigs to the provision of dietary AA in different forms, namely intact proteins, or free AA and small peptides from either an extensively hydrolysed AA source or as purified free AA. The technical properties of EHF allowed us to use it as a model for both AA balance and form studies. In this section of the dissertation, we justify the different experimental models and techniques we used to answer our scientific questions.

2. Model protein source

The thesis was done in collaboration with BCF Life Sciences (Pleucadeuc, France) because of the scientific interest in using EHF as a model protein source. This product comes from the extensive hydrolysis of feathers and even though its AA profile is quite unbalanced, the process completely transforms the protein fraction of EHF into free AA and small peptides. These properties make it an ideal protein source for this thesis. Firstly, it is made up of feathers, a protein source which is also commercially available as an intact protein (feather meal). Secondly, and probably more importantly, it allows us to use purified free AA to balance its AA profile without worrying about transient AA imbalances caused by the difference in the rate of plasma appearance of intact proteins and free AA.

3. The postprandial metabolism of animals approached by plasma concentrations of amino acids, other metabolites, and insulin

Our main goal was to study the effect of the balance and form of AA in the diet on AA bioavailability and metabolism. Our approach was through measuring the changes in the plasma concentrations of AA, other metabolites, and insulin after feeding pigs equipped with a jugular catheter to facilitate the sampling procedure and to handle the pig with minimal stress and pain. The surgery was done accounting for ethical standards and with experienced operators. The experiments were done in compliance with the ARRIVE animal experimentation guidelines and the EU directive 2010/63/EU for animal experiments.

3.1. Postprandial plasma metabolite and hormone kinetics

At the whole animal level, protein and AA metabolism can be approached using various experimental techniques like measurement of plasma metabolites, protein balance, protein deposition, and growth performance. Our approach was to study the evolution of plasma AA concentrations over time (or kinetics) after feeding. Compared to the other mentioned methods, the advantage of the technique is that it affords us to study the dynamic metabolic response of the pigs to dietary AA balance and form. Apart from being able to study the bioavailability of dietary AA, pairing the postprandial plasma concentrations of AA to that of other metabolites and hormones can also give us an estimate of the overall postprandial metabolic response. Thus, we also measured postprandial plasma glucose, insulin, and urea. Insulin is the main anabolic hormone of the body that stimulates cellular AA uptake and protein synthesis (van Loon et al. 2000; Davis et al. 2002). Its release is affected by the presence of nutrients in the plasma, most notably of AA and glucose. Both these nutrients can independently trigger its release (Patti et al. 1998; Saltiel and Kahn 2001), so postprandial plasma glucose may also play a role in AA metabolism, and vice-versa. Meanwhile, plasma urea is produced by the liver after its catabolism of AA, its concentration is frequently used by experiments to reflect the inefficient use of AA (Chen et al. 1995; Regmi et al. 2018). Furthermore, making plasma analyses allows

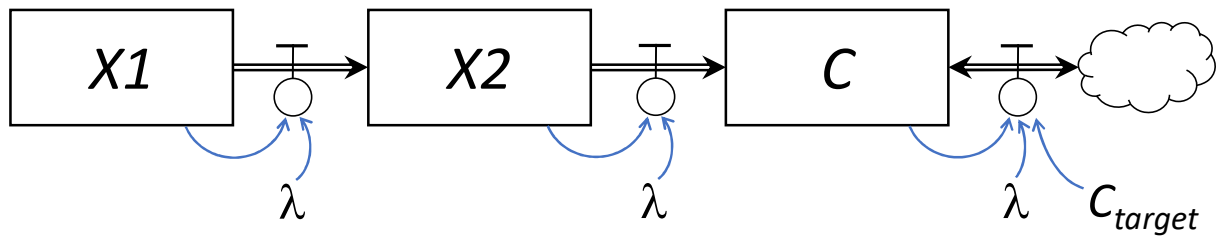


Figure 9. Forrester diagram of the conceptual model where the plasma concentration of amino acids (C , μM) is controlled by a target concentration (C_{target} , μM) and the ingestion of a diet provokes a change in the flow rate (λ , min^{-1}) of amino acids in the plasma via two prior compartments ($X1$ and $X2$)

us to use the same animal multiple times with the possibility of testing different experimental treatments, allowing us to use individual animals as their own control and does not require many animals.

3.2. Modelling postprandial plasma kinetics

Postprandial plasma AA kinetics have been frequently used to describe dietary AA bioavailability by studying the differences in concentrations between experimental diets using a repeated-measures model or a pairwise comparison of each sampling point. However, this technique ignores the underlying biological interactions between two subsequent sampling points. In pharmacokinetics, the bioavailability of administered drugs is instead studied by calculating the area under the curve (AUC), which corresponds to the “metabolic exposure” to the drug that’s being studied. Calculating for the AUC will be more insightful than doing pairwise comparisons of postprandial sampling points to study the metabolic response of pigs to different experimental diets. This calculation can be done using the integration process if the equation of the curve and its boundaries are known. However, unlike in pharmacokinetics, this technique is harder to implement for plasma AA as their concentrations are never zero.

To better characterise and understand our results, we developed a conceptual model in parallel to the thesis (van Milgen et al. 2022). The model incorporated the possible underlying biological mechanisms in play after ingestion of a meal that summarises postprandial plasma concentration data in a limited number of parameters (see Annex 2 for more information). It works with the assumption that there is a homeostatic control of plasma AA concentration that is maintained through a target concentration (Figure 9). This is a rather strong assumption because the plasma AA concentration may not be controlled directly, but could also be the result of the independent control of the influx (i.e., appearance of dietary AA, *de novo* synthesis) and the outflux (i.e., protein synthesis, AA catabolism) of AA in the plasma. Still, using the

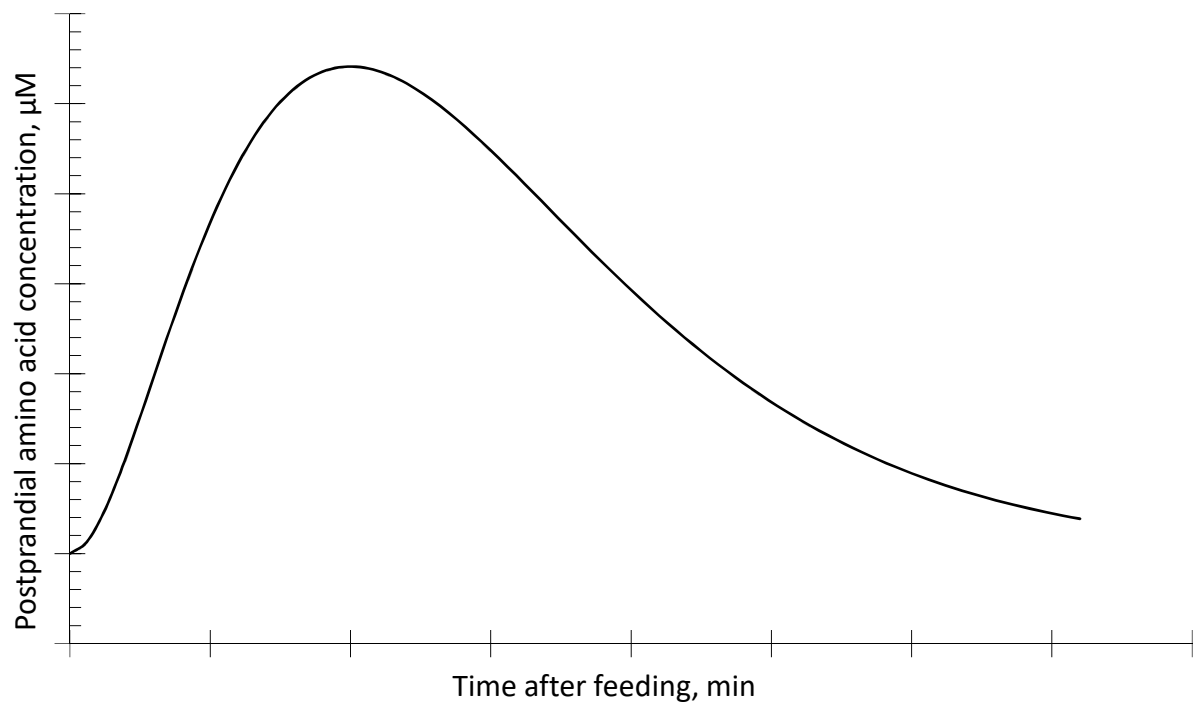


Figure 10. Example of a postprandial plasma amino acid kinetic curve

developed model allowed us to re-describe our data and interpret the changes in the postprandial kinetics using only a few parameters with biological meaning.

To better imagine postprandial plasma AA kinetics and what it typically looks like, an example is illustrated in Figure 10. There is typically an increase in concentrations after feeding, followed by a decrease, where the shape follows a bell-shaped curve. The elephant in the room is that plasma concentrations and their postprandial kinetics are not metabolic fluxes. Further complicating this is the fact that even though the appearance of dietary AA in the plasma is a big part of the increase in concentration after feeding, a portion of this concentration comes from *de novo* synthesis and mobilization from tissue proteins. Moreover, we also cannot distinguish whether the decrease in plasma AA concentrations is because of incorporation in body proteins or because of catabolism. Despite these limitations, this does not preclude us from making certain interpretations in terms of postprandial utilization of AA, as some are more plausible than others. For example, an increase in plasma concentrations after feeding is more likely because of increased influx from the diet than either increased mobilization or decreased outflux. A second example is the comparison between adult and growing pigs who have contrasting AA utilization and potential for protein deposition. In the period right after the meal, protein synthesis is assumed to be much higher in growing pigs that deposit protein than in adult pigs whose protein synthesis is mostly done to replenish the tissue AA pool lost through obligatory mobilization rather than for protein deposition.

4. Digestive response

The small intestine is at the interface between the diet and metabolism. It regulates the availability of dietary AA for the organism through digestion of proteins, absorption of AA, and first pass utilization of AA. Furthermore, supplying AA as free AA or as intact proteins is expected to cause adaptations that include morphological changes of the surface of absorption measured by the morphology of the small intestine mucosa and the capacity to transport AA measured by the expression of AA transporters. All these physiological changes, along with the inherent bioavailability of nutrients in the diet, contribute to nutrient digestibility.

4.1. Digestibility measurements using the slaughter technique and indigestible markers

Digestion of protein results in the release of its constituent AA followed by their subsequent absorption of epithelial cells. Digestibility is the coefficient of disappearance of dietary nutrients from the intestinal lumen after feeding (Rivest et al. 2000). For pigs, even though the digestion process starts in the stomach, the bulk is done through the digestion by pancreatic enzymes released in the duodenum. The absorption of AA and small peptides mainly happens in the jejunum until the ileum. Hence, the digestive and absorption processes are closely tied to the physiology of the small intestine, which suggests that any changes in intestinal physiology can also have an effect on AA digestibility. Feeding intact proteins that needs to be digested would have lower digestibility than dietary free AA and small peptides as AA in these forms does not need to be digested. Measurement of AA digestibility can give an insight on the physiological effects of the form of AA in the diet of pigs.

In the thesis, we measured digestibility using the slaughter and marker method. This involved feeding pigs a diet with indigestible markers (titanium dioxide and cobalt-EDTA) after an overnight fast. We chose to slaughter our pigs and collect digesta samples 3 hours after the meal as our experimental diets are soluble (it is mostly made up of starch) and we know that the average retention time the soluble fraction (liquid) of the digesta is around 3 to 4 hours (Wilfart

et al. 2007; Schop et al. 2019). The digesta samples and the feed were analysed for the content of both AA and indigestible markers that were then used to compute for digestibility. We chose this method as it is relatively convenient to do and other more precise techniques like cannulation may affect intestinal physiology.

4.2. Intestinal morphology

The luminal surface of the small intestine is lined with multiple finger-like projections called villi that increases the total surface area of the small intestine significantly. The main function of these vascular structures is to absorb nutrient. The invaginations between villi are called crypts, the most basic functional unit of the small intestine where the synthesis of new epithelial cells and their differentiation mainly occurs. As the cells in the crypt are not yet differentiated, there is virtually no absorption happening in this structure (Xia et al. 2005; Ma et al. 2019). By measuring the morphology of these structures in the small intestine, we get an idea of the total surface area for absorption. Villi size, height, and area serve as indicators of the total available surface for absorption while deep crypts can typically mean damage to the small intestine and lower capacity for absorption (Al-Masri et al. 2015). We expect that these properties will differ in pigs fed intact proteins and free AA.

4.3. Gene expression of amino acid transporters

The absorption of AA and small peptides in the intestinal lumen is typically done by the various AA transporters with different specificities and a peptide transporter (PepT1) that transport all combinations of small peptides (di- and tri-peptides). These transporters are situated in the enterocyte membrane. The abundance of these transporters in the intestine can be directly proportional to substrate abundance which is also dependent on the form of AA in the diet (Kilberg et al. 2005; Morales et al. 2015). One classical way to check for the abundance of transporters in the small intestine is through measurement of the expression of genes coding for these transporters. Indeed, for part of this thesis, we fed pigs with different forms of AA that

we expect to be available at different times and at different sections of the small intestine. This should then result in differences in the expression of different AA transporters across the different sections.

Another factor that can affect the gene expression of different AA transporters in the small intestine of pigs is the AA profile of the diet (Zhang et al. 2013). Indeed, the AA profile of the model protein source that we used in the experiments is quite unbalanced with very low levels of certain essential AA like Lys, Met, and His. Thus, it can be used to study the effect of feeding a diet with limiting amounts of essential AA.

In Exp. 2B, we selected four AA transporters and the peptide transporter PepT1 to analyse in the different sections of the small intestine. The AA transporter, ASCT2, is a sodium-dependent transporter that transports all neutral proteinogenic AA except for Asp, Arg, Glu, Lys, and His (Bröer and Fairweather, 2018), while those under the SLC7 family, CAT-1, y⁺LAT1, and b^{0,+}AT, transport cationic AA namely, Arg, His, and Lys (Yin et al., 2014). However, b^{0,+}AT is also implicated in the transport of neutral AA (Yin et al., 2014; Bröer and Fairweather, 2018). So, among the AA transporters whose expression was measured, ASCT2 and b^{0,+}AT have broad specificities and have the ability transport most proteinogenic AA (at least 14 AA). However, since b^{0,+}AT can also transport Lys, an essential AA, that is supplied in low amounts in the diet, we hypothesized that this may lead the animal to express more of this transporter than ASCT2 as the animal adapts and tries to capture as much Lys as possible.

EXPERIMENTAL RESULTS

EXPERIMENT 1 (Exp. 1)

Postprandial plasma amino acid and metabolite kinetics of adult and growing pigs fed a diet with free amino acids and small peptides with a balanced or unbalanced amino acid profile[†]

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1. Abstract

Feeding diets with an unbalanced amino acid (AA) profile can reduce the postprandial AA utilization for protein synthesis. Growing pigs use dietary AA mainly for protein accretion, whereas adult pigs use AA mainly for maintenance. The requirement for AA for growth is much larger than that for maintenance and growing pigs may therefore be more affected by a diet with an unbalanced AA profile than adult pigs. This study aimed to compare the postprandial plasma AA and metabolite concentrations of adult and growing pigs after feeding a diet with either an unbalanced (UNB) or a balanced AA profile (BAL). The postprandial plasma concentrations of AA were used to study the influence of AA balance on postprandial AA metabolism. Extensively hydrolysed feathers (EHF) were used as an AA source. Both BAL and UNB contained EHF supplemented with L-Ala, L-Asp, L-Glu, Gly, and L-Trp while BAL was also supplemented with L-His, L-Ile, L-Lys, L-Met, and L-Tyr. Four growing and four adult pigs were fitted with a jugular catheter and received each diet as a meal test thrice. The meal test consisted of giving a small meal after an overnight fast followed by serial blood collection for 360 min. A non-linear regression model was used to describe the postprandial plasma AA kinetics. Plasma kinetics of adult and growing pigs fed BAL resulted in a higher area under the curve (AUC) for the AA that were used to balance the diet. For the other AA, feeding BAL resulted in lower AUC, suggesting faster metabolic utilization of AA for protein synthesis. The apparent quantity of dietary AA appearing in the plasma after feeding was lower in adult pigs, suggesting higher first-pass AA utilization in the intestine and liver. For adult and growing pigs, balancing the AA profile of the diet resulted in faster overall metabolic utilization of AA as seen in the generally lower AUC of BAL compared to UNB.

Key words: amino acid, amino acid balance, metabolism, pig, plasma metabolite

2. Introduction

The metabolism of dietary amino acids (AA) is affected by the dynamics of protein digestion and AA absorption and by the balance between the different AA and other nutrients. For animals, an imbalance occurs when AA arrive in different quantities and at different times relative to their utilization for protein synthesis (Yen et al. 2004). In a diet with an unbalanced AA profile, a limiting supply of one or more AA results in a relative excess of other AA that reduces the overall efficiency of AA utilization (Regmi et al. 2018) and increases nitrogen excretion (Gloaguen et al. 2012). Specifically, it results in lower postprandial plasma concentrations of the limiting AA, while the non-limiting AA are found in higher concentrations compared to feeding a diet with a balanced AA supply (Henry et al. 1992).

The consequences of feeding unbalanced dietary AA can be assessed by measuring protein balance, protein deposition, or animal growth during a few days or weeks. An alternative is to focus on the postprandial metabolism, which constitutes a period of high protein anabolism due to the stimulation of protein synthesis by insulin and AA (Davis et al. 2002). This technique is usually approached by analysing the postprandial plasma AA curve to quantify the bioavailability of dietary AA and estimate metabolic fluxes (Cynober 2002). The arrival of dietary AA in the plasma is affected by dietary supply and the first-pass utilization of the intestine and liver (Wu et al. 2005), while AA disappearance is affected by their utilization by peripheral tissues and the rate of AA catabolism (Kampman-van de Hoek et al. 2016). Although controlled by fluxes, plasma AA concentrations cannot be used as direct measures of metabolic fluxes (Cynober 2002). Nevertheless, indirect interpretations can be made from postprandial changes in plasma AA concentrations.

The utilization and metabolic fluxes of dietary AA can be affected by both the balance among dietary AA and the form in which dietary AA are provided as. Indeed, feeding readily absorbable free AA seems to induce a lower postprandial AA utilization than when provided as

proteins that first need to be digested (Batterham and Bayley 1989; Yen et al. 2004). In the study presented here, the metabolic consequence of dietary AA balance is assessed, while the study of Eugenio et al. (2022, submitted) focuses on the dietary form of AA. In both experiments, feather protein was used as a model source of AA. Feather protein has a very low content of Lys and a high content of Cys compared to muscle protein (Grazziotin et al. 2006). Consequently, feather protein can be used as a model source of unbalanced AA, especially for growing pigs with a high potential for muscle protein deposition. Furthermore, extensively hydrolysed feathers (**EHF**) supply AA in free form and as small peptides and have a digestibility close to 100% (Kersanté et al. 2021). Therefore, the AA profile of a diet with EHF as the only AA source can be balanced by supplementation with free AA without the confounding effect of providing dietary AA in different forms.

In this experiment, we compared the changes in postprandial plasma concentrations of AA, other metabolites, and insulin of adult and growing pigs after feeding a diet with an unbalanced AA supply (**UNB**) with EHF with a diet where EHF was supplemented with free AA to balance its AA supply (**BAL**). We hypothesised that feeding BAL would result in lower postprandial plasma concentrations of AA, most notably for AA that were supplied in the same amount in both diets, as more AA would be used for protein synthesis. Furthermore, feeding UNB is expected to have a more marked effect in growing pigs that have higher needs for AA to support muscle protein deposition than in adult pigs.

3. Materials and Methods

3.1. Animals, housing, diets, and experimental design

Six adult Yucatan mini-pigs (38.7 ± 2.9 kg) of 12 months of age and six growing pigs (Pietrain x [Large-White x Landrace]; 48.9 ± 0.8 kg) of 18 weeks of age from the experimental herd of UE3P (INRAE, Saint-Gilles, France <https://doi.org/10.15454/1.5573932732039927E12>) were surgically fitted with a jugular catheter under general anaesthesia. All pigs in the experiment were non-castrated males. After an overnight fast, pigs were cleaned and premedicated by intramuscular injection of 15 mg/kg body weight of ketamine (Imalgene 1000, Merial, Lyon, France). Anaesthesia was maintained with aerosolized isoflurane (Isoflurane, Baxter, Maurepas, France) mixed with oxygen (3 - 5%) and administered using a gas mask. An indwelling silicone catheter was then inserted through a collateral vein in the right external jugular and externalized through the back of the pig's neck and then kept in a strengthened-elastic pocket kept directly above the site of externalization. The wound was cleaned and disinfected using an iodine solution every other day. Furthermore, 1 mL per 10 kg body weight of amoxicillin (Duphamox, Zoetis, Malakoff, France) on the second and fourth day after the operation was provided. Feed allowance was gradually increased for three days after surgery. The catheters were flushed every two days with a 5 mL saline solution mixed with heparin (1%).

The growing and adult pigs were housed successively in the same experimental facilities with 1 week in between to clean and disinfect the room and pens. Pigs were kept in individual pens (1.0 x 3.5 m) enriched with toys and plastic floor mats. Water was available *ad libitum*. The room was environmentally controlled with temperature maintained at 26 °C, relative humidity at 60%, and 12 h of light daily. For each stage, the experiment lasted 4 weeks, including 1 week of recovery from surgery and adaptation to the experimental diets.

Table 8. Nutrient and amino acid content of extensively hydrolysed feathers and analysed composition (as fed) of the balanced (BAL) and unbalanced diets (UNB) fed to adult and growing pigs¹

Items	EHF ²	Adult pigs		Growing pigs	
		BAL	UNB	BAL	UNB
Composition, %					
EHF		17.0	17.0	17.0	17.0
Corn starch		65.9	66.0	65.9	66.1
Sugar		2.00	2.00	2.00	2.00
Vegetable oil		4.00	4.00	4.00	4.00
Synthetic fibre		5.00	5.00	5.00	5.00
Limestone		0.50	0.50	0.50	0.50
Dicalcium phosphate		2.00	2.00	2.00	2.00
Vitamin and mineral premix ³		0.50	0.50	0.50	0.50
Refined iodized salt		0.60	0.60	0.50	0.50
Purified amino acids					
L-Ala		0.15	0.79	0.01	0.77
L-Asp		0.17	0.30	0.07	0.31
L-Glu		0.18	0.30	0.07	0.31
L-Gly		0.16	0.78	0.01	0.78
L-Trp		0.20	0.20	0.20	0.20
L-His		0.20		0.27	
L-Ile		0.50		0.50	
L-Lys		0.74		0.97	
DL-Met		0.20		0.21	
L-Tyr				0.25	
Analysed content					
Dry Matter, %	98.0	90.1	89.2	89.2	88.7
Total nitrogen, %	12.4	2.83	2.85	2.85	2.90
Gross energy, MJ·kg ⁻¹	18.0	15.7	15.6	15.6	15.7
Amino acids, g·kg ⁻¹					
<i>Only added in BAL</i>					
His	5.62	2.45	0.91	3.43	1.00
Ile	43.7	9.86	5.39	10.3	5.57
Lys	17.4	6.73	2.47	9.37	2.79
Met	5.64	3.06	0.89	2.91	0.91
Tyr	6.52	1.30	1.17	3.51	1.24
<i>Mostly added in UNB</i>					
Ala	42.7	8.29	13.5	7.25	14.6
Asp	64.6	11.0	11.5	11.1	13.9
Glu	97.3	16.4	16.6	16.7	19.3
Gly	71.7	13.5	19.9	11.8	18.8
<i>Equal amounts in BAL and UNB</i>					
Arg	59.5	10.01	9.11	9.38	9.41
Cys	19.2	1.37	1.33	1.31	1.81
Leu	74.1	11.5	10.9	11.2	11.3
Phe	45.1	7.94	7.19	7.23	7.29
Pro	95.1	16.2	15.6	15.9	16.0
Ser	117	19.7	18.6	18.8	19.0
Thr	43.5	7.57	7.14	7.23	7.31
Trp	0.00	2.11	1.87	1.63	2.05
Val	73.8	8.86	8.31	8.70	8.63

¹Diets were offered as mash

²EHF: extensively hydrolysed feathers; cystine and tyrosine were partially removed after extensive hydrolysis of feathers; Trp is destroyed by the process of extensive acid hydrolysis of feathers

³Provided per kg of complete diet: vitamin A, 1,000,000 IU; vitamin D3, 200,000 IU; vitamin E, 4,000 mg; vitamin B1, 400 mg; vitamin B2, 800 mg; calcium pantothenate, 2,170 mg; niacin, 3,000 mg; vitamin B12, 4 mg; vitamin B6, 200 mg; vitamin K3, 400 mg; folic acid, 200 mg; biotin, 40 mg; choline chloride, 100,000 mg; iron (sulfate), 11,200 mg; iron (carbonate), 4,800 mg; copper (sulfate), 2,000 mg; zinc (oxide), 20,000 mg; manganese (oxide), 8,000 mg; iodine (iodate), 40 mg; cobalt (carbonate), 20 mg; and selenium (selenite), 30 mg

The experimental diets were formulated for adult and growing pigs and either had a balanced (National Research Council 2012) or unbalanced ideal AA profile, using EHF as the main AA source (Table 8). In all diets, L-Trp was added as EHF is devoid of this AA. The free non-essential AA L-Glu, L-Asp, L-Gly, and L-Ala were added to have iso-nitrogenous diets. The AA profile of BAL was balanced by supplementing the diets with L-Lys, DL-Met, and L-His. To have the concentrations of all branched chain AA balanced with each other, L-Ile was added in BAL. Also, L-Tyr was added in BAL for growing pigs as they have higher Tyr requirements than adult pigs. All free AA were bought from Sigma Aldrich Chimie (St. Quentin Fallavier, France). The pigs were fed with BAL throughout the experiment except for the days when meal tests and serial blood sampling were performed. Feed was distributed twice daily amounting to 800 g/day for adult pigs and 1 200 g/day for growing pigs.

3.2. Meal tests and serial blood sampling

After an overnight fast, a first blood sample was collected. Then, a small meal (300 g for adult pigs and 450 g for growing pigs) of either BAL or UNB was given, which ensured that the pigs ate all the meal in less than 10 min (Le Floc'h et al. 2019). Blood was collected at 10, 20, 30, 40, 50, 60, 75, 90, 105, 120, 150, 180, 210, 240, and 360 min postprandially. During this period, the pigs had no access to feed but had access to water. For each sampling, 4 to 5 mL of blood was collected via the catheter and transferred in tubes lined with heparin. The catheters were rinsed with sterile saline after each sample. The tubes with collected blood were immediately placed on an ice bed before being centrifuged (3 000 g for 10 min at 4 °C) to separate plasma. Plasma samples were then stored until analyses. Six meal tests, three times for each diet, for four adult and four growing pigs, were carried out. Pigs received the meal test of BAL and UNB in alternating order at intervals of two meal tests per week for three weeks so that each pig served as its own control. The two remaining pigs were kept for the duration of the experiment to serve as a replacement in the event of health problems or malfunctioning of the catheters.

3.3. Feed analyses

Total nitrogen in feed was analysed by combustion in an automated analyser (LECO FP-428 Nitrogen Determinator, LECO, St. Joseph, MI). For AA analysis, feed samples were hydrolysed in 6N hydrochloric acid for 24 h (48 h for branched-chain AA) at 110 °C with constant agitation. For Met and Cys analysis, oxidation with performic acid for 4 h was done before hydrolysis for 18 h. The Trp content was determined after an alkaline hydrolysis with barium hydroxide for 16 h at 120 °C. The AA were analysed after derivatization by AccQtag (AccQ-Fluor reagent kit, Waters, Guyancourt, France) using an HPLC system (Alliance System 2690, Waters, Guyancourt, France). Gross energy content of the diets was measured in an adiabatic bomb calorimeter (IKA, Staufen, Germany).

3.4. Plasma amino acid, metabolite, and insulin assays

The concentrations of AA in plasma were analysed using a UPLC system (Waters Acquity Ultra Performance Liquid Chromatography system, Waters, Guyancourt, France) with the MassTrak AAA column after derivatization of samples using the AccQtag Ultra method (Waters, Milford, MA, USA). Plasma glucose (Glucose (HK), Thermo Fisher Scientific, Vantaa, Finland), urea (Urea kit, Thermo Fisher Scientific, Asnieres-Sur-Seine, France), and free fatty acids (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany) were analysed with commercially available kits using colorimetric methods through an automatic analyser, Konelab 20i (Thermo fisher scientific, Courtaboeuf, France). Plasma concentrations of insulin were measured using a radioimmunoassay kit (Insulin-CT, CisBio Bioassays, Codolet, France) and an AIA-1800 device (Automated Immunoassay Analyzer; TOSOH Bioscience, Tokyo, Japan).

3.5. Calculations and statistical analysis

Data for adult and growing pigs were computed and analysed separately. All statistical analyses were done using R version 3.6.2 (R Core Team 2019). A generalised additive mixed model of

the gam package of R (Hastie 2019) was used as a first analysis to assess the effect of time and diet on plasma concentrations of metabolites and insulin, with the pig as the experimental unit. Only the 20 proteinogenic AA were considered. The model includes time and diet as fixed effects and pig and meal tests nested within pig as random effects. Time was declared as a repeated measure and a smoothing variable. For all statistical analyses performed, a $P < 0.05$ was considered significant.

Plasma AA concentrations were also analysed using non-linear regression. A modified Erlang function was used to analyse the postprandial plasma kinetics of AA (van Milgen et al. 2022). The model describes the postprandial kinetics of plasma AA concentrations over time ($C(t)$) and assumes the existence of a target AA concentration during fasting. Ingestion of the meal results in a temporary increase in plasma AA concentration, and may also change the target concentration due to ingestion of the meal:

$$C(t) = C_0 + (1/2 AUC \lambda^3 t^2 \exp(-\lambda t)) + (C_\delta(1 - \exp(-\lambda t)))$$

Where t (min) is the time of sampling, C_0 ($\mu\text{mol/L}$) is the target AA concentration during fasting (i.e, at $t = 0$), C_δ ($\mu\text{mol/L}$) the change of the target concentration relative to C_0 due to the ingestion of the meal, AUC ($\mu\text{mol/L} \cdot \text{min}$) the area under the curve, and λ (1/min) the fractional flow rate.

Parameter estimates were obtained using the nls2 function (Grothendieck 2013). Fitting of the model was done for each meal test for each pig, so that the response was expressed as a set of four parameters (C_0 , C_δ , λ , and AUC). The effect of diet on the parameters was tested using the lmer function of the lmerTest package (Kuznetsova et al. 2017) with diet as fixed effect and pig and meal test as random effect. The C_δ can be positive, negative, or not different from zero. A t -test with the ls_means function (lmerTest package) was used to test if C_δ differed from zero. The AUC represents the metabolic exposure of the animal to dietary AA from which the

apparent quantity of dietary AA arriving in the jugular plasma (Q ; $\mu\text{mol/L}$) can be calculated as $AUC \times \lambda$ (van Milgen et al. 2022). Differences in Q between the two diets may be due to differences in dietary AA supply or to differences in first-pass AA metabolism of the intestine and the liver. This was assessed as $Q_{BAL:UNB}/diet_{BAL:UNB}$ (as percentage), where $Q_{BAL:UNB}$ is the ratio between the average Q when feeding BAL relative to feeding UNB, while $diet_{BAL:UNB}$ is the ratio between the AA supply of BAL over UNB. The $Q_{BAL:UNB}/diet_{BAL:UNB}$ is thus indicative for the extent to which a different dietary AA supply between the two diets (i.e., through $diet_{BAL:UNB}$) results in a different apparent quantity of AA appearing in the plasma (i.e., through $Q_{BAL:UNB}$). A $Q_{BAL:UNB}/diet_{BAL:UNB}$ of 100% means that the difference in the apparent quantity of AA appearing in the plasma between the diets equals the difference in the dietary supply.

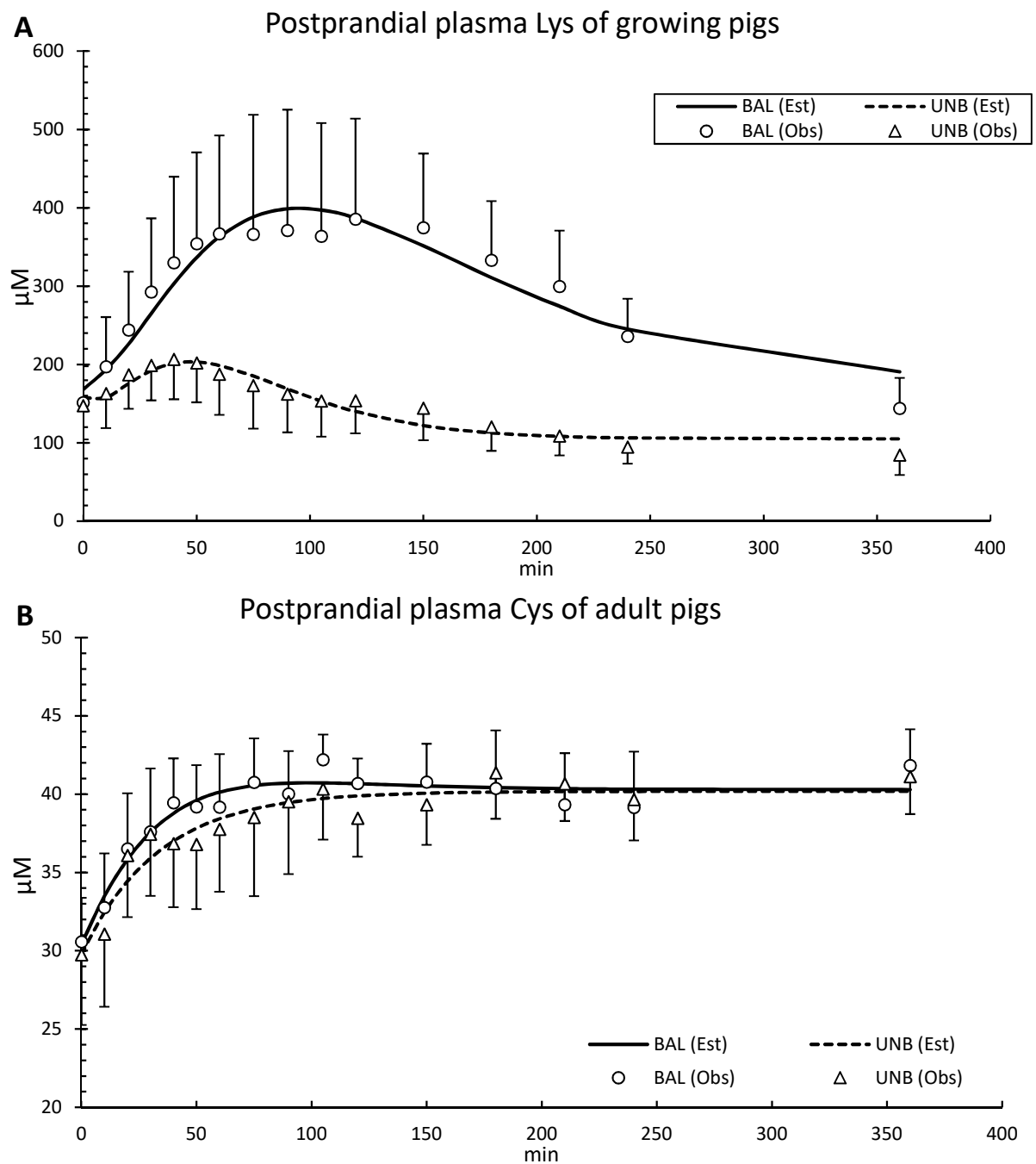


Figure 11. Observed (Obs; mean \pm SD) and estimated (Est) postprandial concentrations (μM) of plasma Lys of growing pigs (A, $n = 12$) and plasma Cys of adult Yucatan mini-pigs (B, $n = 12$) after feeding a diet with an extensively hydrolyzed feathers as a source of an unbalanced amino acids (UNB) or a diet with extensively hydrolyzed feathers supplemented with synthetic amino acids (BAL)

4. Results

4.1. General observations

All animals appeared healthy and there were no problems with feed intake and blood collection except for one adult pig who developed fever for 3 days. This pig was therefore replaced by another pig. For all diets, Cys was around 30% lower than anticipated but the impact on results should be similar since this is consistent for all diets. Apart from Cys, the AA content of BAL were close to the targeted AA.

4.2. Postprandial plasma concentrations

The kinetics of postprandial plasma AA concentration was analysed through a non-linear regression model. The model used was capable of fitting different response types (Figure 11), which included curves for which the plasma concentration followed an asymmetrical bell-shaped curve (Figure 11A for Lys in growing pigs) or an asymptotic curve (Figure 11B for Cys in adult pigs). The capacity to fit different types of curves is due to the three terms of the equation. The first term is the target AA concentration after fasting, the second term represents the asymmetrical bell-shape curve, and third term reflects the change in the target concentration due to ingestion of the meal. These aspects are additive in the equation and can be present to different degrees in the response. For example, the asymmetrical bell-shaped curve is hardly present for Cys. Likewise, when growing pigs are fed UNB, the target Lys concentration decreased from around 150 μM during fasting to 100 μM after ingestion of the meal. Parameter estimates for C_0 , C_δ , AUC , and λ for adult and growing pigs are given in Tables 9 and 10, respectively. For all AA, diet did not affect C_0 for adult and growing pigs and C_0 was higher in adult than in growing pigs.

For both adult and growing pigs, feeding BAL resulted in a higher AUC for most of the AA only added in BAL (i.e., His, Ile, Lys, and Met, and also for Tyr for growing pigs). For AA that were mostly added in UNB, only the AUC of Gly for adult pigs and Ala for growing pigs were

Table 9. Parameter estimates describing the postprandial kinetics of plasma amino acids of adult pigs fed diets containing extensively hydrolysed feathers with either a balanced or unbalanced amino acid profile¹

	C_0 , μM				C_0 , μM				AUC , $\mu\text{M}\cdot\text{min} \times 10^3$				λ , $1/\text{min} \times 10^{-2}$			
	BAL	UNB	RSD	P_{Diet}	BAL	UNB	RSD	P_{Diet}	BAL	UNB	RSD	P_{Diet}	BAL	UNB	RSD	P_{Diet}
<i>Only added in BAL</i>																
His	69.3	65.4	7.09	0.20	-12.1 [†]	-7.95	10.2	0.34	6.53	2.52	2.62	<0.01	2.62	3.43	1.82	0.29
Ile	182	165	29.1	0.17	-24.6	-37.0	28.4	0.62	46.3	34.8	24.7	0.28	1.82	1.97	0.53	0.54
Lys	205	186	25.4	0.07	-8.45	-58.2 [†]	53.1	0.18	41.9	17.6	22.1	0.01	1.89	2.87	1.61	0.15
Met	30.9	31.1	4.89	0.95	-8.53 [†]	-12.9 [†]	6.59	0.11	4.35	1.82	2.12	0.01	2.89	4.21	2.22	0.16
<i>Mostly added in UNB</i>																
Ala	313	331	43.8	0.35	-11.0	-29.0	20.4	0.67	81.7	104	38.4	0.19	1.68	1.55	0.39	0.44
Asp	9.94	9.68	2.24	0.78	-11.6 [†]	-13.6 [†]	11.4	0.68	5.86	7.27	5.10	0.52	1.37	1.30	0.37	0.66
Glu	104	114	21.6	0.30	-23.3	-41.2 [†]	13.2	0.10	17.4	20.7	12.7	0.56	1.81	3.60	4.72	0.37
Gly	1,101	1,085	123	0.77	-259 [†]	-337 [†]	223	0.42	216	307	91.8	0.02	1.42	1.29	0.36	0.41
<i>Equal amounts in BAL and UNB</i>																
Arg	97.6	101	14.5	0.60	-10.6	-28.5	24.1	0.35	31.3	36.3	18.9	0.54	1.76	1.71	0.49	0.80
Asn	33.4	31.9	3.68	0.33	-11.8 [†]	-11.6 [†]	3.84	0.92	0.87	0.87	0.22	0.94	5.78	5.13	1.16	0.20
Cys	29.5	31.3	3.67	0.25	9.42 [†]	8.68 [†]	4.43	0.70	0.54	1.69	3.02	0.37	3.17	2.63	2.01	0.52
Gln	428	415	40.7	0.45	-46.6	-7.06	24.4	0.10	32.3	47.1	61.0	0.56	2.16	1.62	0.97	0.22
Leu	168	167	28.9	0.95	-16.6	-46.6	83.6	0.40	36.5	50.0	25.6	0.23	1.98	1.89	0.66	0.75
Phe	89.4	93.6	9.50	0.29	1.60	-12.5	15.2	0.20	13.6	18.7	8.14	0.15	1.84	1.77	0.54	0.78
Pro	340	360	46.6	0.31	-19.6	-84.6	116	0.20	137	169	47.5	0.14	1.48	1.36	0.30	0.37
Ser	169	190	25.2	0.07	23.7	157 [†]	129	0.04	92.6	162	59.2	0.01	1.37	1.10	0.30	0.04
Thr	198	203	19.1	0.52	-18.5	-37.4	40.1	0.38	49.3	57.6	18.4	0.31	1.60	1.51	0.41	0.60
Trp	51.3	51.1	6.31	0.94	3.46	1.46	3.17	0.64	4.73	5.44	2.45	0.51	2.03	2.90	1.90	0.28
Tyr	84.9	86.4	11.5	0.75	-20.0 [†]	-19.6 [†]	5.52	0.95	4.53	4.75	2.55	0.83	3.67	3.10	2.39	0.56
Val	488	477	52.8	0.63	29.9	-9.72	19.0	0.44	79.3	98.1	41.0	0.29	1.61	1.61	0.48	0.99

¹Parameter estimates (least square means) for the target concentration after fasting (C_0), the change in target concentration due to ingestion of the meal (C_0), the area under the curve (AUC), and the fractional flow rate (λ) of the modified Erlang function (see text for details) from four pigs fed a diet with either a balanced (BAL) or unbalanced (UNB) amino acid profile three times each. RSD – residual standard deviation

[†] C_0 different from 0 ($P < 0.05$)

higher. For the AA that were supplied in equal amounts by the diets, the *AUC* of Ser was higher when feeding UNB in both adult and growing pigs, whereas it was lower for Arg in growing pigs. Furthermore, the *AUC* of adult pigs was generally higher than that of growing pigs, especially for UNB. In adult pigs, the λ was not affected by diet, except for Ser, which was higher in BAL. In growing pigs, the λ of His, Lys, Met, and Cys was affected by the diet, all of which were lower in BAL. The λ of adult pigs was generally lower than that of growing pigs.

For adult pigs, λ was not affected by diet, except for Ser, for which λ was higher in BAL. For growing pigs fed BAL, three of the five AA that were only added in BAL (i.e., His, Lys, and Met) had lower λ . For AA that were supplied in equal amounts in BAL and UNB, only the λ of Cys was lower in growing pigs fed BAL compared to UNB. The λ of adult pigs was generally lower than that of growing pigs.

The difference in the apparent quantity of AA appearing in the plasma due to a difference in dietary AA supply (i.e., $Q_{BAL:UNB}/diet_{BAL:UNB}$) is given in Figure 12. For adult pigs, the $Q_{BAL:UNB}/diet_{BAL:UNB}$ was less than 100% for all AA except Ala, Gly, and Tyr, indicating that the difference in the AA supply between the diets resulted in a lower change in the apparent quantity of AA appearing in the plasma. For growing pigs, the $Q_{BAL:UNB}/diet_{BAL:UNB}$ was greater than 100% for most AA, except for His, Lys, Met, Tyr, Asp, and Ser, the first four of which were added only to BAL. For AA that were provided in equal amounts in both diets, adult and growing pigs differed largely in their plasma response.

There was a significant effect of time ($P < 0.01$) on the postprandial plasma concentrations of insulin, glucose, urea, and free fatty acids (Supplementary Figure 1) and diet did not affect these concentrations ($P > 0.10$). On average, the urea concentration was 285 mg/L in adult pigs and 206 mg/L in growing pigs. The insulin peak, roughly occurring 50 min postprandially, was 68.2 μ U/mL in adult pigs and 49.8 μ U/mL in growing pigs.

Table 10. Parameter estimates describing the postprandial kinetics of plasma amino acids of growing pigs fed diets containing the extensively hydrolysed feeders with either a balanced or unbalanced amino acid profile¹

	C_0 , μM				C_δ , μM				AUC , $\mu\text{M}\cdot\text{min} \times 10^3$				λ , $1/\text{min} \times 10^{-2}$			
	BAL	UNB	RSD	P_{Diet}	BAL	UNB	RSD	P_{Diet}	BAL	UNB	RSD	P_{Diet}	BAL	UNB	RSD	P_{Diet}
<i>Only added in BAL</i>																
His	43.9	45.5	6.35	0.53	3.97	-14.8 [†]	10.0	<0.01	6.76	1.88	2.05	<0.01	2.60	4.30	0.80	<0.01
Ile	137	139	26.4	0.85	84.1	8.14	63.4	0.01	43.1	22.8	16.8	0.01	3.73	3.26	1.40	0.42
Lys	168	155	34.9	0.37	-5.30	-55.2 [†]	52.1	0.12	48.9	10.1	16.8	<0.01	2.76	4.04	1.10	0.01
Met	28.9	27.7	4.74	0.52	6.02	-12.6 [†]	10.3	<0.01	5.54	1.35	2.59	<0.01	3.50	5.54	1.25	<0.01
Tyr	54.4	52.9	13.5	0.79	6.26	-17.7 [†]	12.0	<0.01	5.41	3.09	2.15	0.02	4.29	4.48	1.19	0.69
<i>Mostly added in UNB</i>																
Ala	294	317	56.2	0.33	-45.6	-8.83	48.0	0.32	39.4	53.7	14.5	0.03	2.44	2.30	0.54	0.54
Asp	7.29	7.66	1.78	0.62	-4.14	-3.10	4.76	0.60	3.30	3.85	1.98	0.50	2.18	2.38	0.71	0.48
Glu	84.8	78.8	16.0	0.39	-27.4	-1.21	30.6	0.06	10.5	10.7	6.40	0.97	3.30	2.78	1.44	0.39
Gly	1,134	1,163	266	0.80	172	89.7	119	0.10	195	213	98.6	0.65	1.53	1.73	0.36	0.17
<i>Equal amounts in BAL and UNB</i>																
Arg	79.0	71.7	10.9	0.12	13.7	35.8	30.2	0.09	27.7	18.0	8.37	0.01	3.38	3.79	0.94	0.30
Asn	34.6	35.1	5.51	0.85	-15.4 [†]	-14.0 [†]	4.42	0.45	1.06	1.06	0.27	0.99	5.42	5.70	0.68	0.33
Cys	23.9	23.1	2.83	0.47	8.85 [†]	12.0 [†]	3.49	<0.05	2.39	0.42	2.58	0.08	3.11	5.10	1.40	<0.01
Gln	390	394	20.7	0.87	-30.9	-18.7	18.6	0.62	15.4	11.6	7.43	0.23	2.76	3.76	1.24	0.06
Leu	115	128	38.1	0.42	41.0	42.4	64.2	0.96	34.5	34.5	15.8	0.99	3.83	2.83	1.49	0.12
Phe	67.0	68.8	14.7	0.76	25.0	27.0	31.1	0.87	14.0	12.9	6.45	0.67	3.41	2.31	1.41	0.08
Pro	240	240	52.5	0.99	13.6	83.1	75.8	0.16	108	90.0	35.2	0.24	2.11	2.21	0.59	0.70
Ser	111	123	39.5	0.47	41.4	191 [†]	74.4	<0.05	66.2	105	26.3	0.02	2.00	1.64	0.63	0.18
Thr	277	272	62.9	0.86	28.6	46.8	30.0	0.69	51.6	48.6	24.2	0.77	2.19	2.32	0.84	0.71
Trp	39.2	36.7	4.87	0.24	7.85	16.6 [†]	11.2	0.08	3.57	2.49	1.91	0.19	3.53	3.88	1.01	0.42
Val	442	463	45.0	0.26	152	118	65.0	0.44	64.8	72.1	29.0	0.55	3.51	2.14	1.63	0.06

¹See table 2 for footnote

[†] C_δ different from 0 ($P < 0.05$)

5. Discussion

The nutritional studies that assess the consequences of a dietary AA balance usually formulate the experimental diets by adding free AA to balance the AA profile of protein sources. However, providing AA both as proteins and in free form in the diet can result in a transient AA imbalance caused by differences in the time of arrival of AA in the plasma (Batterham and Bayley 1989; Yen et al. 2004). This is because proteins first need to be digested while free AA are readily absorbable and appear quickly in the plasma after feeding (Batterham and Bayley 1989). To overcome the confounding effect of digestion kinetics, EHF was used here as a model source of rapidly absorbable AA with an unbalanced AA profile. Eighty-two percent of the AA in EHF are in free form; the remainder are small peptides (Kersanté et al. 2021).

The postprandial kinetics of most AA followed a bell-shaped curve with an increase in plasma concentration during the first hour after meal ingestion, followed by a decrease. The decrease in plasma concentration coincided with the postprandial peak of insulin (Supplementary Figure 1A), an anabolic hormone known to stimulate protein synthesis and intracellular AA transport (Davis et al. 2002), which was observed 50 min postprandially. Furthermore, it also coincided with the postprandial increase in plasma urea concentration (Supplementary Figure 1C) that resulted from the hepatic deamination of AA (Bos et al. 2003). The postprandial plasma AA kinetics were described and summarized in a limited number of parameters with biological meaning. The model was capable to summarize all the observed shapes of postprandial plasma AA kinetics. It is based on the idea that plasma concentrations are controlled intrinsically by homeostasis through target concentrations during fasting (C_0) and after feeding ($C_0 + C_\delta$). However, the concept of AA homeostasis remains a simplification of biological reality, and plasma AA concentrations are not constant over time, even after prolonged fasting (Bertolo et al. 2000). Plasma AA concentrations are not necessarily controlled intrinsically, but may be the result of changes in influx (i.e., the appearance of dietary AA after the first-pass metabolism of

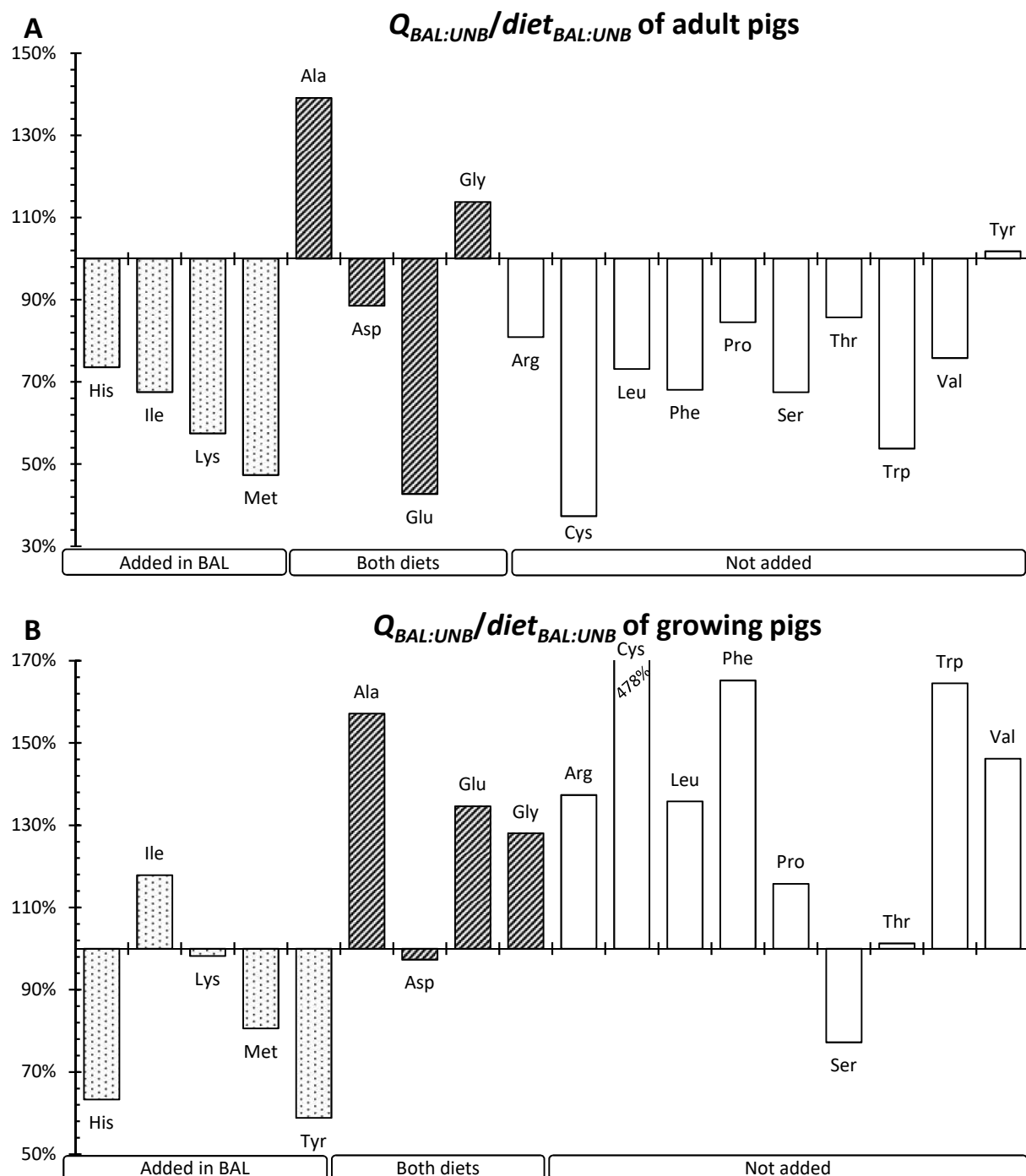
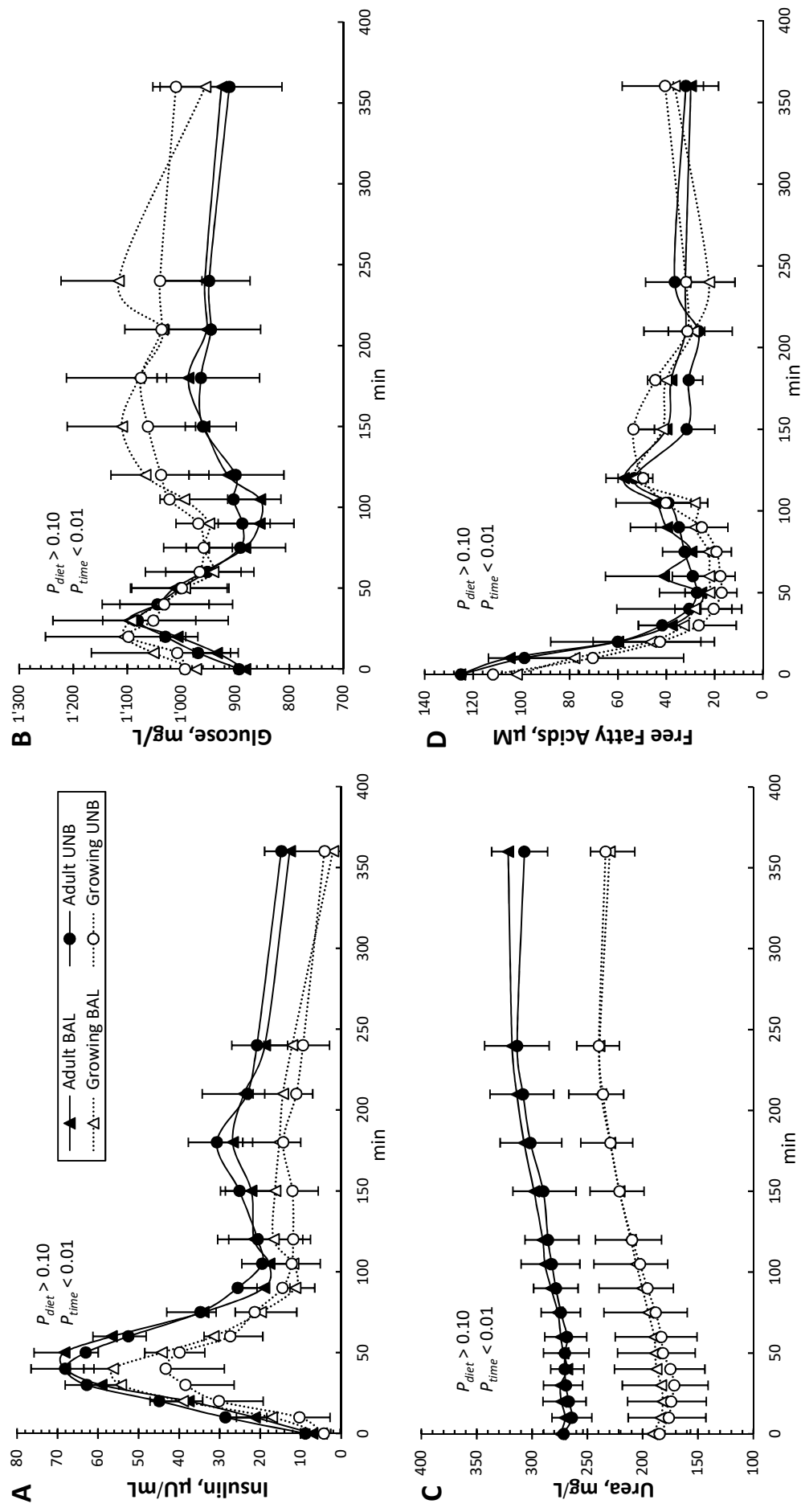


Figure 12. Difference between diets in the apparent quantity of amino acids arriving in the plasma after ingestion of the meal ($Q_{BAL:UNB}$; μM) relative to the difference in diet supply ($diet_{BAL:UNB}$) in adult Yucatan mini-pigs (A; $n = 12$) and growing pigs (B; $n = 12$).

the intestine and the liver, *de novo* synthesis, body protein breakdown) and outflux (i.e., for protein synthesis, AA catabolism), both of which may be controlled independently (van Milgen et al. 2022). However, opposing mechanisms may result in the same change in plasma concentration. For example, either an increase in AA influx or a decrease in outflux would result in the same plasma response (i.e., an increase in AA concentration). Although changes in plasma AA concentrations should not be interpreted directly as changes in AA fluxes, certain mechanisms are more plausible than others (van Milgen et al. 2022). For example, the postprandial increase in plasma concentration is likely initiated by the increase in the influx of AA from the ingestion of a meal, and not to a decrease in AA outflux.

In the model, the parameter C_0 corresponds to the target AA concentration that the animal tries to maintain during fasting. However, it can also be interpreted as the initial plasma concentration and the estimated C_0 were close to the measured AA concentrations before the test meal, and were in line with those reported for adult and growing pigs (Mohamed et al. 2019; Campos et al. 2019). As anticipated, C_0 was not affected by diet. It was assumed that the target concentration may be affected by meal intake but, for most AA, C_δ did not differ from zero indicating that postprandial AA concentrations generally went back to initial concentrations. However, there were cases where there was a change in the target AA concentration. For example, the C_δ for Lys was negative when feeding UNB and this can be interpreted as the result of an enduring demand for Lys for protein synthesis while the dietary Lys supply was limiting.

The *AUC* can be interpreted as the metabolic exposure of the animal to a dietary AA as it appears in the plasma after absorption, uptake, and metabolism by the intestine and liver (influx) until it disappears either due to its incorporation into protein or after being catabolised (outflux). The *AUC* of AA that were only added in BAL as free AA (i.e., His, Ile, Lys, Met, and Tyr) were significantly higher in BAL compared to UNB. For AA that were supplied in equal



Supplementary Figure 1. Kinetics of postprandial plasma concentration (mean \pm SD) of insulin (A), glucose (B), urea (C), and free fatty acids (D) of adult (solid lines; $n = 4$) and growing (dotted lines; $n = 4$) pigs for 360 min after feeding a diet with an extremely hydrolyzed feather (EHF) as a sole protein source with either a balanced (BAL, triangle) or unbalanced (UNB, circle) amino acid profile.

amounts in both diets (i.e., Ala, Asp, Glu, and Gly), the *AUC* was lower for BAL, probably indicating a higher utilization of these AA for protein synthesis. The *Q* is the apparent quantity of AA arriving in the plasma after first-pass metabolism by the intestine and the liver (van Milgen et al. 2022). It is an apparent quantity because the blood flow is unknown and *Q* can therefore not be compared directly with the dietary intake. However, since all pigs were fed with the same amount of feed during the experiment, it can be assumed that the blood flow was similar for both diets (Vaugelade et al. 2000; Val-Laillet et al. 2011). Thus, the $Q_{BAL:UNB}/diet_{BAL:UNB}$ reflects the extent to which a difference in the amount of AA supplied by BAL over UNB resulted in an equivalent difference in *Q*. For both adult and growing pigs, the values of $Q_{BAL:UNB}/diet_{BAL:UNB}$ of Ala and Gly were above 100%. This is consistent with the synthesis of these AA by the intestine and liver (Fujita and Yanaga 2007; Sarabhai and Roden 2019). Contrary to UNB, His, Ile, Lys and Met (and Tyr for growing pigs) were not limiting in BAL. The $Q_{BAL:UNB}/diet_{BAL:UNB}$ was lower than 100% for most of these AA, suggesting that more of these AA were metabolized in the first-pass by the intestine and the liver.

In growing pigs, the $Q_{BAL:UNB}/diet_{BAL:UNB}$ was greater than 100% for all non-limiting AA except for Asp and Ser, suggesting that the first-pass metabolism in the intestine and liver of most AA was lower for BAL than for UNB. Conversely, in adult pigs, $Q_{BAL:UNB}/diet_{BAL:UNB}$ was less than 100% for most non-limiting AA except for Ala, Gly, and Tyr. This suggests that the first-pass metabolism of the intestine and liver plays a relatively important role in AA homeostasis in adult pigs, because differences in the dietary AA supply between BAL and UNB result in smaller differences in plasma concentrations. The difference in the fate of the dietary AA between adult and growing pigs may explain their contrasting responses. In adult pigs, dietary AA are mainly used for renewal of body proteins and catabolism whereas in growing pigs, AA are mainly used for muscle protein synthesis and accretion (Moehn et al. 2004; Park 2006). This is consistent with the lower *AUC* of growing pigs than that of adult pigs, especially for

UNB. The utilisation of these AA for muscle protein synthesis is a plausible explanation, as growing pigs have higher AA requirements for protein deposition compared to adult pigs. Moreover, the stark contrast in AA utilization between adult and growing pigs is also seen in the postprandial plasma urea concentration (supplementary Figure 1A), that was lower for growing pigs indicating they were catabolising less AA in the liver than adult pigs (Bröer and Bröer 2017). In growing pigs, Phe was provided in approximately the same amount in both diets, whereas Tyr was provided in higher amounts in BAL. The $Q_{BAL:UNB}/diet_{BAL:UNB}$ was lower than 100% for Tyr and greater than 100% for Phe. This suggests that growing pigs fed UNB diet catabolized Phe in the liver to synthesize Tyr. In adult pigs, Tyr and Phe were provided in equal amounts in both diets. The $Q_{BAL:UNB}/diet_{BAL:UNB}$ was roughly equal to 100% for Tyr, but less than 100% for Phe, suggesting that Phe was metabolized more in the first-pass in adult pigs fed diet BAL than those fed diet UNB.

6. Conclusion

Balancing the AA profile of diets by adding free AA affected the postprandial plasma AA concentrations of almost all AA. The first-pass metabolism of AA in the intestine and liver seemed to contribute to control plasma AA homeostasis in response to dietary AA balance. Adult pigs utilise more dietary AA in the first-pass than growing pigs, but growing pigs have higher utilisation of plasma AA, most likely for muscle protein synthesis. For adult and growing pigs, balancing the AA profile of EHF resulted in higher and faster overall utilization of postprandial plasma AA for protein synthesis.

7. Declarations

Funding

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Conflicts of interest/Competing interests

The authors have no relevant financial or non-financial interests to disclose

Availability of data and material

Data is available at: <https://doi.org/10.15454/XFN7Z7>

Authors' contributions

FA Eugenio: Writing – original draft preparation.

N Le Floch, J van Milgen, J Duperray, and R Sergheraert – Conceptualization, formal analysis and investigation, methodology, project administration, supervision, resources, funding acquisition, writing – review & editing

Ethics approval

The experiment followed the ARRIVE animal experimentation guidelines and the EU directive 2010/63/EU for animal experiments. The protocol was approved by the regional ethical committee and the experiment was granted authorisation (APAFiS # 2019041610073508) by the Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation.

EXPERIMENT 2A (Exp. 2A)

Feeding pigs amino acids as protein-bound or in free form influences postprandial concentrations of amino acids, metabolites, and insulin[†]

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1. Abstract

Dietary proteins need to be digested first while free amino acids (AA) and small peptides are readily available for absorption and rapidly appear in the blood. The rapid postprandial appearance of dietary AA in the systemic circulation may result in inefficient AA utilisation for protein synthesis of peripheral tissues if other nutrients are not available at the same time. The objective of this experiment was to compare the postprandial concentrations of plasma AA and other metabolites after the ingestion of a diet that provided AA either as proteins or as free AA and small peptides. Twenty-four male growing pigs (38.8 ± 2.67 kg) fitted with a jugular catheter were assigned to one of three diets that provided AA either in protein form (INT), free AA and small peptides (HYD), or as free AA (FAA). After an overnight fast and initial blood sampling, a small meal was given to each pig followed by serial blood collection for 360 min. Postprandial concentrations of plasma AA, glucose, insulin, and urea were then measured from the collected blood. Non-linear regression was used to summarize the postprandial plasma AA kinetics. Fasting concentrations of urea and other AA were higher ($P < 0.05$) while postprandial plasma insulin and glucose were lower ($P < 0.01$) for INT than for HYD and FAA. The area under the curve was lower for INT for most AA ($P < 0.05$), resulting in a flatter curve compared to HYD and FAA. This was the result of the slower appearance of dietary AA in the plasma when proteins are fed instead of free AA and small peptides. The flatter curve may also result from more AA were being metabolised by the intestine and liver when INT was fed. The metabolism of AA of the intestine and liver was higher for HYD than FAA. Providing AA as proteins or as free AA and small peptides affected the postprandial plasma kinetics of AA, urea, insulin, and glucose. Whether the flat kinetics when feeding proteins has a positive or negative effect on AA metabolism still needs to be explored.

Keywords: protein-bound amino acids, free amino acids, metabolism, pig, plasma metabolites

2. Introduction

Feeding livestock requires the use of limited resources like land, water, and energy that contribute to the pressure on the environment. Using free, non-bound amino acids (AA) in animal diets instead of traditional protein sources like soybean meal is a way to address increasing concerns regarding the negative environmental impact of animal husbandry (Garcia-Launay et al. 2014). Apart from the reduction of the dependency on protein sources that are resource-intensive, an alternative is to use by-products of animal production as they contributes to a circular economy and the lessening of wastes (Garcia-Launay et al. 2014). Furthermore, using free AA allows for formulating diets with a reduced crude protein content with an AA profile close to the requirement, an approach that reduces nitrogen excretion (Chrystal et al. 2020b). Even though providing low crude protein diets with supplemental free AA has been a well-established practice in animal nutrition (Gloaguen et al. 2014; Selle et al. 2020), some studies reported that feeding animals with diets with large amounts of free AA reduces the efficiency of AA utilization for growth (Batterham and Bayley 1989; Yen et al. 2004). Since free AA and small peptides can be absorbed directly in the small intestine without the need for digestion (Wu 1998; Krehbiel and Matthews 2003), they appear rapidly in the plasma. Differences in the rate of appearance between AA, other nutrients, or energy may cause a temporary imbalance at the sites of protein synthesis and result in increased AA catabolism compared with feeding AA as proteins (Bos et al. 2003; Yen et al. 2004).

The efficiency by which dietary AA can be used for protein synthesis depends on both the form and balance of dietary AA. In a previous experiment, the effect of the AA balance on their utilisation was studied (Eugenio et al., submitted). In this experiment, the focus is on the form by which AA are supplied, using feather proteins processed in varying degrees as a model protein source. Presently, feathers are mainly valorised in the animal feed sector as feather meal by simple physical processing through steam and pressure that leaves the protein intact but

makes it accessible to digestive enzymes. A more intensive process like extensive acid hydrolysis transforms the protein fraction of feathers into free AA and small peptides (Kersanté et al. 2021). In this study, we compared the postprandial plasma concentrations of AA, metabolites, and insulin in pigs fed either feather meal, extensively hydrolysed feathers composed of 84% of free AA and 16% small peptides, or a mix of free AA following the AA profile of the extensively hydrolysed feathers. We hypothesised that pigs fed a diet with proteins have lower postprandial plasma concentrations of AA and insulin compared to pigs fed a diet with the extensively hydrolysed feather or the mix of free AA.

Table 11. Composition and nutrient analysis (as fed) of the three experimental diets fed to growing pigs¹

	INT	HYD	FAA
Ingredients, %			
Limestone	0.90	0.90	0.90
Monocalcium phosphate	2.20	2.20	2.20
Vitamin and mineral premix ²	0.50	0.50	0.50
Refined iodized salt	0.60	0.60	0.60
Sugar	2.00	2.00	2.00
Extensively hydrolysed feathers		17.0	
Feather meal	17.0		
Vegetable oil	4.00	4.00	4.00
Corn starch	67.8	67.7	69.7
Synthetic fibre	5.00	5.00	5.00
L-Trp		0.10	0.10
Amino acid mix ³			15.0
Analysed content			
Moisture, %	10.3	11.1	9.66
Total nitrogen, %	2.58	2.28	2.10
Gross energy, MJ/kg	16.6	15.8	16.4
Total amino acids, g/kg	143	150	136
Amino acids, g/kg			
Ala	7.40	6.70	5.40
Arg	9.50	8.40	7.60
Asp	9.80	9.60	9.40
Cys	4.80	12.2	12.2
Glu	15.8	14.9	13.1
Gly	11.8	11.1	9.70
His	0.90	1.00	0.50
Ile	7.20	7.10	6.60
Leu	12.3	13.4	11.4
Val	11.7	11.4	10.3
Lys	2.60	2.50	3.10
Met	0.60	0.80	0.80
Phe	7.30	7.40	7.40
Pro	13.6	13.4	13.2
Ser	16.9	17.9	14.1
Thr	6.60	6.70	5.20
Tyr	0.90	0.70	0.80
Trp	3.50	5.10	4.90

¹Experimental diets were offered to the pigs as mash. INT – intact protein; HYD – extensively hydrolysed protein; FAA – free amino acids

²Provided per kg of complete diet: vitamin A, 1 000 000 IU; vitamin D3, 200 000 IU; vitamin E, 4 000 mg; vitamin B1, 400 mg; vitamin B2, 800 mg; calcium pantothenate, 2 170 mg; niacin, 3 000 mg; vitamin B12, 4 mg; vitamin B6, 200 mg; vitamin K3, 400 mg; folic acid, 200 mg; biotin, 40 mg; choline chloride, 100 000 mg; iron (sulfate), 11 200 mg; iron (carbonate), 4 800 mg; copper (sulfate), 2 000 mg; zinc (oxide), 20 000 mg; manganese (oxide), 8 000 mg; iodine (iodate), 40 mg; cobalt (carbonate), 20 mg; and selenium (selenite), 30 mg

³Amino acid mix consisted of L-Lys, DL-Met, L-Thr, L-Val, L-Arg, L-His, L-Ile, L-Leu, L-Glu, L-Tyr, L-Asp, L-Gly, L-Ala, L-Cys, L-Phe, L-Pro, and L-Ser

3. Materials and methods

3.1. Animal housing, diets, and experimental design

The experiment was carried out at UE3P (INRAE, Saint-Gilles, France <https://doi.org/10.15454/1.5573932732039927E12>). Thirty male Piétrain x Large-White x Landrace growing pigs were selected at 12 weeks of age (33.7 ± 4.1 kg). They were surgically fitted with a jugular catheter following the procedure of Eugenio et al. (submitted). Throughout the experiment, pigs were housed in individual pens (1.0 x 2.0 m) equipped with a nipple drinker and a feeding trough. The room was environmentally controlled with temperature maintained at 26 °C, relative humidity at 60%, and 12 h of light daily. After the surgery, pigs were allotted to one of three dietary treatments where AA were provided as feather meal as a model of proteins (**INT**), as extensively hydrolysed feathers providing AA as free AA and small peptides (**HYD**), or as purified free AA with the same AA profile as that of HYD (**FAA**). The ingredient and nutrient composition of the experimental diets are given in Table 11. Apart from the AA source, the ingredient composition of the three diets was almost identical. L-Trp was added in HYD (and also in FAA) as it is destroyed by the extensive hydrolysis process. All free AA were bought from Sigma Aldrich Chimie (St. Quentin Fallavier, France) except for L-Cys and L-Tyr, which were supplied by BCF Life Sciences (Pleucadeuc, France). Experimental diets were offered as mash. A daily feed allowance of around 1 200 g/day was distributed twice daily as meals (morning and afternoon). Throughout the experiment, the pigs were offered a mixture of the experimental diet (at 90%) and a regular, pelleted starter diet (at 10%) as the pigs did not eat well when fed exclusively with the experimental diet.

3.2. Meal tests and serial blood sampling

One week after catheter implantation, pigs were submitted to a meal test that consisted of giving each pig a small meal followed by serial blood collection. After an overnight fast, a first blood sample (4 - 5 mL) was collected. Then, a small meal (300 g) was given to ensure the pigs ate

all the offered experimental diet in less than 10 min. From 10 to 360 min after the meal, blood was collected at 10, 20, 30, 40, 50, 60, 75, 90, 105, 120, 150, 180, 210, 240, and 360 min. Blood was collected in tubes lined with heparin and immediately placed on an ice bed before being centrifuged (3 000 g for 10 min at 4 °C) to separate plasma. Plasma samples were then stored in freezers (-80 °C or -20 °C) until further analyses. The catheters were rinsed with sterile saline after each blood sampling. During the blood sampling procedure, pigs had no access to feed but had access to water. This procedure was done for 24 pigs (8 pigs per treatment). The other pigs were kept for the duration of the experiment to serve as a replacement in the event of health problems or malfunctioning of the catheters.

3.3. Feed analyses

The nitrogen concentration in the feed was analysed by combustion in an automated analyser (LECO FP-428 Nitrogen Determinator, LECO, St. Joseph, MI). For AA analysis, feed samples were hydrolysed in 6N HCl for 24 h at 110 °C with constant agitation to release AA. For Met and Cys analysis, oxidation with performic acid was done for 4 h before hydrolysis for 18 h. The Trp content was determined after alkaline hydrolysis with barium hydroxide for 16 h at 120 °C. The AA content was analysed after derivatization by AccQtag (AccQ-Fluor reagent kit, Waters, Guyancourt, France) using an HPLC system (Alliance System 2690, Waters, Guyancourt, France). The gross energy content was measured in an adiabatic bomb calorimeter (IKA, Staufen, Germany).

3.4. Plasma amino acids, metabolites, and insulin assays

The plasma concentrations of AA were analysed using a UPLC system (Waters acquity ultra performance liquid chromatography system, Waters, Guyancourt, France) with the MassTrak AAA column after derivatization of samples using the AccQtag Ultra method (Waters, Milford, MA, USA). Plasma glucose (Glucose (HK), Thermo Fisher Scientific, Vantaa, Finland) and urea (Urea kit, Thermo Fisher Scientific, Asnieres-Sur-Seine, France) were analysed with

commercial kits using colorimetric methods in an automatic analyser, Konelab 20i (Thermo fisher scientific, Courtaboeuf, France). Plasma concentrations of insulin were measured using a radioimmunoassay kit (Insulin-CT, CisBio Bioassays, Codolet, France) and an AIA-1800 device (Automated Immunoassay Analyzer; TOSOH Bioscience, Tokyo, Japan).

3.5. Calculations and statistical analysis

All statistical analyses were done using R version 3.6.2 (R Core Team 2019). The pig was the experimental unit. The average postprandial plasma concentrations of AA, glucose, urea, and insulin were analysed using a generalized additive mixed model (GAMM function) of the gam package of R (Hastie 2019). The model included time of blood sampling and diet as fixed effects and the pig as the random effect. Time was declared as a repeated measure and a smoothing variable. A difference was considered to be significant at $P < 0.05$ for all statistical analyses done.

Plasma AA concentrations were further analysed using non-linear regression with a modified compartmental Erlang function (van Milgen et al. 2022). The model assumes the existence of a target AA concentration that the animal tries to maintain. This target concentration may be affected by the ingestion of the test meal, resulting in a model with either three or four parameters (van Milgen et al. 2022). The 3-parameter model was used to describe postprandial plasma kinetics when the initial and final concentrations were similar while the 4-parameter model was used when these concentrations differed. Depending on the AA, the models used in the experiment were:

$$\textbf{3-parameters: } C_{(t)} = C_0 + (1/2 AUC \lambda^3 t^2 \exp(-\lambda t))$$

$$\textbf{4-parameters: } C_{(t)} = C_0 + (1/2 AUC \lambda^3 t^2 \exp(-\lambda t)) + (C_\delta(1 - \exp(-\lambda t)))$$

Where $C_{(t)}$ (μM) is the plasma AA concentration at the time of sampling (t , min), C_0 (μM) the target AA concentration during fasting (i.e., at $t = 0$), C_δ (μM) the change in the target AA

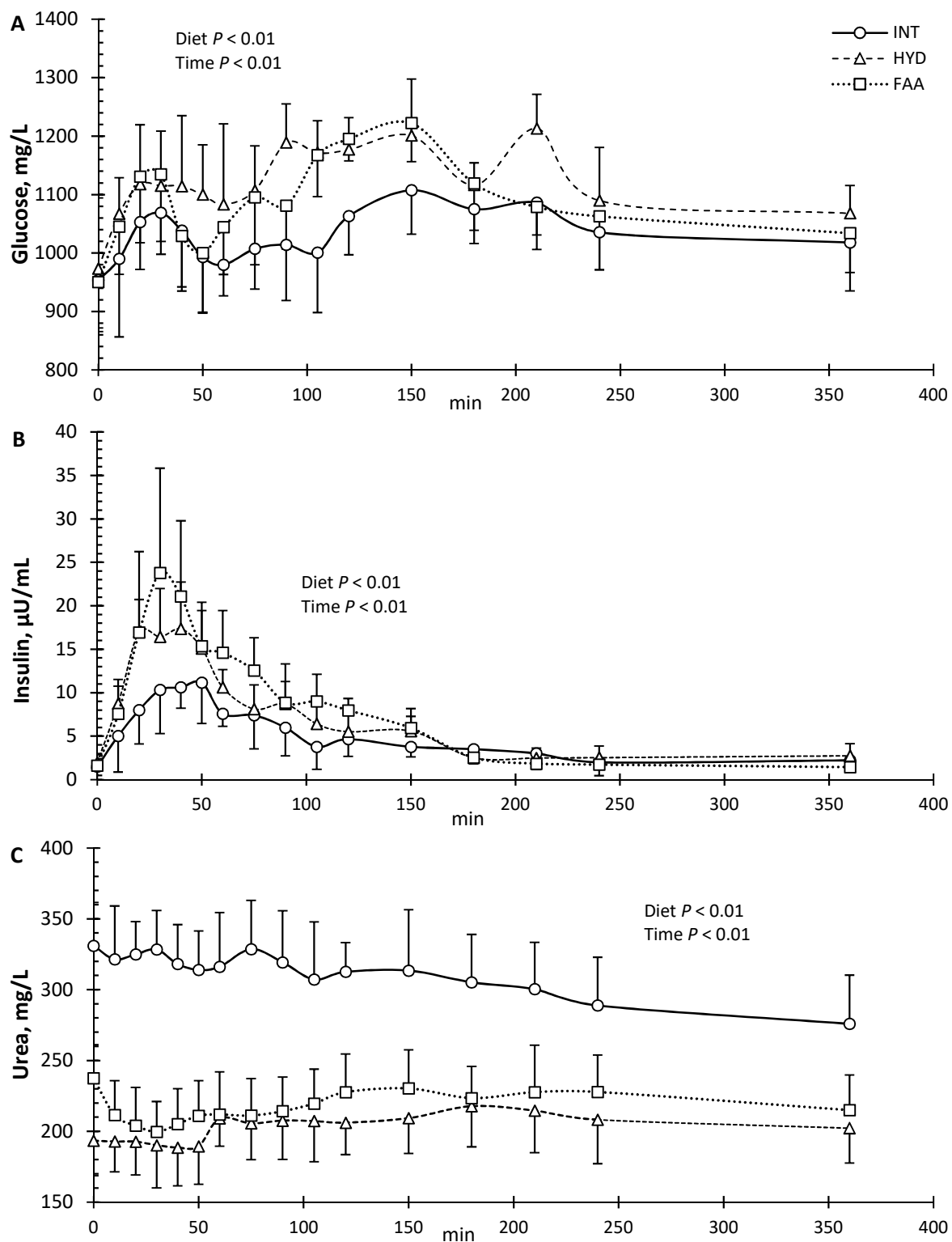


Figure 13. Postprandial plasma kinetics (mean \pm SD) of glucose (A), insulin (B), and urea (C), of pigs ($n = 24$) after feeding a diet whose amino acids originated from intact protein (INT), extensively hydrolysed protein (HYD), or free amino acids (FAA)

concentration relative to C_0 , AUC ($\mu\text{M}\cdot\text{min}$) the area under the curve over and above the target AA concentration, and λ (1/min) the rate of the postprandial change in AA concentration. For the 4-parameter model, λ is also the rate of change of the target AA concentration.

The choice of the model was done on a per AA basis to compare the values of parameters using the same model between diets. First, the individual plasma concentrations of the 20 proteinogenic AA were fitted using the 4-parameter model using the NLS2 function (nls2 package (Grothendieck 2013)) of R. Regardless of the diet, if C_δ differed from zero for at least half the pigs (≥ 12 pigs) for a particular AA, the 4-parameter model was used. In the other cases, the plasma concentrations were refitted using the 3-parameter model. The effect of the diet on each parameter was tested using the LMER function using the lmerTest package (Kuznetsova et al. 2017) with diet as a fixed effect and pig as a random effect.

The apparent appearance of dietary AA in the plasma after ingestion of the meal (Q ; μM) was calculated as $AUC \times \lambda$. The differences in Q between two experimental diets may be due to a difference in AA supply or to a difference in the first-pass metabolism of dietary AA by the intestine and the liver. This was assessed by computing for the ratio between the Q of two treatments and the ratio between the AA supplied by the two dietary treatments. This ratio estimates the extent to which dietary AA are metabolised differently in the first pass between two diets. This ratio was computed for INT over HYD ($Q_{\text{HYD:INT}}/\text{diet}_{\text{HYD:INT}}$), for INT over FAA ($Q_{\text{FAA:INT}}/\text{diet}_{\text{FAA:INT}}$), and for HYD over FAA ($Q_{\text{HYD:FAA}}/\text{diet}_{\text{HYD:FAA}}$), and expressed as percentages.

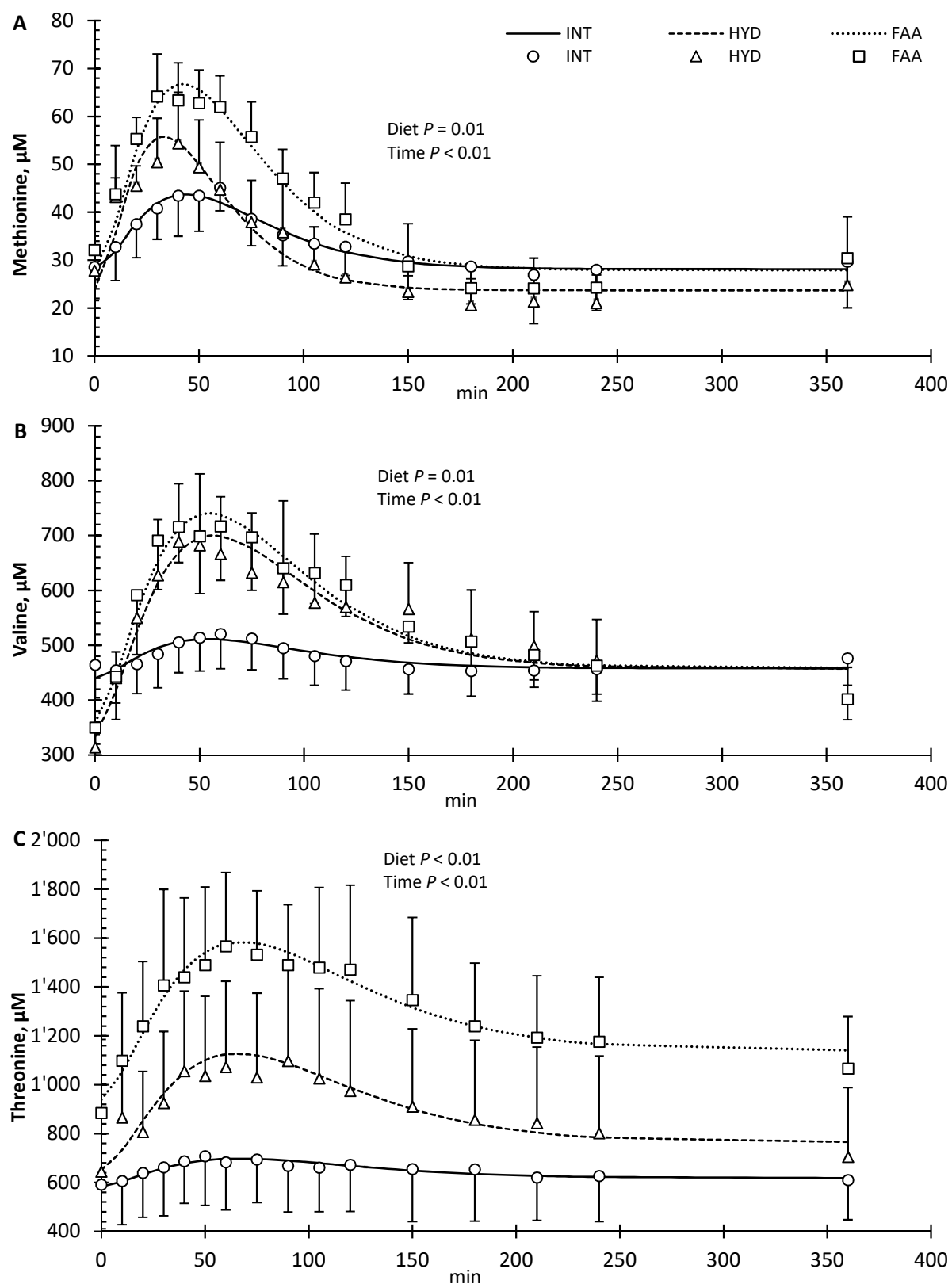


Figure 14. Observed (symbols; mean \pm SD) and estimated (lines) postprandial concentrations of plasma methionine (A) using the 3-parameter model and plasma valine (B) and threonine (C) using the 4-parameter model of pigs ($n = 24$) after feeding a diet whose amino acids originated from intact protein (INT), extensively hydrolysed protein (HYD), or free amino acids (FAA)

4. Results

4.1. General observations

Except for one pig that needed to be replaced, most of the pigs appeared healthy during the experiment. The AA profiles of the diets were similar to expected values. Although for FAA, some AA were somewhat lower than expected (i.e., Glu, Gly, Ser, and Thr).

4.2. Average postprandial plasma concentrations of metabolites and insulin

The average postprandial plasma concentrations of glucose, insulin, and urea are given in Figure 13. The postprandial plasma glucose concentration was lower while that of urea was higher for pigs fed INT compared to pigs fed HYD or FAA. The postprandial plasma insulin concentration was highest for FAA and lowest for INT. The concentration of urea after an overnight fast was higher for INT compared to the other diets.

4.3. Postprandial kinetics of plasma amino acids

The 4-parameter model was used for Arg, Cys, Gln, Glu, Ile, Leu, Val, Phe, Pro, Ser, Thr, and Tyr, which all had a positive C_δ . For the other proteinogenic AA, the 3-parameter model was used. The fitting of the 3-parameter model is illustrated in Figure 14A for the postprandial plasma concentrations of Met where the ingestion of the meal induced a bell-shaped curve and initial fasting concentration was similar to the final concentration. Figures 14B and 14C show the kinetics of Val and Thr and illustrate how feeding INT resulted in a relatively flat curve, whereas feeding HYD or FAA results in an asymmetrical bell-shaped curve where the final concentration was higher than the initial concentration (i.e., $C_\delta > 0$).

Tables 12 and 13 present the estimates for C_0 and C_δ , and for AUC and λ , respectively. Pigs fed INT had higher values of C_0 for Arg, His, Ile, and Val than the two other diets. Pigs fed HYD had the highest C_0 of Trp while those fed FAA had the highest C_0 for Cys and Thr. Pigs fed INT had generally lower estimates of C_δ , AUC , and λ . For C_δ , HYD had a higher estimate for Leu while FAA had the highest C_δ for Pro and Thr. The AUC of Trp was highest for FAA and

Table 12. Estimates of the initial concentration (C_0) and change in target concentration (C_δ) of the postprandial plasma amino acid kinetics of growing pigs fed a diet with amino acids originating from intact protein (INT), extensively hydrolysed protein (HYD), or free amino acids (FAA)¹

Amino acid	C_0 , μM					C_δ , μM				
	INT	HYD	FAA	RSD	P_{Diet}	INT	HYD	FAA	RSD	P_{Diet}
Ala	322	314	361	42.4	0.08
Arg	116 ^a	70.1 ^b	82.0 ^b	21.6	<0.01	46.6	44.0	39.4	27.0	0.86
Asn	46.4	47.6	50.8	7.03	0.46
Asp	5.24	5.26	5.20	1.04	0.99
Cys	26.3 ^{ab}	22.3 ^b	33.0 ^a	6.74	0.01	7.65 ^b	15.9 ^a	17.7 ^a	7.89	0.04
Gln	392	405	401	49.5	0.87	55.8	45.8	33.3	42.7	0.57
Glu	73.9	68.9	70.3	14.3	0.81	6.06	6.60	0.75	6.5	0.18
Gly	1,103	1,138	1,405	252	0.07
His	59.9 ^a	51.7 ^{ab}	42.7 ^b	12.4	0.04
Ile	118 ^a	87.9 ^b	98.2 ^{ab}	16.7	<0.01	15.5	30.0	15.1	17.2	0.17
Leu	149	122	137	22.2	0.07	10.9 ^b	55.2 ^a	40.0 ^{ab}	29.4	0.02
Val	440 ^a	334 ^b	359 ^b	49.4	<0.01	17.8 ^b	123 ^a	99.5 ^a	60.0	<0.01
Lys	55.9	48.4	45.3	22.7	0.64
Met	28.1	23.7	27.9	5.64	0.24
Phe	96.4	91.5	83.8	21.2	0.50	23.1	49.9	40.5	23.8	0.10
Pro	194	203	217	28.9	0.29	89.8 ^b	117 ^{ab}	173 ^a	61.0	0.04
Ser	161	180	178	63.4	0.81	51.5 ^b	128 ^a	134 ^a	62.2	0.03
Thr	584 ^b	652 ^{ab}	939 ^a	238	0.02	34.3 ^b	113 ^{ab}	200 ^a	86.3	<0.01
Trp	34.0 ^b	44.0 ^a	40.9 ^{ab}	5.88	<0.01
Tyr	59.4	68.8	61.9	15.8	0.48	17.6 ^b	50.5 ^a	64.5 ^a	21.4	<0.01

¹Parameter estimates (least square means) for the target concentration after fasting (C_0) and the change in target concentration due to ingestion of the meal (C_δ) of the modified Erlang function (see text for details) from 24 pigs fed either INT, HYD, or FAA; ^{a,b} within a row with different superscript letters differ ($P < 0.05$) from each other; RSD – residual standard deviation

lowest for INT. The λ of Ala, Asn, and Gln was highest for HYD and lowest for INT. The C_0 of Cys, Gly, and Thr was higher for pigs fed FAA than for those fed HYD. The AUC of Arg, Asn, Gly, His, Leu, Met, and Trp was higher for the pigs fed FAA, while the λ of Met was higher for pigs fed HYD. The $Q_{HYD:INT}/diet_{HYD:INT}$ and $Q_{FAA:INT}/diet_{FAA:INT}$ were all above 100% for all AA (Figure 15). Also, the $Q_{HYD:FAA}/diet_{HYD:FAA}$ was below 100% for all AA, except for Cys (Figure 16).

Table 13. Estimate of the area under the curve (*AUC*) and the fractional flow rate (λ) of the postprandial plasma amino acid kinetics of growing pigs fed a diet with amino acids originating from intact protein (INT), extensively hydrolysed protein (HYD), or free amino acids (FAA)¹

Amino acid	<i>AUC</i> , $\mu\text{M}\cdot\text{min} \times 10^2$					λ , $1/\text{min} \times 10^{-2}$				
	INT	HYD	FAA	RSD	P_{Diet}	INT	HYD	FAA	RSD	P_{Diet}
Ala	220	285	364	112	0.06	2.53 ^b	3.33 ^a	2.93 ^{ab}	0.58	0.04
Arg	61.1 ^b	157 ^a	227 ^a	55.7	<0.01	3.21	4.56	3.82	1.29	0.14
Asn	18.1 ^b	19.1 ^b	31.3 ^a	6.67	<0.01	3.05 ^b	5.23 ^a	4.31 ^{ab}	1.39	0.02
Asp	0.63 ^b	5.81 ^a	4.94 ^a	2.71	<0.01	9.41	3.28	4.23	4.89	0.06
Cys	13.1 ^b	105 ^a	35.9 ^{ab}	67.6	0.04	5.43	2.75	4.30	2.43	0.11
Gln	95.4	107	146	94.9	0.55	2.60 ^b	4.28 ^a	3.77 ^{ab}	1.23	0.04
Glu	12.3	32.5	29.8	17.3	0.08	7.46	4.04	4.62	3.36	0.15
Gly	943	772	1,306	459	0.09	0.92 ^b	2.87 ^a	2.19 ^a	1.04	0.01
His	16.3 ^b	23.4 ^{ab}	32.2 ^a	7.55	<0.01	3.15	4.57	4.12	1.17	0.07
Ile	18.4 ^b	110 ^a	136 ^a	29.3	<0.01	4.12	5.02	4.62	0.94	0.19
Leu	28.1 ^b	175 ^a	233 ^a	49.0	<0.01	4.51	5.09	4.48	1.06	0.45
Val	53.2 ^b	254 ^a	285 ^a	72.5	<0.01	3.93	4.11	3.96	0.83	0.90
Lys	72.8 ^b	77.3 ^b	115 ^a	25.8	<0.01	3.36 ^b	4.85 ^a	4.48 ^a	0.60	<0.01
Met	12.6 ^b	19.5 ^b	30.2 ^a	5.69	<0.01	4.65	6.65	4.78	1.70	0.05
Phe	27.4 ^b	111 ^a	151 ^a	40.7	<0.01	3.19	4.18	3.42	1.12	0.21
Pro	112 ^b	512 ^a	628 ^a	177	<0.01	2.24 ^b	2.85 ^a	2.86 ^a	0.50	0.03
Ser	21.8 ^b	413 ^a	455 ^a	210	<0.01	3.11	3.00	2.83	0.79	0.78
Thr	98.7 ^b	441 ^a	558 ^a	167	<0.01	3.34	3.51	3.16	0.96	0.77
Trp	9.86 ^c	18.8 ^b	25.9 ^a	4.96	<0.01	4.46	4.73	4.39	0.72	0.62
Tyr	14.8 ^b	72.0 ^a	86.3 ^a	27.9	<0.01	3.65	3.89	3.48	0.99	0.71

¹Parameter estimates (least square means) for the area under the curve (*AUC*), and the fractional flow rate (λ) of the modified Erlang function (see text for details) from 24 pigs fed either INT, HYD, or FAA; ^{a,b} within a row with different superscript letters differ ($P < 0.05$) from each other; RSD – residual standard deviation

5. Discussion

The objective of this study was to assess the form of the dietary AA supply on the postprandial plasma concentrations of AA, urea, glucose, and insulin in growing pigs. To gain insight in the interpretation of the postprandial plasma AA kinetics, a model was used that described the changes in plasma concentration over time using three or four parameters with biological meaning (van Milgen et al. 2022). It was hypothesised that the appearance of AA from proteins in the plasma was more gradual because of the need of proteins to be digested, while free AA and small peptides are quickly absorbed and readily available for absorption (Wu 1998; Krehbiel and Matthews 2003; Bos et al. 2003). To ensure that pigs ate the experimental diets, a small amount of standard starter diet was mixed with the experimental diets and this was the same for all diets (10%). Nevertheless, 85% of the AA intake was provided by the experimental diets and most of the observed responses would be due to the experimental diets. The measured plasma concentrations of AA as well as the other metabolites and insulin were in accordance with other experiments conducted in growing pigs (Bertolo et al. 2000; Zem Fraga et al. 2021). Despite the fact that the three diets were formulated to provide the same amount of each AA, we observed some deviations between the expected and analysed AA contents. The concentration of Cys was lower in INT compared to the other two diets, which had more than double the amount of this AA. This can be explained by the processing involved in the production of feather meal that converted some Cys to lanthionine (Papadopoulos et al. 1986). The concentration of Trp was also lower for INT as feather meal is low in this AA. L-Trp was not added in diet INT as this would have meant providing it in free form, which could impact some of the measured traits, particularly the *AUC*. The *AUC* can be interpreted as the metabolic exposure of the animal induced by the appearance of dietary AA in the plasma after first-pass metabolism by the small intestine and the liver and clearance of AA due to metabolism by peripheral tissues like the skeletal muscle. Metabolism includes the incorporation of AA into

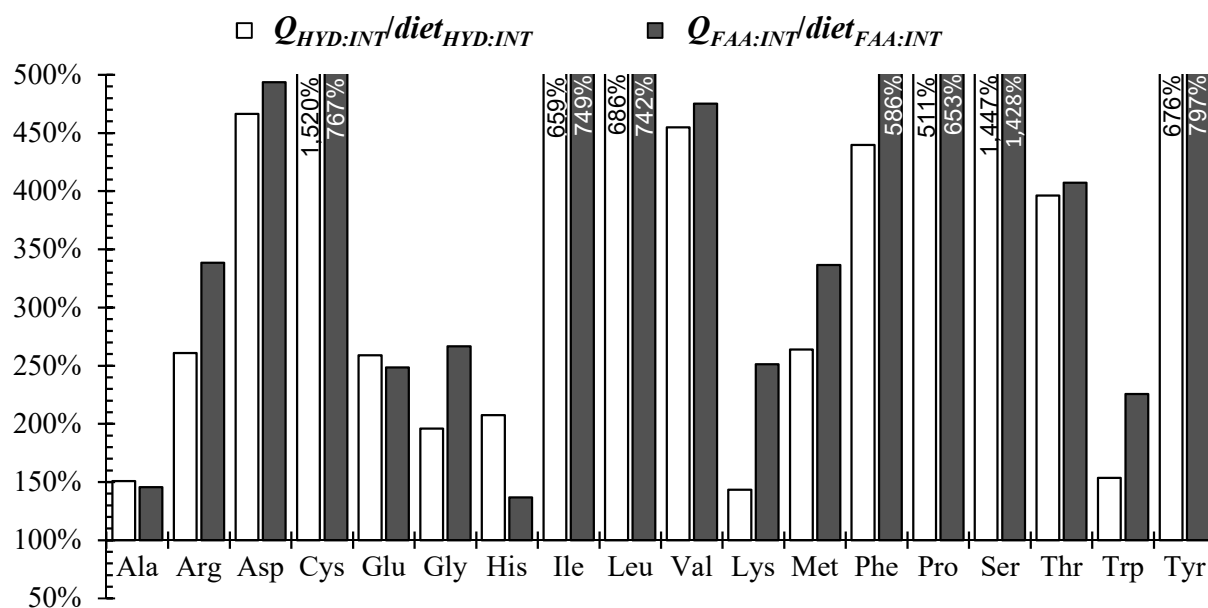


Figure 15. The change in the apparent quantity of amino acids arriving in the plasma after feeding over the change in the dietary amino acid supply of diet INT over either diet HYD ($Q_{HYD:INT}/diet_{HYD:INT}$) or diet FAA ($Q_{FAA:INT}/diet_{FAA:INT}$)

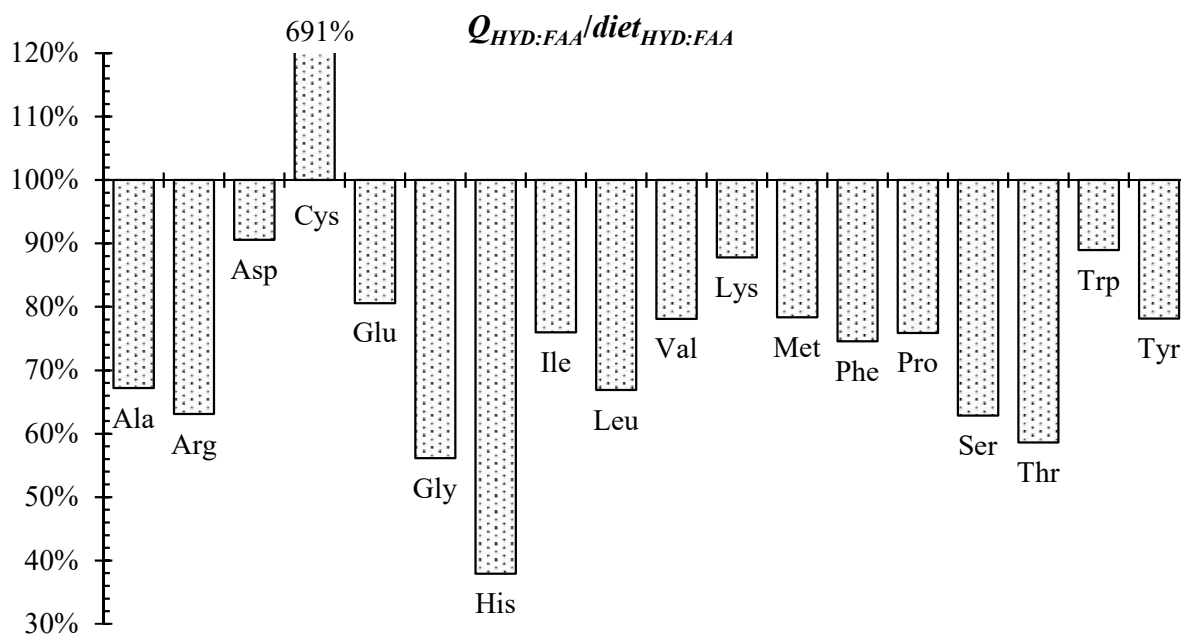


Figure 16. The change in the apparent quantity of amino acid arriving in the plasma after feeding over the change in the dietary amino acid supply of diet HYD over diet FAA ($Q_{HYD:FAA}/diet_{HYD:FAA}$)

proteins, AA catabolism, or their conversion to other metabolites. The lower *AUC* of Cys and Trp for INT fed pigs than the other treatments probably resulted from a lower Cys and Trp intake.

Feeding proteins induced a modest and gradual increase in plasma AA concentrations that resulted in a low *AUC* compared to pigs fed HYD and FAA. These observations are consistent with the low digestibility of AA from feather meal (Grazziotin et al. 2006; Sulabo et al. 2013). Other studies found that the appearance of dietary AA in the plasma is less and slower for proteins than for free AA (Koopman et al. 2009). The parameter λ characterises the change in AA concentrations and a low value of λ results in a more gradual increase and decrease of AA concentration. The λ of INT was lower than those of HYD and FAA. Apart from the low digestibility of INT, the rate of AA appearance in the plasma may be influenced by the first-pass metabolism of AA in the intestine and the liver. The ratio of the average Q and dietary supply between two diets is indicative for the difference in first-pass AA metabolism in the intestine and liver between two diets. The observation that $Q_{HYD:INT}/diet_{HYD:INT}$ and $Q_{FAA:INT}/diet_{FAA:INT}$ was higher than 130% (see Figure 15) indicates that more AA were used in the first-pass by the intestine and liver when feeding diet INT compared to diets HYD or FAA, which agrees with results of others (Metges et al. 2000; Daenzer et al. 2001; Wu 2009). The rapid absorption of AA from HYD and FAA seemed to be associated with a lower intestinal and hepatic metabolism in the first-pass. This observation may explain the higher urea concentrations measured in the fed state in pigs fed INT compared to those fed FAA and HYD. The concentration of urea in the plasma is an indicator of AA catabolism occurring mainly in the liver (Brown and Cline 1974). In the fed state, it reflects the catabolism of dietary AA whereas in the fasted state, it reflects the catabolism of AA released by protein breakdown. Accordingly, pigs fed proteins may be mobilising more AA during an overnight fast, which also explains the higher C_0 for most AA in INT. Hence, after an overnight fast, pigs fed INT

seemed to be in a more catabolic state than pigs fed FAA and HYD, similar to the findings of other studies (Kampman-van de Hoek et al. 2016).

Feeding HYD and FAA induced higher concentrations and a higher peak of plasma insulin compared to INT. Insulin is the main anabolic hormone that induces muscle protein synthesis (Davis et al. 2002; O'Connor et al. 2003). The insulin concentration only marginally increased after feeding INT. The post-prandial change in AA concentration was more prominent (i.e., larger *AUC* and a higher peak concentration) for most AA of HYD and FAA than for INT and this may be attributed to differences in absorption rates rather than to rates of AA utilization for protein synthesis. It is difficult to assert how the shape (i.e., flat or bell-shaped) or rate of appearance in the plasma (i.e., slow or rapid) is associated with the rate of protein synthesis. However, AA are not only constituents of body proteins, but also act as anabolic signals (Groen et al. 2015). A more marked postprandial AA concentration may therefore result in a higher signal for protein synthesis. However, this was not confirmed in broilers where no difference in the net protein synthesis after feeding proteins and free AA was observed (Chrystal et al. 2020b). Otherwise, the metabolic signals of feeding proteins and free AA start not only as AA appear in the plasma, but also in the stomach and the intestine. The tertiary and quaternary structures of the protein may be detected by the intestinal tissues, which may affect the post-absorptive metabolism. However, there is limited information about this in the literature (Jahan-Mihan et al. 2011; Piumetti 2022).

Even though feeding HYD and FAA induced very similar postprandial responses, there were some noticeable differences. Feeding FAA generally resulted in a greater *AUC* compared to feeding HYD. This was even more apparent in the $Q_{HYD:FAA}/diet_{HYD:FAA}$ ratio, which was lower than 100% for most AA (see Figure 16). An explanation might be because of the small peptides present in HYD, which may have been more metabolized in the intestine and liver compared to free AA (Hara et al. 1984; Ten Have et al. 2007). Actually, peptides are not transported out of

the intestinal cells and they first need to be hydrolysed into their constituent AA before being transported to the peripheral tissues (Krehbiel and Matthews 2003).

6. Conclusion

Providing AA in protein form or as free AA and small peptides affects the postprandial kinetics of AA, urea, insulin, and glucose. Feeding INT results in a relatively flat plasma kinetics and lower plasma concentrations of AA compared to feeding HYD and FAA. This means that the form of AA in the diet of pigs has an influence in their bioavailability, but it remains unclear if the response of feeding proteins is to be interpreted positively (lower metabolic exposure and higher AA utilization for protein synthesis) or negatively (higher urea concentrations, lower insulin concentrations).

7. Declarations

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Conflicts of interest/Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Authors' contributions

FA Eugenio: Writing – original draft preparation.

N Le Floc'h, J van Milgen, J Duperray, and R Sergheraert – Conceptualization, formal analysis and investigation, methodology, project administration, supervision, resources, funding acquisition, writing – review & editing.

Ethics approval

The experiment followed the ARRIVE animal experimentation guidelines and the EU directive 2010/63/EU for animal experiments. The protocol was approved by the regional ethical committee and the experiment was granted authorisation (APAFiS # 27196-2020091509061764 v2) by the Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation.

EXPERIMENT 2B (Exp. 2B)

Expression of intestinal amino acid transporters, gut morphology, and amino acid digestibility in pigs fed proteins or free amino acids¹

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1. Abstract

Dietary amino acids (AA) supplied as protein or in free form are digested and absorbed at different rates, but can also induce differences in the intestinal physiology of pigs. We compared the AA digestibility, gene expression of AA transporters, and intestinal morphology of pigs fed diets providing different forms of AA. Thirty growing pigs (33.7 ± 4.1 kg) were fed one of three experimental diets that provided AA as protein from feather meal (INT), as free AA and small peptides obtained by extensive hydrolysis of feathers (HYD), or as a mix of individual purified AA with the same AA profile as HYD (FAA). Pigs were fed the same quantity of feed, energy, and AA. After 14 days, pigs were slaughtered 3 h after feeding a meal with indigestible markers. Digesta and tissue were collected from different sections of the small intestine. Jejunal digesta was used to measure AA digestibility. Samples of the duodenum, jejunum, and ileum were analyzed for the gene expression of intestinal AA transporters and intestinal morphology. The AA digestibility of INT was lower compared to HYD and FAA ($P < 0.05$). Digestibility of Cys, Gly, His, Met, and Pro was lower for FAA compared to HYD ($P < 0.05$). This may be due to the small peptides in HYD, which are absorbed faster than individual AA. The villi area in the ileum of HYD fed pigs was the highest ($P < 0.05$) among the treatments, which may be associated with the reabsorption of endogenous proteins, which occurs mostly in the ileum. In the duodenum, INT expressed more PepT1 ($P < 0.01$) probably due to the rapid transit time of digesta when HYD and FAA are fed. Pigs fed HYD expressed more ASCT2 ($P = 0.02$) and CAT-1 ($P = 0.04$) in the jejunum compared to the pigs fed the other diets. The expression of these transporters along the intestine depended on the relative abundance of dietary absorbable AA. The results showed that the dietary AA form can have an influence on the morphology and on the expression of different AA transporters along the different sections of the small intestine.

Keywords: amino acid transporter, digestibility, free amino acid, intact protein, intestinal morphology, pig

2. Introduction

In pig feeds, amino acids (AA) are mostly supplied as proteins principally from protein sources like soybean meal and cereals. Purified free AA are also added to reduce the crude protein content of the diet while ensuring that the AA supply meets the animal's requirement. Also, alternative protein sources including by-products of animal production are becoming increasingly available (Holkar et al., 2018). These alternative protein sources require technological processes to be sanitized and to improve digestibility. Feathers are typically pressured cooked to produce feather meal whose AA are still protein-bound (Papadopoulos et al., 1986), while extensive acid hydrolysis transforms the protein into free AA and small peptides (Hou et al., 2017).

The dynamics of protein digestion can affect the availability of AA for the animal and subsequently their metabolism. Pre-hydrolyzed dietary proteins have a higher digestibility than proteins (Vente-Spreuwenberg et al., 2004; Grazziotin et al., 2006) and free AA and small peptides do not need to be digested and can be rapidly absorbed after feeding (Weijzen et al., 2022). Both the reduction and the lack of a need for digestion induce differences in the digestive and absorptive capacities of the small intestine (Dai et al., 2013; Morales et al., 2017). These differences can be seen through changes in the morphology and the expression of different AA and peptide transporters in the intestine of pigs (Ma et al., 2019).

The objective of this experiment was to compare the intestinal morphology and gene expression of AA transporters in the three sections of the small intestine and the jejunal AA digestibility of pigs after feeding a diet that supplies AA either as proteins or as free AA and/or small peptides from an extensively hydrolyzed protein or as purified AA. We hypothesized that the higher availability of absorbable AA in the proximal part of the small intestine would result in an increased surface area of absorption and increased expression of AA transporter genes in the proximal part compared to the latter part of the small intestine.

Table 14. Composition and nutrient analysis (as fed) of the three experimental diets fed to growing pigs¹

Item	INT	HYD	FAA
Ingredients, %			
Limestone	0.90	0.90	0.90
Monocalcium phosphate	2.20	2.20	2.20
Vitamin and mineral premix ²	0.50	0.50	0.50
Refined iodized salt	0.60	0.60	0.60
Sugar	2.00	2.00	2.00
Extensively hydrolysed feather		17.0	
Feather meal	17.0		
Vegetable oil	4.00	4.00	4.00
Corn starch	67.8	67.7	69.7
Synthetic fibre	5.00	5.00	5.00
L-Trp		0.10	0.10
Amino acid mix ³			15.0
Analysed content			
Moisture, %	10.3	11.1	9.66
Total nitrogen, %	2.58	2.28	2.10
Gross energy, MJ/kg	16.6	15.8	16.4
Total amino acids, g/kg	143	150	136
Amino acids, g/kg			
Ala	7.40	6.70	5.40
Arg	9.50	8.40	7.60
Asp	9.80	9.60	9.40
Cys	4.80	12.2	12.2
Glu	15.8	14.9	13.1
Gly	11.8	11.1	9.70
His	0.90	1.00	0.50
Ile	7.20	7.10	6.60
Leu	12.3	13.4	11.4
Val	11.7	11.4	10.3
Lys	2.60	2.50	3.10
Met	0.60	0.80	0.80
Phe	7.30	7.40	7.40
Pro	13.6	13.4	13.2
Ser	16.9	17.9	14.1
Thr	6.60	6.70	5.20
Tyr	0.90	0.70	0.80
Trp	3.50	5.10	4.90

¹Experimental diets were produced in dry form and were offered to the pigs as mash; INT – intact protein; HYD – hydrolysed protein; FAA – free amino acid

²Provided per kg of complete diet: vitamin A, 1 000 000 IU; vitamin D3, 200 000 IU; vitamin E, 4 000 mg; vitamin B1, 400 mg; vitamin B2, 800 mg; calcium pantothenate, 2 170 mg; niacin, 3 000 mg; vitamin B12, 4 mg; vitamin B6, 200 mg; vitamin K3, 400 mg; folic acid, 200 mg; biotin, 40 mg; choline chloride, 100 000 mg; iron (sulfate), 11 200 mg; iron (carbonate), 4 800 mg; copper (sulfate), 2 000 mg; zinc (oxide), 20 000 mg; manganese (oxide), 8 000 mg; iodine (iodate), 40 mg; cobalt (carbonate), 20 mg; and selenium (selenite), 30 mg

³Amino acid mix consists of L-Lys, DL-Met, L-Thr, L-Val, L-Arg, L-His, L-Ile, L-Leu, L-Glu, L-Tyr, L-Asp, L-Gly, L-Ala, L-Cys, L-Phe, L-Pro, and L-Ser

3. Materials and methods

3.1. Ethics approval for animal experimentation

The experiment followed the ARRIVE animal experimentation guidelines and the EU directive 2010/63/EU for animal experiments. The protocol was approved by the regional ethical committee and the experiment was granted authorisation (APAFiS #27196-2020091509061764 v2) by the Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation.

3.2. Animal housing, diets, and experimental design

Thirty male Piétrain x Large-White x Landrace growing pigs (33.7 ± 4.1 kg) were selected at 12 wk of age and were randomly assigned to one of three experimental diets during this 14 d experiment. The animals used in the experiment were all fitted with jugular catheters because they were also used in another experiment (Eugenio et al., submitted) involving a meal test followed by serial blood sampling. This procedure was done in the middle of this experiment (at 7 d). The experiment was done in the facilities of UE3P (INRAE, Saint-Gilles, France <https://doi.org/10.15454/1.5573932732039927E12>), where the pigs were housed in individual pens (1.2 x 2.0 m) equipped with a nipple drinker and a feeding trough in an environmentally controlled room (temperature: 26 °C; relative humidity: 60%; 12 h of light). The experimental diets provided AA either as protein from feather meal (**INT**), as free AA and small peptides from extensively hydrolyzed feathers (**HYD**), or as free AA with the same AA profile as HYD using purified AA (**FAA**). The ingredient and nutrient composition of the experimental diets are given in Table 14. Purified L-Trp (Sigma Aldrich Chimie, St. Quentin Fallavier, France) was also added in HYD and FAA as the extensively hydrolyzed feather was completely devoid of this essential AA. Apart from L-Cys and L-Tyr, which were provided by BCF Life Sciences (Pleucadeuc, France), all purified AA used to formulate FAA were obtained from Sigma Aldrich Chimie (St. Quentin Fallavier, France). Experimental diets were offered as mash. A daily feed allowance of 1,200 g was distributed twice daily as meals (morning and afternoon).

For each meal, the pigs were offered a mixture of the experimental diet (at 90%) and a regular, pelleted starter diet (at 10%) as there were palatability issues when exclusively feeding the experimental diets. The inclusion of the starter diet was marginal, and only a minor and similar impact on the measured responses for the three diets was expected. On the 14th day of the experiment, pigs were offered a small meal (300 g) of their respective experimental diets mixed with indigestible markers titanium dioxide (**TiO₂**) and cobalt-EDTA (**Co-EDTA**) that were each added at 4%.

3.3. Slaughter and tissue sample collection

Three hours after feeding the diet with indigestible markers, pigs were sedated with general anesthesia by injecting 10 mL per kg body weight of ketamine (Imalgene 1000, Merial, Lyon, France) then euthanized by injection of 5 mL per 50 kg body weight of a cocktail of embutramide, mebezonium, and tetracaine (T61, MSD Santé Animale, Beaucoz  , France) via the catheter followed by exsanguination. Immediately after opening the abdominal cavity, the stomach until the distal ileum was removed, and the cardiac, pyloric, and ileocecal sphincters were immediately clamped. The small intestine was divided in three parts corresponding to the duodenum, jejunum, and ileum. The duodenum was defined as the first 70 cm of the small intestine, the ileum as the last 70 cm (Al-Masri et al., 2015), and the jejunum as the segment in-between. The contents of each intestinal segment were collected, weighed, and frozen. The frozen intestinal contents were freeze-dried later, ground, and stored in a cold room for future analysis. For the tissue samples, the middle part of each segment was collected and rinsed with saline water and placed on a cold plate rinsed with RNase-away (Invitrogen Corp, Carlsbad, USA). This sample was then divided into two parts. One sample of around 3 to 4 cm was immediately placed in 10% neutral buffered formalin and kept in a cold room. The other sample was finely cut and around 2 g of tissue was collected in microtubes and immediately placed in liquid nitrogen before being stored at -80   C for future analysis.

3.4. Intestinal morphology

The tissue samples from the duodenum, jejunum, and ileum that were fixed in formalin were transferred to 70% ethanol in less than 48 h. Two small samples from each tissue sample were cut and embedded in paraffin. Small bands of the paraffin embedded tissue were finely cut (5 μ M) using a microtome (HistoCore Multicut, Leica Biosystems, Illinois, USA) and mounted on glass slides. The samples were then stained with hematoxylin and eosin and examined under a light microscope (Eclipse E400, Nikon, Champigny-sur-Marne, France) using an image analysis software (NIS-Elements AR 3.0; Nikon Instruments). The measurements on villi (height and area) and crypts (depth) were done on at least 20 villi and 20 crypts that were well oriented for each sample with x 40 magnification. From these measurements, the villi height to crypt depth ratio was calculated.

3.5. Gene expression of amino acid and peptide transporters

Approximately 70 mg of frozen (in liquid nitrogen) duodenum, jejunum, and ileum samples were pulverized (Precellys 24, Bertin Instruments, Montigny-le-Bretonneux, France) with the Trizol reagent (Invitrogen Corp, Carlsbad, USA) and a commercial ribonucleic acid (RNA) isolation kit (NucleoSpin RNA, Machery-Nagel, Hoerdts Cedex, France) to obtain purified RNA samples following the manufacturer's recommendations. These were then eluted with 150 μ L RNA-free water. Total RNA was determined using an automated spectrophotometer (DeNovix DS-11, DeNovix Inc., Delaware, USA). The quality of total RNA was measured using the automated Bioanalyzer 2100 (Agilent Technologies, Massy, France) with the RNA 6000 Nano Assay version II kit (Agilent Technologies, Massy, France). The samples were only kept when the RNA integrity number generated using Bioanalyzer 2100 Expert software (Agilent Technologies) was at least 7.

Complementary deoxyribonucleic acid (DNA) was synthesized from 2 μ g of total RNA in a reverse transcription reaction using a commercial high capacity RNA-to-DNA kit (Applied

Table 15. Primers for the real-time quantitative PCR analyses of cDNA derived from mRNA of different target genes of intestinal amino acid and peptide transporters and the reference genes used¹

Gene symbol	Gene name	Sequence (5' to 3')	Number of amino acids transported	Product, bp	Accession number
Target genes					
SLC15A1	PepT1	Forward: CCCAGGCTTGCTACCCAC Reverse: ACCCGATGCACCTTGACGA	144	144	NM_214347.1
SLC1A5	ASCT2	Forward: GCCAGCAAAGATTGTGGAGAT Reverse: GAGCTGGATGAGGTTCCAAA	14	206	DQ231578
SLC7A9	b ^{0,+} AT	Forward: CCTTTCAGAAACCTGCCCT Reverse: GAAGGCCACGAAAGAGTGGAA	16	144	NM_001110171
SLC7A7	y ⁺ LAT1	Forward: TCAAGTGGGGAACCCCTGGTA Reverse: ATGGAGAGGGCAGATTCTT	5	259	NM_001110421
SLC7A1	CAT-1	Forward: ATCTCTGCCTGAACAAATGCCA Reverse: ATGCAGTCAAAGCCCCACGAA	3	138	NM_001012613
Reference genes					
HSPCB	Heat shock 90kD protein 1, β	Forward: GGCAGAAAGACAAGAGAGAAC Reverse: CAGACTGGGAGGTATGGTAG	131	131	Q9GLE9_PIG
PPIA	Peptidylpropyl isomerase A	Forward: AGCACTGGGGAGAAAAGGATT Reverse: AAAAAGTGGGAACCGTTTGTG	220	220	NM_214353

¹PepT1 – intestinal peptide transporter; ASCT2 – sodium⁽⁺⁾-neutral amino acid exchanger; b^{0,+}AT – cationic amino acid plus cysteine transporter; y⁺LAT1 – cationic and neutral amino acid transporter; CAT-1 – high affinity cationic amino acid transporter

Biosystems, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) and incubated in a thermocycler (PTC-100 Thermal Cycler, MJ Research, Massachusetts, USA) for 60 min at 37 °C. The reaction was stopped by elevating the temperature to 95 °C for 5 min. The primers for the peptide transporter, PepT1 (SLC15A1), and the AA transporters ASCT2 (SLC1A5), b⁰⁺AT (SLC7A9), and y⁺LAT1 (SLC7A7) have been described previously (Sun et al., 2015; Morales et al., 2017) while for CAT-1 (SLC7A1), a specific primer was designed using their published sequences at the Genbank. Two reference genes, peptidylprolyl isomerase A (**PPIA**) and heat shock 90kD protein 1 β (**HSPCB**), were used to normalize the variations on messenger RNA levels (Park et al., 2015). The primers and their sequences are summarized in Table 15.

A calibration curve for each primer, including that of reference genes, was generated using a pooled complementary DNA sample (calibrator) with an equal representation of each diet and each tissue that was diluted seven times (16, 4, 1, 0.25, 0.06, 0.02, and 0.003 ng· μ L⁻¹) and plotting its crossing threshold (*C_t*) against the log₁₀ value of each dilution after a polymerase chain reaction (**PCR**; Applied Biosystems, California, USA) run. The arbitrary efficacy of each primer was then calculated using the log₁₀ of the slope (linear regression) for each gene.

The expression of the target genes in the duodenum, jejunum, and ileum was estimated using a quantitative PCR assay using the Fast SYBR Green Master Mix (Applied Biosystems). Briefly, a mixture consisting of 2.5 ng of the complementary DNA samples, 0.5 μ M of each forward and reverse primer for each target gene, 6.25 μ L of Fast SYBR Green Master Mix, and RNA free water to complete a final volume of 12.5 μ L was prepared in PCR plates (Thermo Scientific) and centrifuged (2,250 g for 30 s at room temperature). In each PCR plate, three calibrators and two negative controls (pooled RNA sample per tissue without any reverse transcription step) and one RNA-free water were also placed. The StepOnePlus software (Applied Biosystems) was programmed to run an amplification protocol consisting of 40 cycles of 3 s at 95 °C for complementary DNA denaturation, followed by 30 s at 60 °C for annealing

and extension reactions to synthesize messenger RNAs. The dissociation protocol consisted of a linear increase of 1 °C per min and was performed from 60 to 95 °C. Throughout this protocol, a continuous fluorescence acquisition was performed to obtain a melting curve. The melting curve of each specific PCR product was analyzed to ensure that no primer dimers or nonspecific DNA products were amplified.

The calibrators were used to compute for relative expression of each target gene for each sample. The NormFinder software was used to compute for the normalization factor for each sample (Andersen et al., 2004). Thus, the gene expression of each target gene was expressed as a normalized, relative value for each sample using the equation:

$$\text{Normalized relative expression} = \frac{\text{Efficacy}^{-(Ct \text{ Sample} - Ct \text{ Calibrator})}}{\text{Normalization factor}}$$

3.6. Chemical analyses of feed and intestinal content samples

The gross energy content of the diets was measured in an adiabatic bomb calorimeter (IKA, Staufen, Germany). Total nitrogen concentrations in the diets and digesta were analyzed by combustion in an automated analyzer (LECO FP-428 Nitrogen Determinator, LECO, St. Joseph, MI). For AA analysis, feed samples were hydrolyzed in 6N HCl for 24 h at 110 °C with constant agitation to release AA. For Met and Cys analysis, oxidation with performic acid for 4 h was done before hydrolysis for 18 h. The Trp content was determined after alkaline hydrolysis with barium hydroxide for 16 h at 120 °C. The AA were analyzed after derivatization by AccQtag (AccQ-Fluor reagent kit, Waters, Guyancourt, France) using a high-performance liquid chromatography system (Alliance System 2690, Waters, Guyancourt, France).

3.7. Marker quantification and digestibility calculations

It was intended to measure the AA digestibility in the duodenum, jejunum, and ileum. However, the collected quantity of digesta from the duodenum and ileum was not enough to perform the AA analysis. Hence, only samples collected from the jejunum were used. The jejunal

digestibility was used as an indicator of the difference in AA digestibility between diets as most of the AA absorption happens in the jejunum (Bröer and Fairweather, 2018).

The indigestible markers were quantified in the feed and jejunal contents using a modified technique by Tsanaktsidou and Zachariadis (2020). Briefly, the TiO_2 and Co-EDTA in the samples were determined using wet acid digestion and mineralization by first boiling the samples in 30 mL of concentrated nitric acid for 2 min followed by addition of concentrated sulfuric acid while heating for 4 min or until the solution becomes clear to white yellowish. The mineralized samples were then allowed to cool by placing the samples in an ice bath and diluted to 100 mL using deionized water (Milli-Q system, Millipore, USA). For each marker, a calibration curve was established using 6-point curve (concentrations: 0.0, 0.1, 0.2, 0.5, 0.8, 1.0, and 2.0) by preparing standard solutions with TiO_2 and Co-EDTA. Using an inductively coupled plasma-atomic emission spectrometer (SpectrAA 220FS, Varian, Grenoble, France), the concentrations (as $\text{mg}\cdot\text{L}^{-1}$) of titanium and cobalt were determined at 336.112 nm and 231.160 nm, respectively. These concentrations were then used to compute the quantities (as $\text{g}\cdot\text{kg}^{-1}$) of TiO_2 and Co-EDTA in the samples.

Jejunal digestibility (%) was computed as:

$$\text{Digestibility} = \left[1 - \left(\frac{\text{Marker}_{\text{Diet}}}{\text{Nutrient}_{\text{Diet}}} \right) * \left(\frac{\text{Marker}_{\text{Digesta}}}{\text{Nutrient}_{\text{Digesta}}} \right) \right] * 100$$

3.8. Statistical analysis

All statistical analyses were done using R version 3.6.2 (R Core Team, 2019). The pig was the experimental unit. Except for jejunal digestibility, statistical analysis was done per intestinal segment. Data were analyzed with a linear mixed model using the lmer function of R in the lmerTest package (Kuznetsova et al., 2017). The model included the main effect of diet with pig and replicate as random effect. Least square means were computed using the emmeans

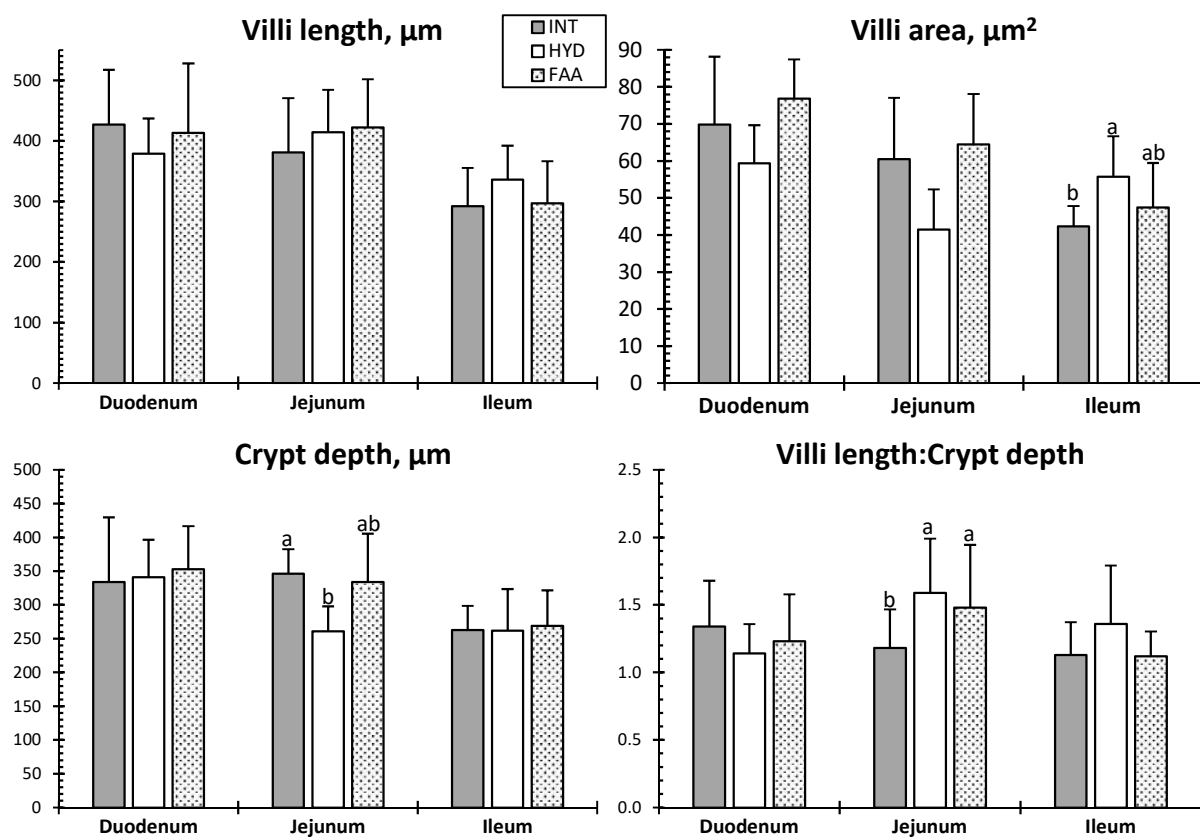


Figure 17. Intestinal morphology of the duodenum, jejunum, and ileum (mean \pm SD) of pigs ($n = 30$) fed a diet whose amino acids originated from intact protein (INT), extensively hydrolysed protein (HYD), or free amino acids (FAA). Different letters within an intestinal segment differ significantly ($P < 0.05$)

function of R under the EMMEANS package (Lenth, 2020). A P -value < 0.05 was considered a statistically significant difference.

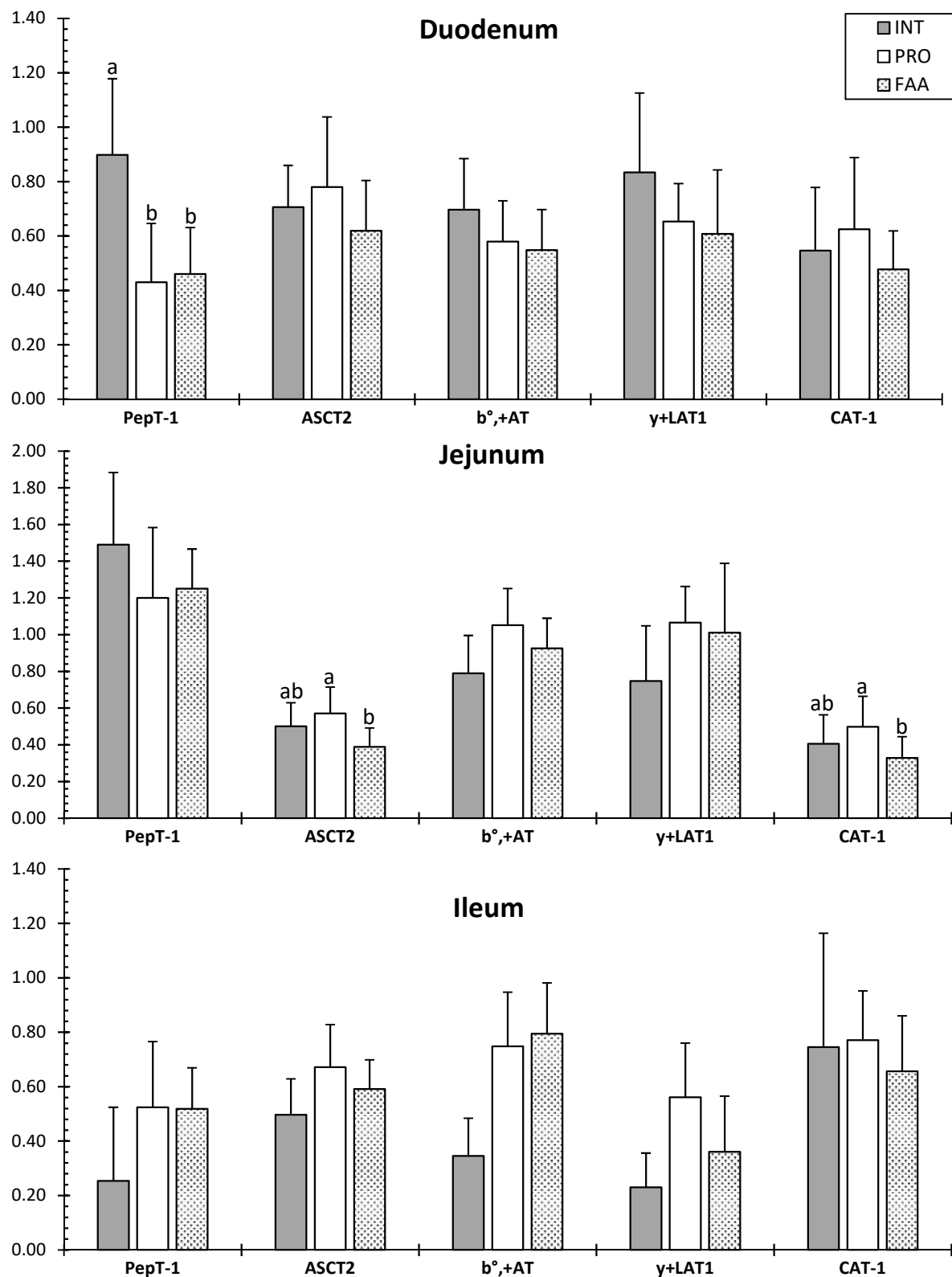


Figure 18. The normalized, relative gene expression (mean \pm SD) of different amino acid transporters and the intestinal peptide transporter of different segments of the small intestine of pigs (n = 30) fed a diet whose amino acids originated from intact protein (INT), extensively hydrolysed protein (HYD), or free amino acids (FAA). HSPCB and PPIA were used as reference genes. Different letters within a gene differ significantly ($P < 0.05$)

4. Results

4.1. General observations

One pig was replaced in the experiment as it did not recover well after the surgery and feed intake did not return to normal. All the other pigs appeared healthy during the experiment. For FAA, some AA were somewhat lower than expected (i.e., Glu, Gly, Ser, and Thr). Nevertheless, the AA profiles of the diets were similar.

4.2. Intestinal morphology of duodenum, jejunum, and ileum

The results of the intestinal morphology of the duodenum, jejunum, and ileum of the pigs are given in Figure 17. The surface area of the villi was the largest for HYD pigs and the smallest for INT pigs in the ileum. Crypt depth was lower in the jejunum for HYD pigs compared to INT and FAA pigs. The ratio of the villi length over crypt depth in the jejunum was lower for INT pigs compared to both HYD and FAA pigs.

4.3. Relative expression of amino acid and peptide transporters along the small intestine

The relative expression of the gene coding for the intestinal peptide transporter PepT1 was higher in the duodenum of INT pigs compared to both HYD and FAA pigs (Figure 18). In the jejunum, the expression of the genes coding for the sodium-neutral AA exchanger, ASCT2, and the high affinity cationic amino acid transporter, CAT-1, was the highest in HYD pigs and the lowest in FAA pigs. Regardless of the diet, the expression of PepT1, y^+LAT1 , and $b^{0,+}AT$ was generally higher in the jejunum compared to that in both duodenum and ileum. The inverse held for ASCT2 and CAT-1, which were the least expressed in the jejunum.

4.4. Jejunal digestibility

The jejunal digestibility of all AA was considerably lower for INT than both HYD and FAA (Table 16). For INT, the digestibilities of His, Val, and Trp were less than 70% and the highest value was for Ile at 89.1%. For HYD and FAA, most digestibilities were higher than 90% with the exception of His for HYD and Cys, Gly, His, Lys, and Pro for FAA. The digestibilities of

Table 16. Digestibility (%) of dietary amino acids measured in the jejunum of pigs fed a diet whose amino acids originated from intact protein (INT), extensively hydrolyzed protein (HYD), or free amino acids (FAA)¹

Amino acid	INT	HYD	FAA	RSD	<i>P</i> -Value
Ala	80.5 ^b	95.5 ^a	91.4 ^a	5.37	<0.01
Arg	81.6 ^b	96.2 ^a	93.0 ^a	5.33	<0.01
Asp	72.8 ^b	94.3 ^a	91.3 ^a	7.09	<0.01
Cys	73.5 ^c	97.8 ^a	83.3 ^b	2.85	<0.01
Glu	83.6 ^b	94.3 ^a	91.6 ^a	3.89	<0.01
Gly	77.7 ^c	93.0 ^a	88.2 ^b	3.60	<0.01
His	66.5 ^b	88.0 ^a	70.1 ^b	9.04	<0.01
Ile	89.1 ^b	97.5 ^a	96.8 ^a	2.68	<0.01
Leu	88.3 ^b	97.5 ^a	96.4 ^a	2.78	<0.01
Lys	72.8 ^b	90.0 ^a	89.4 ^a	5.87	<0.01
Met	70.6 ^b	93.0 ^a	91.2 ^b	5.99	<0.01
Phe	78.6 ^b	97.1 ^a	96.6 ^a	2.73	<0.01
Pro	78.0 ^c	95.1 ^a	87.3 ^b	4.60	<0.01
Ser	88.9 ^b	98.3 ^a	97.2 ^a	2.24	<0.01
Thr	84.7 ^b	96.3 ^a	93.9 ^a	3.13	<0.01
Tyr	73.1 ^b	96.6 ^a	95.8 ^a	3.50	<0.01
Val	69.7 ^b	97.6 ^a	96.8 ^a	2.52	<0.01
Trp	60.9 ^b	90.9 ^a	90.3 ^a	4.38	<0.01
Total amino acid	74.2 ^b	96.0 ^a	93.2 ^a	3.71	<0.01

¹Digestibilities were measured using the slaughter method where the digesta was collected three hours after feeding a diet with indigestible markers (TiO₂ and Co-EDTA); ^{a,b} within a row with different superscript letters differ (*P* < 0.05); RSD – residual standard deviation

all AA were numerically higher for HYD compared to FAA, with the digestibilities for Cys, Gly, His, Met, and Pro being statistically higher.

5. Discussion

This experiment aimed to study the changes in the intestinal physiology of pigs that can be induced when feeding AA either as proteins and/or free AA and small peptides. Other studies found that the replacement of proteins by hydrolyzed proteins in the diet of weanling pigs to increase the villi height to crypt depth ratio in the proximal part of the small intestine (Ma et al., 2019). Furthermore, the provision of free AA in the diet has been shown to increase the expression of intestinal AA transporters in different sections of the intestine (Zhang et al., 2013; Morales et al., 2017). Both these studies suggested that the availability of readily absorbable substrates affected the intestinal capacity to absorb AA.

Apart from the intrinsic digestibility of proteins in the diet, digestibility can also be affected by transit time. The transit time of digesta from the stomach to the end of the small intestine is comprised between 5 to 6 h for the solid fraction and between 3 to 4 h for the liquid fraction (Schop et al., 2019). In our experiment, we assumed that no AA from the last meal remained in the intestine after an overnight fast for any of the experimental diets when pigs were slaughtered to collect digesta. Digestibilities were calculated from digesta samples taken 3 h after the meal, but only little digesta was found in the duodenum or ileum. However, this does not preclude that digesta was still present in the stomach to arrive in the duodenum later on, or that digesta would appear later in the ileum. Hence, the digestibility values reported here are probably affected by the time between the meal and slaughter. If the animals were slaughtered earlier, reported digestibility values would probably have been lower. Consequently, the values reported in Table 16 are to be interpreted as relative values to be compared between diets. Compared to proteins that need to be digested to release AA, free AA and small peptides are immediately absorbed. As all the AA in HYD and FAA were small peptides and/or free AA, we expected their higher AA digestibility compared to INT and this was confirmed in the study. The AA digestibility of HYD was systemically higher than that of FAA with, on average, a 4%

difference. This is surprising because 18% of the AA in HYD were supplied as small peptides. Indeed, dietary peptides are known to stimulate endogenous secretions more than free AA (Butts et al., 1993) and endogenous secretions contribute to underestimating the “true” digestibility (Stein et al., 2007). However, this was not confirmed by the results on crypt depth. The hyperplasia of crypt cells usually signals damage to the small intestine (Haschek et al., 2010) or increased endogenous secretions (Xu et al., 2003). As the pigs in the experiment were in good health, the lower crypt depth of HYD pigs than FAA pigs in the jejunum may be interpreted as lower endogenous secretions when feeding HYD compared to FAA, which is not in accordance with their AA digestibility. Alternatively, the absorption of small peptides may be faster than that of individual AA (Shiraga et al., 1999; Bröer and Fairweather, 2018), because at least two AA are absorbed by a single peptide transporter.

The measured traits to assess the morphology of the three sections of the small intestine are in line with those reported by other experiments (Park et al., 2020). Both villi length and crypt depth can be used as indicators of digestion and absorption capacity of the small intestine (Xia et al., 2005). Part of this is because the villi have a structural function and increase the surface area of the small intestine (Xia et al., 2005; Guay et al., 2006). Another part is because the villi is the main unit of AA absorption in the small intestine with the AA transporters located on its surface (Gao et al., 2000; Salgado et al., 2002). A higher villi height thus reflects an increased total intestinal surface area for nutrient absorption. Contrary to villi, crypts have very little absorptive functions as it has virtually no AA transporters (O’Loughlin et al., 1996; Umar, 2010). Hence, an increased villi size and villi height to crypt depth ratio was interpreted as a higher capacity for intestinal absorption (Zhang et al., 2017). Our results confirm the observations of Yun et al. (2005) and Park et al. (2020) who found that feeding pigs with highly digestible diets resulted in higher villi height and villi height to crypt depth ratio as these indices were higher in the jejunum of HYD and FAA compared to INT. Conversely, the lower villi

length to crypt depth ratio in the jejunum of pigs fed INT could be associated with the presence of proteins that are still being digested. Indeed, a deeper crypt may be the outcome of increased total endogenous secretions (schulze, 2006; Stein et al., 2007). Some studies found more endogenous secretions when feeding diets with protein compared to diets where the supplied AA are immediately absorbable (Chung and Baker, 1992). The increased presence of endogenous protein in the jejunum may have also contributed to the lower digestibility of INT which was systematically lower than HYD and FAA. Most endogenous proteins are re-absorbed in the ileum (Lobley and Lapierre, 2003), and an increased surface area of the ileum for INT could be expected. However, it was unclear why the villi area of HYD pigs that was the highest among the experimental diets in the ileum, followed by FAA.

The expression of PepT1 was the highest in the duodenum of INT fed pigs. This was unexpected because there should be less absorbable peptides in the duodenum of INT than in HYD pigs, especially given their digestibility. Another surprising finding was that the expression of PepT1 was not different from HYD and FAA. We hypothesized that the release of the gastric acid in the stomach may have hydrolyzed the small peptides in HYD into its constituent AA, meaning there were virtually no dietary peptides arriving in the small intestine for both HYD and FAA. Otherwise, as the transit time of the soluble part of the digesta containing free AA and peptides is very quick in the proximal part of the small intestine (Schop et al., 2019), this may have not given enough time for peptides from HYD to be absorbed in the duodenum compared to those in INT. This might be the reason why we saw higher expression of PepT1 in the jejunum compared to the duodenum across all the experimental diets, but it may be more evident for HYD, whose PepT1 expression in the jejunum was around twice to that of the duodenum. Furthermore, because digestive enzymes were mostly released in the duodenum, their eventual breakdown (to be re-absorbed as they were not used) may have caused an “infusion” of peptides which may be why we saw a higher expression of PepT1 in the jejunum of FAA (and possible

HYD) fed pigs compared to their duodenum. Meanwhile, the increased expression of PepT1 in the jejunum compared to the duodenum of INT fed pigs was expected as this was the result of normal protein digestion, and therefore increased abundance of small peptides. The expression of ASCT2 and CAT-1 in the jejunum was the highest for pigs fed HYD. The ASCT2 transports most proteinogenic AA except for Asp, Arg, Glu, His, and Lys (Bröer and Fairweather, 2018) whereas CAT-1 transports the cationic AA Arg, His, and Lys (Yin et al., 2014; Bröer and Fairweather, 2018). These two AA transporters are able to transport most proteinogenic AA in the diet, except for Asp and Glu, and their higher expression in pigs fed HYD might explain why AA digestibility was marginally higher for HYD compared to FAA.

6. Conclusion

The results of this experiment showed that feeding AA as free AA and small peptides instead of proteins results in changes in the intestinal physiology of pigs. Substrate availability is probably the main determinant of these changes, which may have been caused by the inherently different dynamics of digestion. However, even though free AA and small peptides in the diet are readily available and do not need digestion, the variation in the digestibility of individual AA is most likely due to the difference in the amount of endogenous proteins produced and the presence of small peptides in HYD that are absorbed faster.

7. Declarations

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Conflicts of interest/Competing interests

The authors have no relevant financial or non-financial interests to disclose

Synthesis of the results

- Extensively hydrolysed feathers can be used as a model of dietary AA source to study the effects of the balance and form of AA in the diet of animals on AA metabolism and digestive physiology.
- When pigs are fed a diet with limiting amounts of some essential AA, the concentrations of the limiting essential AA goes below fasting concentrations six hours after meal ingestion. Balancing the AA profile of diets by the supplying the limiting AA using synthetic AA would result in higher overall AA utilization during the postprandial period.
- The AA utilization of adult and growing pigs is different and there seems to be different levels of its control. The first-pass metabolism of the intestine and the liver has the ability to control the amount and profile of dietary AA released in the systemic circulation.
- The difference in the AA digestibility of intact proteins and free AA and small peptides affects the rate of appearance of dietary AA. This influences the postprandial metabolic utilization of AA and the overall metabolic status of pigs as seen in the postprandial kinetics of urea and insulin.
- The bioavailability of AA from intact proteins and free AA is not the same because the former needs to be digested before they can be available to the pig. However, even though both free AA and small peptides in the diet are readily absorbed by the animal, they do not have the same bioavailability.
- The form of AA in the diet influences substrate availability and affects the physiology of the small intestine of pigs like the surface area of the mucosa and the gene expression of different AA and peptide transporters.

GENERAL DISCUSSION

The aim of the thesis was to study the metabolism of AA and the digestive physiology of pigs after feeding a diet whose AA profile is either balanced or unbalanced or a diet whose AA is supplied either as intact proteins or as free AA and small peptides. Our strategy was to split this objective into two scientific questions that we attempted to answer with two animal experiments using EHF as a model AA source. In the first experiment (Exp. 1), we assessed the postprandial metabolism of AA of adult and growing pigs after feeding a diet with a balanced or unbalanced AA profile. In the second experiment, we determined if the form of AA in the diet, supplied either as intact protein or as free AA and small peptides, has an impact on the postprandial metabolism of AA (Exp. 2A) and the intestinal physiology (Exp. 2B) of pigs. As we have already discussed the results of the experiments in the previous section, the goal of this section is to add new insights to data produced in the two experiments.

The responses of AA metabolism and digestive physiology to feeding AA are complex and depend on factors like the profile of AA in the diet, the dietary form of AA, the balance with the other nutrients, the frequency of feeding, and the physiological stage of the animal. After feeding, dietary AA are mainly used for protein synthesis. The first-pass metabolism of the intestine plus the liver takes precedence and these tissues first take the AA that they need from the diet before making the rest available for the muscle and other extra-intestinal tissues. Once in the plasma, the metabolic fate of dietary AA is to be incorporated into proteins (Bos et al. 2003) whereas the excess AA will be catabolised. Protein synthesis is controlled by AA availability and the fact that feeding is also known to stimulate the secretion of insulin, an anabolic hormone stimulating protein synthesis (O'Connor et al. 2003). Insulin greatly contributes to the response of protein metabolism of pigs by driving the uptake of AA by peripheral tissues (Davis et al. 2001, 2002). However, protein synthesis can only occur when all the proteinogenic AA are present and in sufficient amounts (Munro 2012). Due to this, protein synthesis is constrained by the most limiting AA and the other non-limiting AA will be

left unused (Dangin et al. 2001; Bröer and Bröer 2017) and will be ultimately catabolised and excreted (Brown and Cline 1974; Gloaguen et al. 2012).

The form in which AA is supplied in the diet influences the rate of intestinal absorption and their eventual appearance in the plasma, which can ultimately affect their bioavailability (Metges et al. 2000; Weijzen et al. 2022). Free AA and small peptides do not need to be digested before being absorbed and therefore appear in the plasma quite rapidly compared to intact proteins. However, the rapid absorption and appearance of AA in the plasma may not necessarily favour protein synthesis and deposition as it can also cause a transient excess of AA in the sites of protein synthesis and result in inefficient use of AA (Batterham and Bayley 1989; Officer et al. 1997). At the intestinal level, the difference in AA availability can also cause changes in the physiology of the small intestine like its morphology and the expression of AA and peptide transporters (Zhang et al. 2013; Morales et al. 2017).

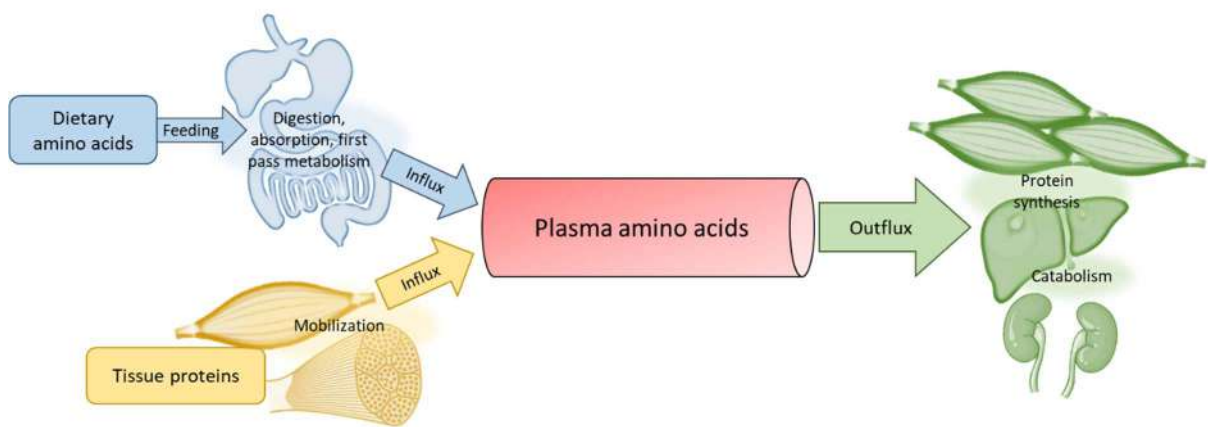


Figure 19. The influx of amino acids from the diet (blue) and tissue mobilization (yellow) into the plasma (what we measure) and its outflux (green) for protein synthesis and catabolism

1. The postprandial amino acid metabolism of pigs

In the thesis, we approached the study of the AA metabolism of pigs in response to dietary AA balance and form using the postprandial measurement of the concentration of AA, other metabolites, and insulin in the systemic plasma (jugular vein). This was done because systemic plasma AA concentrations can reflect the metabolic status of the animal (Leung et al. 1968; Liao et al. 2018) and constitute a pool readily accessible for blood sampling with minimally invasive techniques. However, plasma AA concentrations do not indicate how and how much AA fluxes change. Part of this is because AA metabolism (i.e., protein synthesis, bioconversion, *de novo* synthesis of non-essential AA, and AA catabolism) is done intracellularly and not in the plasma (see Figure 2). For that purpose, we developed a conceptual model that summarises and describes postprandial plasma AA kinetics after a meal in three or four parameters where these concepts are taken into account (van Milgen et al. 2022). Ultimately, it was the parameters of the model that we used to interpret the postprandial kinetics in response to a meal.

To draw our conclusions, we made strong assumptions regarding the sequence of metabolic events in the postprandial period. For instance, we assumed that after feeding, the increase in plasma AA concentrations can be attributed to the influx of dietary AA, even though release of AA from the mobilisation of tissue proteins may also contribute to this influx. The decrease in plasma concentrations, and how it can be used to interpret fluxes is much more complex. After feeding, there are two possible sources of AA influx in the plasma (Figure 19), but the fluxes contributing to outflux cannot really be distinguished (with our technique). We could have been conservative and said that all outfluxes of AA from the plasma are just the uptake by peripheral tissues, most notably the muscle. However, we went quite far in attributing the response of pigs to various metabolic responses like inefficient AA metabolism, changes in the first-pass metabolism of the intestine and liver, or increased catabolism due to relative excesses and metabolic imbalances that may have been caused by our experimental treatments without

explicitly measuring outfluxes. Even so, our confidence that our interpretations are correct is high as we understood the limitations of the model plus we were able to relate the parameters and the effects of our diets, to known biological mechanisms.

One of the intrinsic qualities of the model is that it assumes that the pig tries to maintain a target concentration of AA. We used two different target concentrations: C_0 or the target concentration before feeding and C_{final} (or $C_0 + C_\delta$) or the target concentration after feeding. The concept of target AA concentrations (both pre- and postprandial) may be arguable as plasma AA concentrations are never constant even with prolonged fasting (Bertolo et al. 2000; Pratt et al. 2002). But in Exp. 1, where we did six meal tests per pig, we observed that fasting concentrations are generally similar intra-pig (i.e., coefficient of variation was just 9.35% for all AA on average). This suggests that fasting concentrations probably reflect the metabolism of each individual pig and that target concentration is closely tied to the animal's metabolic status between meal tests. With the way the model works, C_0 and the measured fasting concentrations are closely related to each other. In Exp. 1, we saw that the C_0 of AA was not affected by the diet whereas we found some differences in the C_0 of certain AA in Exp. 2A. This is easily explainable as in Exp. 1, all animals were fed the same diet before each meal test contrary to Exp. 2A. Fasting concentrations can indeed be affected by both the metabolic status and the previous meal of animals (Harker et al. 1968; Højfeldt et al. 2020). This clearly shows that we can rely on the meaning and interpretation we gave to C_0 .

The concept of C_δ defined as the difference between C_{final} and C_0 is necessary for the model because not all postprandial AA concentrations returns to their fasting concentrations for the duration of the measurements (360 min). In Exp. 1, feeding pigs with a diet with limiting essential AA (i.e., UNB) results in the C_{final} of the limiting AA going below C_0 which we think is because the pigs were fed a diet with no limiting AA (i.e., BAL) prior to the meal test. This is also seen by other studies (Hill and Olsen 1963; Regmi et al. 2018) and exemplifies how

animals may react to feeding diets with limiting essential AA and how we can assess this using the model. Meanwhile in Exp. 2A, we saw that some AA did not require the model to include C_δ to properly fit their postprandial kinetics. For us, this validates the biological significance of C_δ . Feeding different diets before and during the meal test can indeed induce differences in the metabolic fluxes of pigs, and C_δ may be used to assess these difference.

Plasma AA concentrations measured in blood collected in the jugular vein is also influenced by the first-pass metabolism of the digestive tract and liver. We used the parameter Q related to the amount of AA in the diet to be indicative of the extent of first-pass metabolism between two situations (diets or physiological status). For example, in Exp. 1, we showed that adult pigs extracted more AA in the first-pass than growing pigs. Our results are in accordance with the finding of Boirie et al. (1997b) who reported increases in the first-pass uptake of Leu with age in humans. Furthermore, the Q of INT in Exp. 2A is also lower than the Q of HYD and FAA as well as that of BAL and UNB in Exp. 1 (i.e., around 70% lower on average between all the mentioned diets). This is consistent with the findings of some experiments that reported that the uptake of AA by the intestine and liver is reduced when feeding free AA (Metges et al. 2000; Daenzer et al. 2001).

Lastly, we think that the model characterised the postprandial AA metabolism of the pigs quite well. In Exp. 1, our hypothesis was that a balanced AA profile may increase the utilization of peripheral tissues compared to a diet with an unbalanced AA profile. We showed that the AUC of BAL was lower than UNB. This is in line with the fact that protein synthesis only occurs when all proteinogenic AA are present and in the right amounts (Munro 2012), meaning the bioavailability of all AA systematically increases as long as we feed a balanced diet to animals. In Exp. 2A, the AUC of HYD and FAA was higher than that of INT, but this may be more indicative of their appearance rate rather than their disappearance rate. So the interpretation of the AUC in Exp. 2A should be different than the interpretation we had in Exp. 1, as we probably

cannot use *AUC* to estimate AA bioavailability for protein synthesis in this scenario. Likewise, a study in broilers found that the postprandial protein synthesis rates were similar for both intact proteins and free AA even though the latter appears in the plasma much quicker (Chrystal et al. 2020b). As AA are not really stored by the body except when used for protein synthesis, they are degraded when they exceed the capacity of the cells for protein synthesis (Quevedo et al. 1994). Hence, the metabolic fate of dietary AA will mainly go towards protein synthesis if the amount of AA in the cells are just enough to not trigger a catabolism response (Millward et al. 1996). So unlike in Exp. 1 where the rate of appearance of AA in the plasma is probably the same for all diets, making conclusions regarding postprandial AA bioavailability is more difficult in Exp. 2A. This shows that we are limited in the interpretations that we can make because we did not measure fluxes. Nevertheless, this does not preclude from drawing viable conclusions based on parameters like as *AUC*, as long as we are aware of its limitations.

Table 17. Dry matter digestibility (%) in the different section of the small intestine of growing pigs fed an experimental diet whose amino acid comes in the form of intact protein (INT), extensively hydrolysed protein (HYD), or as free amino acids (FAA)^a

Parameter	INT	HYD	FAA	RSD	P_{Diet}
n	9	7	7		
Duodenum ^b	11.2	27.8	48.4	27.8	0.11
Jejunum ^c	40.1	67.5	69.1	38.0	0.09
Ileum ^d	69.9 ^b	88.1 ^a	79.8 ^a	10.2	0.03

^aData is presented as least square means. Statistics were done in R using the LMER function where diet was the fixed effect and repetition was the random effect. RSD – residual standard deviation. We only chose samples where we had enough samples to at least measure the dry matter for a single sample

^bAll samples for INT were analysed in duplicates; only 6 samples from HYD were analysed in duplicate; only 4 samples from FAA were analysed in duplicate

^cAll samples were analysed in duplicate

^dOnly 5 samples each for INT and HYD were analysed in duplicate while only 3 samples from FAA were analysed in duplicate

2. Physiological consequences of feeding pigs with different forms of amino acids

The intestine is a tissue that has various important functions, including digestion and absorption of nutrients (Boudry et al. 2002; Blanco and Blanco 2017). Digestion of dietary proteins is mainly done by enzymatic hydrolysis of intestinal proteases and peptidases and is necessary to have constituent AA available to the animal (Switzar et al. 2013; Wang et al. 2021a), while dietary AA and small peptides are immediately absorbed and transported by various AA and peptide transporters (Brosnan 2003; Bröer and Fairweather 2018). Both digestion and absorption can be largely influenced by the physiology of the intestine (Deng et al. 2018; Ma et al. 2019). Being a very adaptable tissue (Boudry et al. 2002; Pan et al. 2020), we can expect that the intestine will adapt to substrate availability in the diet.

As clearly shown in Exp. 2B, the AA digestibility of HYD and FAA is much higher than INT. Unfortunately, we were only able to report on AA digestibility in the jejunum because we did not get enough samples from the duodenum and ileum for AA analysis. However, we were able to measure dry matter digestibility of the diets across the three sections of the small intestine (Table 17) for few samples ($n < 10$). We showed an effect of diet on dry matter digestibility in the ileum which was found to be lower ($P = 0.03$) for INT compared to both HYD and FAA. These results were in line with the results of other experiments (Jeong et al. 2016; Ma et al. 2019). Furthermore, the dry matter digestibility in the ileum was numerically higher for HYD than FAA. This may be related to what we saw in Exp. 2B where the AA digestibility of HYD was systematically higher than that of FAA. We hypothesize that it was explained by the presence of peptides in HYD, which can be absorbed quicker than individual AA (Bröer and Fairweather 2018).

Feeding free AA and small peptides instead of intact proteins was found to affect the intestinal morphology and the expression of intestinal AA transporters across the three sections of the small intestine of pigs, similar to the results of other experiments (Guay et al. 2006; Morales et

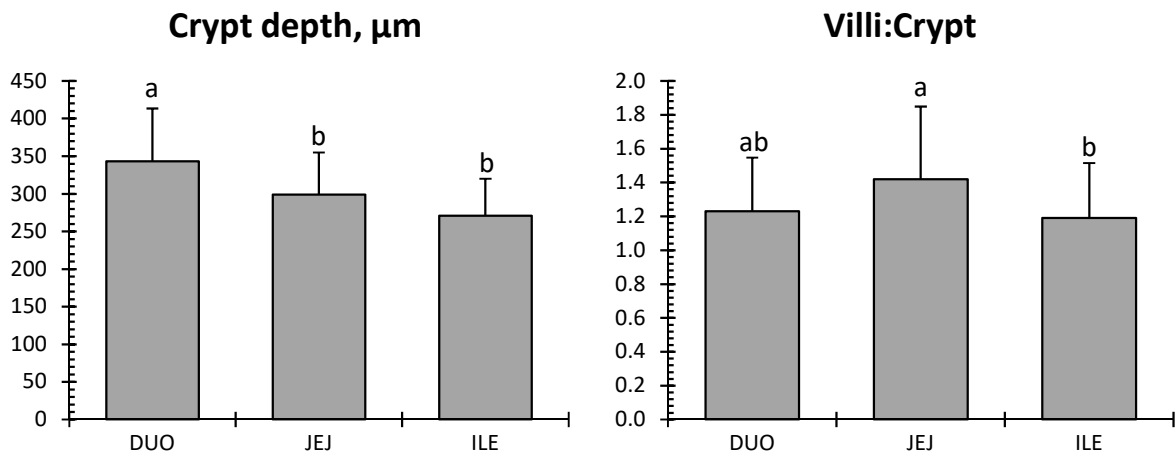


Figure 20. The morphology (mean \pm SD) of the duodenum (DUO), jejunum (JEJ), and ileum (ILE) of pigs in Experiment 2B ($n = 30$). Different letters within a section differ significantly ($P < 0.05$)

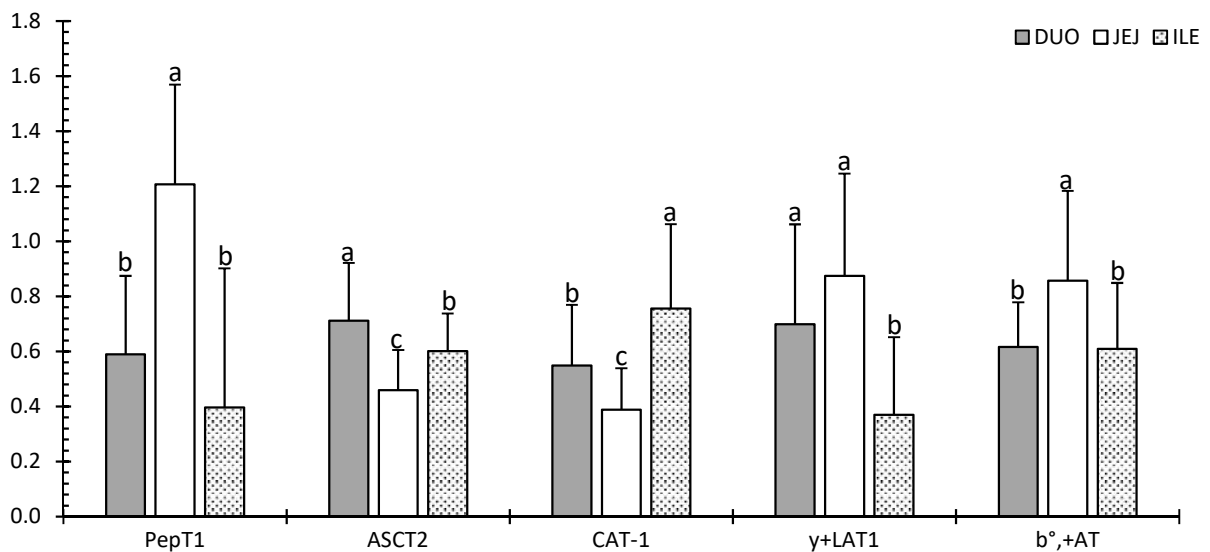


Figure 21. The normalized, relative gene expression (mean \pm SD) of different amino acid transporters and the intestinal peptide transporter in the three sections of the small intestine of pigs in Experiment 2B ($n = 30$). HSPCB and PPIA were used as reference genes. Different letters within a section differ significantly ($P < 0.05$)

al. 2017). These effects are anchored to the differences in the digestive dynamics of dietary AA. However, it seems that the function of each section is also a major determinant regarding both intestinal morphology and expression of transporters. Indeed, the release of digestive enzymes occurs in the duodenum (Montoya et al. 2006) and we see in Figure 20 that the crypt depth in the duodenum was highest ($P < 0.05$) among the three sections of the small intestine of pigs. The villi length to crypt depth ratio, a frequently used index of the absorption capacity of the small intestine, was the highest ($P < 0.05$) in the jejunum. This is most likely because the bulk of absorption takes place in this section (Xia et al. 2005; Gilbert et al. 2008b). It also explains the highest expression of PepT1, y⁺LAT1, and b^{0,+}AT ($P < 0.05$) in the jejunum (Figure 21). We hypothesize that the limiting Lys may be the reason for the lower expression of ASCT2 ($P = 0.02$) and CAT-1 ($P < 0.01$) in the jejunum. Because b^{0,+}AT (can transport Lys) is already highly expressed, there might be a reduced need for CAT-1, which prefers Lys transport, and ASCT2, another AA transporter with broad specificity but cannot transport Lys (Jungnickel et al. 2018; Bröer and Fairweather 2018).

As already mentioned, in Exp. 2, pigs were fed with a small amount of standard starter diet mixed with the experimental diets to limit the feed refusal of pigs, especially those fed EHF. The effect of mixing a standard diet with the experimental diet on intestinal physiology is probably negligible, or at least equivalent between the three diets. We did not encounter this problem in Exp. 1 despite the experimental diets being quite similar. We think that it may have something to do with the fact that the AA profile of the diets in Exp. 2B were deliberately left unbalanced. Furthermore, the period during which the pigs were fed the experimental diets may have been too short for their intestinal physiology to be affected by the diet. Although other studies also fed the pigs for 14 days and found dietary effects, they were using young piglets just after weaning (Zhang et al. 2013; Morales et al. 2017) whose intestines may have been more responsive to dietary interventions than growing pigs.

3. Future perspectives

Although the experiments allowed us to answer the scientific questions of the thesis, we think that there are still some areas that should be explored by future studies. We mainly studied the postprandial AA metabolism response of pigs to dietary AA balance and form. In doing so, we precluded certain aspects of the overall AA metabolism in the responses we measured, particularly the long-term protein and AA metabolism of pigs. This has usually been studied by the nitrogen or AA balance studies that measure nitrogen or AA retention or through measurement of growth performance and feed intake in response to dietary treatments.

We have mentioned many times in the dissertation that the study of AA metabolism, whether in the first-pass or at the systemic level, is not complete without the study of AA flows and metabolic fluxes. Former studies measured these fluxes using invasive techniques like permanent catheters in the portal, hepatic, and other various gastric veins to study first-pass AA metabolism and the combination of jugular vein and local catheters in the vein and artery of one tissue (i.e., femoral muscle) to study regional AA metabolism. These techniques are more difficult to justify and perform now and even more in the future due to evolving views and regulations regarding ethical animal experimentation. Currently, there is a lot of effort being done to develop alternative methods to study animal metabolism while limiting the catheterisation of living animals. Collecting tissues after slaughtering (i.e., for AA deposition, measurement of gene expression, etc.) is still an option. There are many experimental approaches using labelled AA with stable isotopes (i.e., ^{13}C or ^{15}N) to study AA metabolism but the principle is generally the same where one would follow the metabolic fate of AA through AA dilution (collecting blood) or protein deposition (i.e., collecting blood and tissues) or AA oxidation (i.e., collecting the urine, breath-test, etc.) for which the methods do not necessarily require catheterisation of deep vessels (Hoskin et al. 2003; Fiorotto et al. 2012). Whatever the technique, the use of tracers to quantify metabolism is based on modelling (i.e. mathematical

Table 18. The fraction of peptides and its contribution to the total amino acid content of the extensively hydrolysed feathers

Peptide size	Fraction of peptides	Fraction of total amino acids
> 4 amino acid residues ^a	7.80%	1.40%
3 – 4 amino acid residues	29.8%	5.36%
2 – 3 amino acid residues	62.4%	11.2%

^aThe molecular weight of all peptides in the extensively hydrolysed feathers is less than 800 daltons

equations) based on assumptions to measure metabolic fluxes. Lastly, *ex vivo* techniques may also be applicable. One technique is the use of Ussing chambers, which allows to measure both the absorption rate and retention of a “live” intestinal tissue (Awati et al. 2009). Future experiments should consider these alternative methods.

Modelling approaches may also be done alone or associated to animal experiments. We have done that, to a lesser degree, in the thesis by modelling the postprandial responses of pigs. However, as already discussed, it still has limitations as long as we do not know fluxes. Although, incorporating already available data (i.e., blood flow, first-pass metabolism, and protein deposition/retention) may be a solution to improve current conceptual models. Apart from metabolic fluxes, we can also imagine fine tuning of the model that was used in the thesis to better analyse postprandial plasma AA concentrations. The model that we used in the thesis uses the same flow rate (λ) for the appearance of dietary AA in the plasma and its eventual disappearance. We know that the postprandial appearance and disappearance rates of plasma AA may be related, but may be controlled independently. Future modelling approaches may try and distinguish the flow rates of the appearance (λ_1) and disappearance (λ_2) of plasma AA.

Something interesting that we have found with using EHF as the model protein source of the thesis is that the metabolic and physiological responses of pigs are different compared to feeding a diet with just purified free AA. We hypothesise that this is mainly caused by the peptides in EHF. Throughout our experiments, we assumed that EHF does not need digestion. However, a preliminary study done by the partner company of the thesis (BCF Life Sciences) has characterised the distribution of the sizes of peptides in EHF (Table 18) and showed that there are still some peptides (> 4 AA residues) that first needs to be digested. Though we may have been incorrect in saying that EHF does not require digestion, its peptide fraction is nevertheless composed of relatively small peptides that may be hydrolysed quickly.

Furthermore, at least 82% of its total AA is still in free form. Both of these mean that its absorption will still be very quick, as we've also seen in our experiments.

Another interesting venture is the characterisation of these peptides. This step is currently ongoing. However, it remains to be seen if the process of production of EHF consistently produces the same peptides. The information will help in trying to understand why it seems to have functional properties in aquatic species. It is currently being used in aquaculture feeds as a palatability booster, which has been repeatedly proven for different species (i.e., shrimp, sea bass, and tilapia). This is not the case in pigs. Furthermore, the partner company has also found evidences that it can improve growth performance and lower the stress response of shrimps. These responses may very well be because of bioactive peptides in EHF, which we will only know after its characterisation. It may even perhaps be extended to monogastric animals, and be used as a model for peptide research.

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List of Publications

Scientific articles

Published

- **Eugenio, F.A.**, J. van Milgen, J. Duperray, R. Sergheraert, and N. Le Floc'h. 2022. Feeding intact proteins, peptides, or free amino acids to monogastric farm animals. *Amino Acids*. 54:157–168. doi:10.1007/s00726-021-03118-0.
- van Milgen, J., **F.A. Eugenio**, and N. Le Floc'h. 2022. A model to analyse the postprandial nutrient concentration in the plasma of pigs. *Anim. - Open Sp.* 1:100007. doi:10.1016/j.anopes.2022.100007.

Submitted

- **Eugenio, F.A.**, J. van Milgen, J. Duperray, R. Sergheraert, and N. Le Floc'h. Postprandial plasma amino acid and metabolite kinetics of adult and growing pigs fed a diet with free amino acids and small peptides with a balanced or unbalanced amino acid profile.
Submitted to *Animal*
- **Eugenio, F.A.**, J. van Milgen, J. Duperray, R. Sergheraert, and N. Le Floc'h. Feeding pigs amino acids as protein-bound or in free form influences postprandial concentrations of amino acids, metabolites, and insulin.
Submitted to *Animal*
- **Eugenio, F.A.**, J. van Milgen, J. Duperray, R. Sergheraert, and N. Le Floc'h. Expression of intestinal amino acid transporters, gut morphology, and amino acid digestibility in pigs fed proteins or free amino acids.
Submitted to *Journal of Animal Science*.

Conference proceedings

1. Le Floc'h, N., **Eugenio, F.A.**, Meniga, M., Duperray, J., Sergheraert, R. and van Milgen, J., 2019. Ingestion of a highly digestible protein hydrolysate induces singular postprandial plasma amino acid kinetics in minipigs. In *EAAP Scientific Series* (pp. 1079-90). Wageningen Academic Publishers.
2. **Eugenio, F.A.**, van Milgen, J., Duperray, J., Sergheraert, R. and Le Floc'h, N., 2020, December. Plasma amino acid profile after feeding a balanced or unbalanced sustainable protein source in pigs. In *71. Annual Meeting of the European Federation of Animal Science (EAAP) (No. 26)*. Wageningen Academic Publishers.

ANNEX 1



Feeding intact proteins, peptides, or free amino acids to monogastric farm animals

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Abstract

For terrestrial farm animals, intact protein sources like soybean meal have been the main ingredients providing the required amino acids (AA) to sustain life. However, in recent years, the availability of hydrolysed protein sources and free AA has led to the use of other forms of AA to feed farm animals. The advent of using these new forms is especially important to reduce the negative environmental impacts of animal production because these new forms allow reducing the dietary crude protein content and provide more digestible materials. However, the form in which dietary AA are provided can have an effect on the dynamics of nutrient availability for protein deposition and tissue growth including the efficiency of nutrient utilization. In this literature review, the use of different forms of AA in animal diets is explored, and their differences in digestion and absorption rates are focused on. These differences affect the postprandial plasma appearance of AA, which can have metabolic consequences, like greater insulin response when free AA or hydrolysates instead of intact proteins are fed, which can have a profound effect on metabolism and growth performance. Nevertheless, the use and application of the different AA forms in animal diets are important to achieve a more sustainable and efficient animal production system in the future, as they allow for a more precise diet formulation and reduced negative environmental impact. It is, therefore, important to differentiate the physiological and metabolic effects of different forms of AA to maximize their nutritional value in animal diets.

Keywords Intact protein · Free amino acid · Hydrolysate · Monogastric farm animal · Animal feeding

Introduction

To become more sustainable, the negative environmental impact of farm animal production has to be reduced. This has led to global adjustments in animal feeding, like the reduction of the crude protein (CP) content in the diet and the better adjustment of dietary supplies to the nutritional requirements of the animal for maintenance and other physiological needs (e.g., growth, reproduction). Soybean meal is the most widely used protein source in diets of monogastric farm animals such as pigs and poultry, due to its favourable amino acid (AA) profile and high digestibility (Cromwell 2000). However, its use around the globe has been questioned because of the carbon and nitrogen footprint related

to its production, which is mainly concentrated in the United States and Brazil. These aspects have contributed to a reduced use of soybean meal in non-producing countries and the need to use alternative, locally sourced ingredients (Flou-Paneri et al. 2014; Garcia-Launay et al. 2014). Also, a considerable part of the protein supply of monogastric farm animals originates from cereals (e.g., corn, wheat, barley). However, the AA composition of cereals and other plant protein sources such as rapeseed meal is not as good as the AA composition of soybean meal to fulfil the AA requirement. This, in combination with the reduction of CP, has led to the use of free AA (e.g., Lys, Met, Thr, Trp, and Val) to ensure a proper supply of AA while making the animal diet cheaper and more convenient to formulate.

The production of animal-derived products (e.g., meat, milk, and eggs) also results in the production of by-products that are not used for human consumption (e.g., feathers, blood, and offals). These by-products have been used as protein sources in animal feed but the risk of pathogen transmission has reduced their use, especially in Europe. To reduce this risk, animal proteins can be hydrolysed into peptides, a

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shorter chain of AA, which also improves the digestibility of proteins that are resistant “by nature” such as feathers. Hydrolysis of protein sources provides an opportunity to valorise these by-products and turn them into viable alternative AA sources in animal diets (Bah et al. 2016; Dieterich et al. 2014).

In monogastric farm animals, dietary AA can be supplied as intact proteins, peptides, or as free AA. Free AA and small peptides can be absorbed directly by the gut while intact proteins and larger peptides have to be hydrolysed through digestion in the gastrointestinal tract (Wu 1998; Krehbiel and Matthews 2003). Consequently, different dietary forms of AA are absorbed at different rates, which can have consequences on the postprandial metabolism of AA. This literature review explores the interests and perspectives of supplying dietary AA in free form, as peptides, or as intact protein, focussing on their use in the diets of monogastric farm animals like pigs and poultry. It may help to understand AA metabolism in a context of using alternative forms and sources of AA in farm animal diets.

Digestion of dietary protein and absorption of amino acids and small peptides

The provision of dietary AA is required for the production of animal-derived products as they are the building blocks of proteins constituting meat, milk, and eggs. Proteins play diverse functions such as serving as structural components (e.g., collagen, myosin, actin), as metabolic substrates, and as catalysts to various chemical reactions like enzymes (Wu 2013a; Cox and Nelson 1942). Although there are about 700 naturally occurring AA, the focus of this review is on the standard proteinogenic AA for animals, which are the 20 AA used for protein synthesis that are encoded for in the standard genetic code (Wu, 2013a; Ambrogelly et al. 2007). Selenocysteine, a rare AA found in some animal proteins (Clark et al. 2013), is not considered in this review. Peptides can be categorized by the number of AA residues they have, which ranges from di- and tri-peptides to longer oligopeptides with up to 20 AA residues. Those with even more AA residues (but less than 50 residues, corresponding to < 10,000 Da molecular weight) are simply called polypeptides, while a common protein has more than 50 AA residues and has a more complex three-dimensional structure (Munro 2012; Ten Have et al. 2007).

Digestion is the breakdown of large macromolecules into absorbable nutrients (Kong and Singh 2008). Digestibility is the coefficient of disappearance of a nutrient from the gastrointestinal tract after ingestion (Sauer and Ozimek 1986). For monogastric farm animals, protein digestion involves the breakdown of dietary proteins and large peptides through hydrolysis in the stomach and small intestine. The resulting

small peptides and free AA are the forms that can then be absorbed by the small intestine. Digestibility of protein in animal diets involves the measurement of what is left of its constituent AA after passing the whole, or a section, of the gastrointestinal tract. Since there is virtually no absorption of AA in the large intestine, experimental methods for estimating “true” protein digestibility until the distal ileum, the last section of the small intestine, have been put in place and the ileal digestibility for many available ingredients used in monogastric animal diets has been determined. The protein and AA digestibility of different intact protein sources varies (Table 1), which may be attributed to the complexity of their protein structure or even their AA profile (Cox and Nelson 1942; Bhattarai et al. 2017). Pre-hydrolysis of proteins increases their digestibility compared to the native (intact) form (Heimbürger et al. 1997).

Absorption of free AA and small peptides from the small intestinal lumen into the systemic circulation involves nutrient transporters located in the enterocyte membrane. Some studies show that the distribution of AA and peptide transporters in the gut is influenced by the form by which the AA are provided (Morales et al. 2017). In pigs, the expression of genes coding for AA transporters is upregulated when AA are provided in free form as opposed to their intact protein counterparts (Morales et al. 2017; Zhang et al. 2013). Likewise, the expression of intestinal PepT1, the main dietary peptide transporter in the small intestine, is upregulated in pigs fed a diet with a high protein content, especially if the protein is highly digestible (Gilbert et al. 2008).

Protein sources with similar digestibility values do not necessarily have the same kinetics of digestion and absorption. The kinetics of digestion and absorption is difficult to study *in vitro* as it involves different and complex mechanisms and, *in vivo*, it requires multiple cannulation along the gut, which can be complicated and raises ethical concerns that limit its use. The appearance of AA in the systemic circulation after feeding may also be used to study the dynamics of digestion and absorption. However, the postprandial plasma concentration of AA reflects not only the combined dynamics of digestion and absorption, but also the metabolism by the splanchnic tissues and the protein turnover and AA metabolism of the whole organism (Liao et al. 2018). After feeding, the concentrations of AA in the peripheral blood steadily increase followed by a gradual decline (Figs. 1 and 2). The magnitude of this increase and decrease is more important when feeding rapidly absorbed AA like free AA and hydrolysed proteins. In rats (Fig. 1), gastric administration of intact proteins, hydrolysates, or free AA with the same AA profile results in a quicker appearance of AA in the plasma for free AA and hydrolysates as opposed to intact proteins. Thus, the plasma AA kinetics of free AA and hydrolysates results in an asymmetric bell-shaped curve while it was rather

Table 1 Protein and amino acid digestibility (%) of different protein sources fed to monogastric farm animals^{a,b}

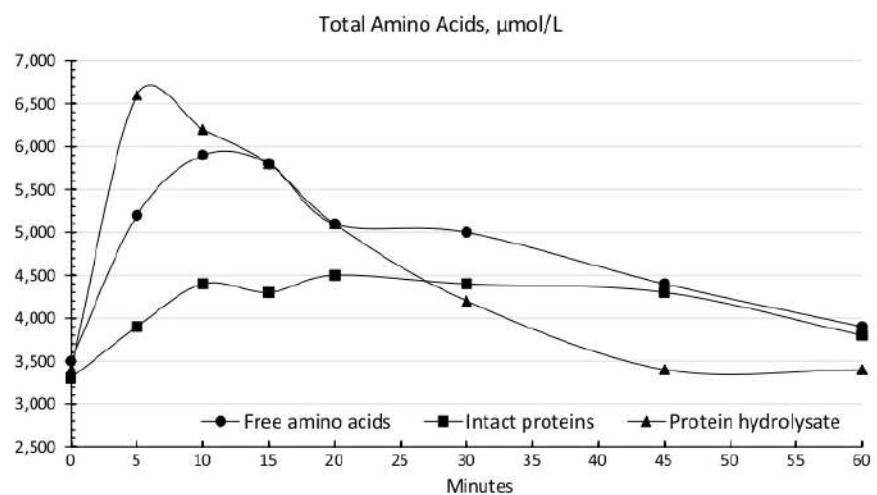
Protein source	CP	LYS	MET	TAA	References
Soybean meal	70.0	79.9	80.1	74.4	Cervantes-Pahm and Stein. (2010) ^d
Fermented soy	70.1	72.7	83.7	76.5	Cervantes-Pahm and Stein. (2010) ^d
Wheat gluten	88.6	77.6	83.2	82.7	Chae et al. (1999) ^c
Fish meal	70.8	82.9	86.0	75.2	Cervantes-Pahm and Stein. (2010) ^d
Whey protein		93.3	89.9		Gottlob et al. (2006) ^c
Meat and bone meal	67.8	56.5	65.6	67.8	Wang et al. (2018) ^d
Spray-dried plasma	81.8	81.2	82.4	82.3	Jeong et al. (2016) ^c
Skim milk	81.7	83.8	85.8	83.6	Chae et al. (1999) ^c
Casein	81.3	92.6	95.1	84.7	Cervantes-Pahm and Stein. (2010) ^d
Feather meal	57.8	62.0	66.5		Kerr et al. (2019) and Grazziotin et al. (2006) ^d
Faba beans	89.0	81.2	55.0		van der Peet-Schwering et al. (2006) ^d
Peas	91.9	90.9	66.8		van der Peet-Schwering et al. (2006) ^d
Canola meal	72.3	66.4	70.3		van der Peet-Schwering et al. (2006) ^d

^aSummary of the ileal digestibility of some dietary protein sources. Free amino acids are not included in the table as they are considered to be completely digestible

^bValues are presented as percentage for crude protein (CP), lysine (LYS), methionine (MET), and total amino acids (TAA)

^cApparent ileal digestible values—measured by the proportion of ingested amino acids left in the distal ileum

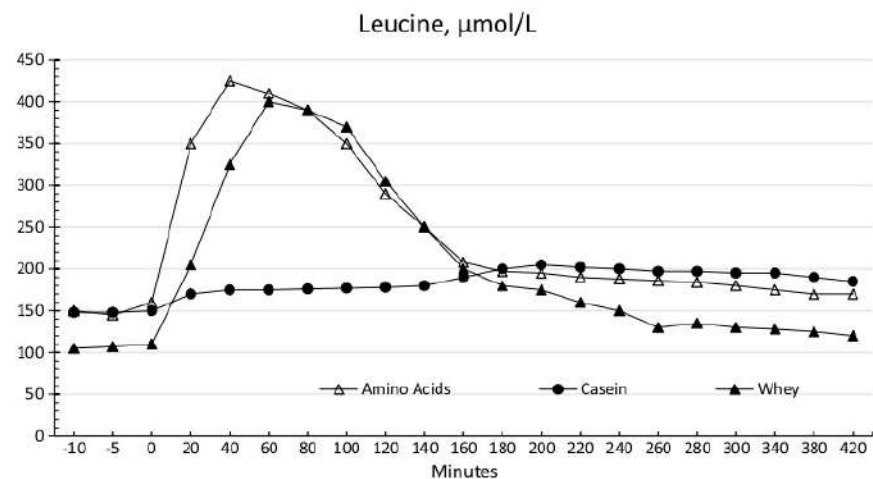
^dStandardized ileal digestible values—apparent ileal digestibility corrected for endogenous losses

Fig. 1 Plasma concentrations of total proteinogenic amino acids (μmol/L) in the portal vein of rats after gastric infusion of intact protein (■), hydrolysate (▲), or free amino acids (●) with the same amino acid profile (Kodera et al. 2006)

flat for intact proteins (Kodera et al. 2006), but this is not the case for all intact protein sources. In young men, the postprandial plasma Leu concentration reaches its maximum within one hour after ingestion of whey protein, with a kinetic curve looking similar to that of feeding free AA and hydrolysates (Fig. 2). However, feeding casein results in a kinetic curve that remained flat throughout the experiment (Dangin et al. 2001), like for intact soy proteins. Though feeding free AA and hydrolysates results in a faster appearance of AA in the plasma, the rate of appearance of AA after the ingestion of different intact proteins

may be variable, which allows to classify intact proteins as so-called fast- and slow-proteins (Dangin et al. 2001). Fast-proteins (e.g., whey) are proteins that are quickly digested and absorbed, and the released AA appear quickly in the blood. Conversely, slow-proteins (e.g., casein, soybean meal) are digested slower and their constituent AA appear with a more delayed increase in plasma postprandial concentrations (Koopman et al. 2009). Therefore, the postprandial AA kinetics of slow-proteins often look flat compared to fast-proteins.

Fig. 2 Postprandial leucine plasma concentration ($\mu\text{mol/L}$) of healthy young men given free amino acids (Δ), whey protein (\blacktriangle), and casein (\bullet) protein sources with identical amino acid profile (Dangin et al. 2001)



Consequences of feeding intact proteins, hydrolysates, or free amino acids on animal metabolism

The postprandial metabolic responses of animals after feeding intact proteins and protein hydrolysates or free AA are summarized in Fig. 3. The kinetics of digestion and absorption determine the metabolic fate of AA by non-digestive tissues (Wang et al. 2021a). More specifically, this fate is mainly due to the rate of AA appearance in the plasma. A rapid increase and high concentrations of AA in the peripheral circulation induced by feeding free AA or hydrolysates result in contrasting outcomes. It increases the rate of AA deamination (catabolism) by the liver (Davis et al. 2002; De Feo et al. 1992) while also enhancing protein synthesis of tissues like the muscle (Dangin et al. 2001). The increase in muscle protein synthesis is also induced by the insulin response after a meal by favouring the utilisation of AA for protein synthesis while decreasing protein breakdown (O'Connor et al. 2003; Paddon-Jones et al. 2004). Although insulin and AA act independently on protein synthesis (O'Connor et al. 2003), they synergistically stimulate muscle protein synthesis after meal ingestion. Feeding free AA or hydrolysates induces a higher postprandial peak of insulin compared to feeding intact proteins (Calbet and Holst 2004) with certain AA like Arg, Leu, Phe, and Gln being known to stimulate insulin secretion (van Loon et al. 2000; O'Connor et al. 2003). Particularly, Leu has been shown to increase the sensitivity of muscle to insulin and stimulates muscle protein synthesis (Anthony et al. 2002). Compared to feeding hydrolysates or free AA, the postprandial insulin secretion and plasma AA concentrations are lower for intact proteins (van Loon et al. 2000), which can lead to lower protein synthesis and retention (Koopman et al. 2009).

Feeding animals diets with free AA and peptides can also increase AA catabolism and therefore result in inefficient

use of AA (Davis et al. 2002; Hou et al. 2017). Some studies found that feeding free AA instead of intact proteins resulted in inefficient use of AA for protein retention (Batterham and Bayley 1989; Batterham and Murison 1981). With a sudden and quick appearance of AA in the plasma after ingestion of free AA, the sites of protein synthesis are assumed to be saturated, which can reduce the uptake and use of AA by tissues (Batterham and Bayley 1989). Since AA are not stored in the body, free AA remain in the plasma and are subject to catabolism (Boirie et al. 1997; Guillet et al. 2004). Conversely, the delayed plasma appearance of AA released by the digestion of intact proteins results in lower catabolism of AA and, even though it results in a lower rate of protein synthesis, may result in a better nitrogen balance after the meal (Boirie et al. 1997).

In practical conditions, farm animals are fed with a mixture of the different forms of AA (i.e., protein-bound and free AA). In pigs, the absorption of total Lys and Thr was lower when pigs were fed a low CP diet supplemented with free Lys and Thr than when these AA were provided in the same amount as intact proteins (Yen et al. 2004). This occurs despite a faster absorption of these two AA when supplied as free form as indicated by the maximum plasma concentrations of Lys and Thr in the portal vein that were observed two hours earlier than for pigs fed intact protein. A more gradual appearance of dietary AA in the systemic circulation as observed after ingestion of intact proteins may lead to greater protein utilization and muscle protein accretion (Reidy et al. 2013; Wang et al. 2021a).

In the lumen of the gut, proteins of non-dietary origin are commonly referred to as "endogenous proteins". Feeding intact proteins results in greater endogenous protein production than with feeding free AA (Nyachoti et al. 1997). The endogenous proteins are nitrogen-containing compounds like digestive enzymes, products of the mucosal membrane (e.g., mucins), and sloughed off epithelial cells (Awati et al.

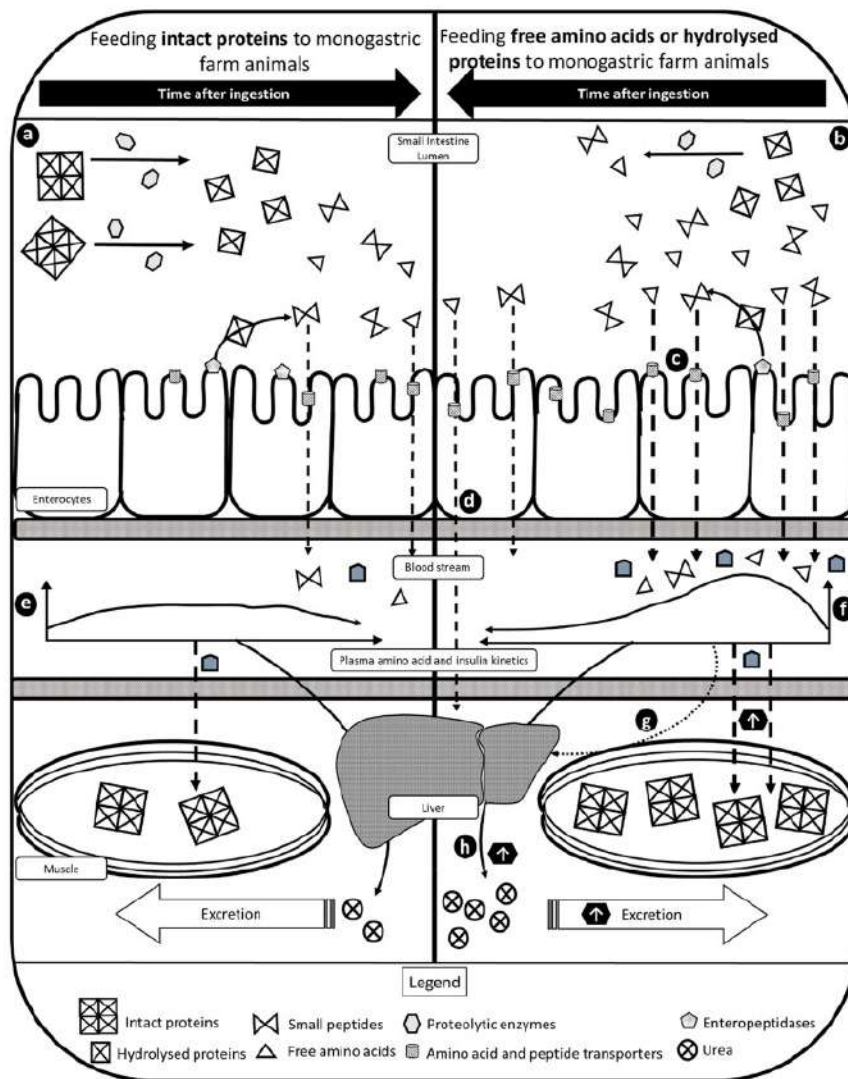


Fig. 3 Model of the different metabolic and physiological effects of feeding monogastric farm animals with different forms of dietary amino acid that is given as intact protein or as hydrolysed protein or free amino acids. In the intestinal level, because intact proteins still needs to be digested, more proteolytic enzymes are released after its ingestion contributing to more endogenous protein losses (a) while feeding hydrolysates or free amino acids lessens the need for these enzymes as hydrolysates are more easily digested and free amino acids are immediately available for absorption (b). Furthermore, due to the greater availability of hydrolysates and free amino acids, there are more amino acid and peptide transporters found in the proximal part of the small intestine (c) although with this increased substrate availability, amino acids may be directed to the liver for deamination/catabolism (d). Since protein digestion takes time, the appearance of its products in the plasma is more constant resulting in a charac-

teristically flat kinetics of amino acids after feeding intact proteins (e). Feeding hydrolysates and free amino acids induces a quicker appearance of plasma amino acids and an asymmetric distribution of postprandial plasma amino acids kinetics that is skewed towards the beginning (f). Plasma amino acids induces the release of insulin that increases the uptake of amino acids by the muscle and increase protein synthesis. However, in this postprandial state, due to the rapid appearance of amino acids in the plasma after feeding hydrolysates or free amino acids, there is a period of temporary imbalance of amino acid uptake of sites of protein synthesis, like the muscle, that causes more amino acids to be catabolized compared to feeding intact proteins (g). As a consequence to this increased rate of amino acid catabolism, more urea is produced by the liver which can also be interpreted as a lower efficiency of use of dietary nitrogen (h)

2009). A significant part of these endogenous proteins (e.g., gastric and pancreatic secretions and enzymes) is produced in response to digesta present in the gastro-intestinal tract (Lobley 2003). Feeding free AA instead of intact proteins,

diminishes the secretion of proteolytic enzymes and could therefore affect the “apparent” protein digestion (Adeola et al. 2016; Butts et al. 1993). In addition, endogenous secretions can be partially re-absorbed and are not necessarily lost

for the animal (Souffrant et al. 1986). Endogenous secretions thus have both a direct (i.e., providing the digestive enzymes) and an indirect effect (i.e., by contributing to protein secretion and absorption in the gut) on digestion and, eventually on plasma AA appearance.

The gut itself also uses dietary AA, thereby affecting the overall bioavailability of these AA. The portal-drained viscera, which include the stomach, intestines, pancreas, and spleen, are estimated to use 30–60% of AA like Thr, Lys, and Phe (Stoll and Burin 2006), while they only account for less than 10% of the body weight in monogastric animals. Some of these dietary AA are metabolized by the gut to produce other molecules. For example, the gut tissue has been shown convert dietary Met to Cys that is one of the three AA constituting glutathione, a major antioxidant in the gut (Li et al. 2014; Riedijk et al. 2007). Apart from the gut tissue, the microbiota found in the small intestinal lumen also use dietary AA (Apajalahti and Vienola 2016). In broilers, the AA requirement of lactic acid producing bacteria (i.e., *Lactobacillus* spp., *Streptococcus* spp., and *Enterococcus* spp.), which is the dominant microbiota population in the small intestine, is very close to the AA requirement of birds (Apajalahti and Vienola 2016; Dai et al. 2013a). This may mean that the gut microbiota can potentially compete with the animal for dietary AA, which might be more critical for essential AA (EAA). Proteins that are not digested in the small intestine are fermented in the large intestine, affecting the microbial ecosystem and favour the proliferation of harmful bacteria (Apajalahti and Vienola 2016). Conversely, feeding free AA or hydrolysed proteins that are absorbed very quickly in the small intestine prevents the proliferation of harmful bacteria (Wang et al. 2016; Zhang and Piao 2021) and may contribute to maintain the absorptive and digestive capacities of the small intestine.

Feeding free amino acids and hydrolyzed proteins to complement intact proteins in animal diets

In terrestrial farm animal nutrition, dietary AA are mostly provided as intact proteins from soybean meal and cereal grains. Other protein sources include cereal by-products, legumes, oil seed meals, and animal-by product meals (Florou-Paneri et al. 2014) and the use of these sources differs between countries and geographic areas. During the last decades, there has been an increased use of free AA, which are often added to supplement and complement low-protein diets to ensure the proper supply of AA and allows formulating diets with a composition close to the requirements of the animal. Dietary AA are commonly classified as nutritionally essential or non-essential AA. The EAA are those that should be provided by the diet because of the

inability of the animal to synthesize the carbon backbone and those that are insufficiently synthesized. Non-essential AA (NEAA) can be synthesized de novo using metabolic intermediates (D'Mello 2003). However, there is a difference between the biochemical and the biological capacity to synthesize NEAA. In young pigs, reducing the dietary supply of NEAA has been shown to limit protein synthesis and growth (Deng et al. 2009). Thus, even though animals may have the capacity to synthesize NEAA, they may have a dietary requirement for these AA (Wu et al. 2013) at certain production stages. For example, during pregnancy, lactation, and weaning Arg, Glu, Gln, Gly, and Pro may become limiting for pigs and require a dietary supply (Wu et al. 2014). Therefore, it is difficult to define the “essentiality” of AA because all AA are conditionally essential (Table 2).

Ideal protein is a concept used in animal nutrition to express the AA requirements of the animal. It refers to the balance of dietary AA that is needed to exactly cover the productive potential of farm animals. It concerns all the EAA and some conditionally EAA. The capacity to synthesize the other NEAA is assumed to be sufficient to cover the requirements (Baker 2000). The AA profile of ideal protein

Table 2 Nutritional classification of proteinogenic amino acids in the diets of monogastric farm animals^a

Mammals ^b			Poultry ^c		
EAA	CEAA	NEAA	EAA	CEAA	NEAA
Cys	Gln	Ala	Arg	Gln	Ala
His	Glu	Asn	Cys	Glu	Asn
Ile	Gly	Asp	Gly	Cys ^e	Asp
Leu	Pro	Ser	His	Tyr ^f	Ser
Lys	Arg ^d		Ile		
Met	Cys ^e		Leu		
Phe	Tyr ^f		Lys		
Thr			Met		
Trp			Phe		
Tyr			Pro		
Val			Thr		
			Trp		
			Tyr		
			Val		

^aTable derived from Wu et al. (2014). Amino acids are listed in their three-letter IUPAC abbreviation. EAA essential amino acids, CEAA conditionally essential amino acids, NEAA non-essential amino acids

^bPigs and ruminants, also applicable for rodents

^cChickens, ducks, quails, and geese

^dArginine is classified as a conditionally essential amino acid for pig by NRC (2012)

^eCan be synthesized in the liver by conversion of methionine and serine, but methionine needs to be in sufficient amount

^fCan be synthesized in the liver by conversion of phenylalanine as long as it is in sufficient amounts

is expressed as a ratio relative to of Lys, which is the first limiting AA in typical diets. However, it ignores possible interactions among AA (e.g., among the branched-chain AA; Kim et al. 2001; van Milgen and Dourmad, 2015). Also, the ideal protein requirement depends on the physiological state of the animal (e.g., weaned piglets, growing pigs, gestating and lactating sows, laying hens) and on the capacity to synthesize certain AA (e.g., Pro and Gly in poultry), and therefore, different ideal protein ratios have been proposed. The concept of ideal protein is simple and has been widely used in formulating diets for monogastric animals.

Use of free amino acids in farm animal nutrition

The concept of ideal protein largely contributed to the use of free AA in animal diets. Furthermore, it is believed that the CP of the diet can be reduced without affecting performance as long as the supply of EAA and NEAA is ensured (Gloaguen et al. 2014; Heo et al. 2009). Thus, the provision of synthetic EAA to complement intact proteins in the diet allows that farm animals can perform according to their genetic potential, as dictated by the ideal protein concept. The highest efficiency of nitrogen utilization is achieved when the supply of all AA exactly matches the requirement. This means that more nitrogen will be used for protein retention and production of animal derived-products and less nitrogen is excreted (Garcia-Launay et al. 2014). Providing protein sources that are not easily digested results in a lower efficiency of nitrogen utilization. Likewise, provision of AA in excess lowers nitrogen efficiency.

Free AA in pure, crystalline form like L-lysine, DL-methionine, L-threonine, L-tryptophan, and L-valine are commonly added to commercial animal diets. The L-enantiomers are produced by fermentation and subsequent purification, whereas DL-methionine is produced through chemical synthesis. Other commercially available AA include L-methionine and hydroxy analogues of methionine. The reduction of the CP content of the diet accompanied by supplementation of free AA has been called the “protein-sparing effect of free AA” (Baker 2009; Han and Lee 2000). However, there are practical limitations to the extent of this effect. Providing a very low CP diet to broilers (4.5% lower CP than the control diet) reduced their growth performance even though the diet was supplemented by all limiting EAA according to the ideal protein concept (Wang et al. 2021b). Similarly, finishing pigs fed with diets with very low levels of CP (11 vs 14%) supplemented with EAA had lower growth performance than those fed a control diet (Tuitoek et al. 1997). Recent studies suggest that the synthesis of NEAA in pigs depends on the availability of EAA and glucose (Hou et al. 2017), both of which are primarily sourced from the diet. Also, direct supplementation of NEAA in free form may alleviate the need

for their de novo synthesis when AA nitrogen is limiting (Gloaguen et al. 2014).

The provision of Lys, Met, and Thr in synthetic form in broiler diets resulted in higher plasma concentrations of these AA than when they were provided as intact proteins (Chrystal et al. 2020). In pigs, the Lys and Thr plasma concentrations remained higher for a longer period when these AA were provided in free form instead of intact proteins (Morales et al. 2020). High plasma concentrations after ingestion of free AA for an extended period may indicate that these AA are not used for protein synthesis, which may be a signal of inefficiency in AA utilization. This may be caused by an AA imbalance in plasma due to the rapid appearance of AA (Yen et al. 2004). Batterham and Bayley (1989) suggested that this imbalance may also result in increased AA catabolism and therefore in a lower AA efficiency of feeding diets with free AA. To summarize, the bio-availability of AA provided in free form by the diet may not be the same as that coming from intact proteins. It remains unknown if there is a ratio between AA coming from intact proteins and free AA that optimizes AA utilisation for protein retention.

There is a growing interest in the targeted utilisation of free AA for roles other than being constituents of body proteins and peptides. These AA are usually provided as free AA instead of intact proteins as their dietary amounts can only be precisely adjusted using free AA. Certain AA (e.g., Asn, Thr, Ser, and Val) have effects on the modulation of metabolic pathways (Wu 2013b, 2010), the regulation of protein synthesis and turnover, carbohydrate, and AA metabolism (Dai et al. 2013b; Wu 2010), and feed intake (Trevisi et al. 2018), while others (e.g., Arg, Gln, Glu, Gly, and Trp) are involved in signalling, immunity, and antioxidative responses (Wu et al. 2009; Wu 2010). The supplementation of Gln in the diet of young broiler chickens has been shown to improve intestinal health with longer villi length, greater intestinal IgG and IgA concentrations, and improved growth performance compared to birds fed a control diet (Chalvon-Demersay et al. 2021; Bartell and Batal 2007). Stress hormones and stress-related behaviour were also reduced in young chicks when given diets that were supplemented with Ala, Ser, and Pro (Kurauchi et al. 2009). Otherwise, AA like Trp, Gln, Leu, and Ala can support health and can be adjusted based on age and the health status of animals (Le Floch et al. 2018).

Hydrolysed proteins or peptides in farm animal nutrition

The hydrolysis of intact protein sources opens up the possibility of using inedible, waste, or by-products in animal diets and therefore reduce competition with the food supply for humans, while also increasing the nutrient value of common

ingredients by making their protein fragment more digestible (Hou et al. 2017). These ingredients are collectively called hydrolysed proteins or hydrolysates, which essentially means that they provide AA as peptides instead of intact proteins. Hydrolysis of intact protein sources improves their nutritional value and also reduces anti-nutritional factors that may be present (Martínez-Alvarez et al. 2015; Pasupuleti and Braun 2008). For soybean meal and other highly digestible materials, the improvement may be marginal, but pre-hydrolysis of materials that are not easily digested improves their nutritional value substantially. Hydrolysis of non-edible materials like feathers (with a protein digestibility of less than 10%) results in a five-fold increase of its protein digestibility while a more intensive hydrolysis process renders the feathers almost completely digestible (Grazziotin et al. 2006). Hydrolysis techniques by chemical (i.e., acidic or basic) or physical means (i.e., steam and pressure) are used for both animal-derived proteins (e.g., casein, whey, blood, meat, mucosa) and plant-based proteins (e.g., soybean, wheat, barley; Pasupuleti et al. 2008; Dieterich et al. 2014). Keratin-based materials (e.g., feathers, wool, and hair) can be processed through these means and can also be treated by controlled bacterial breakdown (Pasupuleti and Braun 2008). Other protein sources can be processed through microbial fermentation techniques that not only improves digestibility but also modifies the AA profile of the material (Cervantes-Pahm and Stein 2010; Jones et al. 2010). Extensive hydrolysis results in a greater proportion of small peptides and free AA compared to those that were only partially or briefly hydrolysed (Bouhamed and Kechaou 2017), but it may also result in the degradation of the AA (Bellagamba et al. 2015; Papadopoulos et al. 1986). Certain hydrolysis conditions induce crosslinking of AA or racemization with a negative impact on digestibility (Bouhamed and Kechaou 2017; Friedman 1999).

Like certain free AA, peptide-rich ingredients can have properties other than being a highly digestible AA source. Peptides with less than 20 AA residues and abundant in Arg, Lys, and Pro residues induce beneficial effects like immune modulation and antimicrobial activities (López-Barrios et al. 2014). Hydrolysed protein products have been tested for their immunomodulation and anti-oxidant properties (Nørgaard et al. 2012; Hou et al. 2017) and as alternatives to antimicrobial to support health and growth performance of animals (Hou et al. 2017). However, the benefits on growth performance of hydrolysed proteins in animal feeds is inconsistent and their functional properties are not fully understood (Nørgaard et al. 2012; Martínez-Alvarez et al. 2015). These functional properties have been attributed to bioactive peptides such as opioid peptides (4–8 AA residues) that alter feeding behaviour by binding to certain receptors in the brain (Hou et al. 2017). In addition, some bioactive peptides exert antimicrobial effects

by damaging the membrane of harmful bacteria or directly affecting their metabolism (Hou et al. 2017; López-Barrios et al. 2014). Protein hydrolysates are usually fed to young animals with high AA requirements and with a less mature immune and digestive system like weaned piglets and chicks (Min et al. 2004; Hou et al. 2017). Young broiler chickens fed a diet supplemented with hydrolysed pig mucosa had greater performance compared to those fed the control diet (Frikha et al. 2014), but no effect was observed in older chickens. This might be because younger animals are more susceptible to the functional properties of hydrolysates as they are still developing their immune and digestive systems. Poudel et al. (2020) reported changes in the faecal microbiota of young pigs fed a peptide-based feed additive and hypothesised that such changes may favour the maturity of the digestive tract.

Chemical and physical hydrolysis techniques involve processes that indiscriminately cut the protein into smaller fragments. As a result, the resulting product may have different peptides in terms of size and AA composition, which may explain the inconsistencies on the observed effects on the animal. Thus, animal performance may vary substantially and enhanced performance cannot be pinpointed to a particular peptide in the hydrolysate. There is a general understanding regarding the positive effects of hydrolysis of protein sources, but further characterisation of the resulting peptides is still needed. This information is important because it may provide “additional value” to these ingredients over and above to the value of a highly digestible source of AA.

Conclusion

Although farm animal diets contain intact proteins, there has been an increasing trend to substitute and complete the diet with free AA or hydrolysates. Though feeding these new forms has advantages, there is still much to be learned. A better characterization of the peptides from currently available sources is needed to identify the nutritional and functional properties of peptides. Future research should identify and quantify how the dietary form of AA affects metabolism. For example, it is unknown if the animal can metabolically differentiate between free and protein-bound dietary AA. Most of the recent studies regarding dietary form of AA deal with their short-term effects, but there is limited information on the long-term effects on metabolism and physiology. For nutritionists, the challenge will be to determine the ideal proportion of AA provided as intact proteins or in free form for optimizing animal performance and health in a sustainable way.

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Research involving human participants and/or animals Not applicable.

Informed consent/consent to participate Not applicable.

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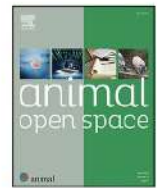
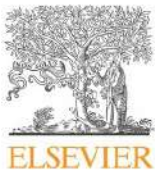
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ANNEX 2



A model to analyse the postprandial nutrient concentration in the plasma of pigs

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ABSTRACT

Changes in the postprandial nutrient concentration in the plasma are the result of the combined effects of intake, digestion, absorption, and metabolism. The concentration typically follows an asymmetrical bell-shaped curve as a function of the time after the meal. Although differences between dietary treatments can be analysed using a pairwise comparison of the observed nutrient concentrations, this provides little insight in the possible underlying biological mechanisms. These mechanisms may be represented in a model that can be used in a regression analysis to summarise the observed data in a limited number of parameters. The objective of this study was to propose equations that can be used in the statistical analysis of postprandial nutrient concentrations. The equations were derived from the compartmental representation of the Erlang function in which the last of a series of compartments was used to represent the nutrient concentration in the plasma. The preceding compartments were used to represent the postprandial response provoked by ingestion of the meal. A homeostatic control mechanism was included based on a target nutrient concentration that the animal seeks to maintain. This target concentration may differ between the fasting state and after ingestion of a meal. The models were developed as differential equations, which were integrated analytically providing equations that can be used for data analysis. The fit of the equations was tested using the postprandial histidine concentration of a pig that received a diet that was either balanced or unbalanced in the amino acid supply. The unbalanced diet was also deficient in histidine. The observed data could be summarised in three or four parameters that describe the target histidine concentration after an overnight fast, the possible change in the target concentration due to ingestion of a meal, the area under curve of the postprandial response (i.e., the "metabolic exposure"), and a rate constant describing the dynamics of the response. The biological interpretation of these and derived parameters is discussed, including the potential pitfalls of interpreting nutrient concentrations as nutrient flows. In conclusion, the models developed here are based on biological concepts and allow to summarise time series of nutrient concentrations in a limited number of parameters. The concepts can be modified depending on how the biological mechanisms involved are perceived and on the type of available data.

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Implications

There are different ways to analyse the effect of an experimental treatment on the postprandial nutrient concentration in the

plasma. A pairwise comparison of observations between treatments ignores the relationship that exists between successive observations. Analysis of the data by a regression model requires to define the (supposed) underlying biological mechanisms and summarises the data as a set of parameters. Differences between experimental treatments can then be summarised in parameters and analysed in relation to the biological representation in the model and the experimental treatments.

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Specification table

Subject	Nutrition
Type of data	Maple V (release 5.1) code and output, data used as example, R code (version 4.1.2) of the non-linear regression models and output.
How data were acquired	Data were obtained from a study in which growing pigs were given, after an overnight fast, a meal with either a balanced or unbalanced supply of amino acids. Pigs had jugular catheters from which blood samples were taken at regular intervals. Plasma was obtained by centrifugation of the blood, and plasma amino acids were analysed by Ultra Performance Liquid Chromatography (UPLC).
Data format	Raw data, analysed data (i.e., estimated model parameters), and output from Maple with the analytical solutions obtained after integrating the differential equations.
Parameters for data collection	Data are recorded as the histidine concentration in the plasma as a function of time after ingestion of a meal.
Description of data collection	The data were collected from a growing pig that was given a meal with a balanced or unbalanced supply of amino acids. The unbalanced diet was deficient in histidine. The meal was given after an overnight fast.
Data accessibility	Repository name: Portail Data INRAE Data identification number: https://doi.org/10.15454/GKFOEH

Introduction

The postprandial nutrient concentration in the plasma is the result of the combined effects of intake, digestion, absorption, and metabolism of the nutrient. After an overnight fast and ingestion of a meal, the concentration typically follows an asymmetrical bell-shaped curve, characterised by a rapid increase from the baseline, to reach a maximum and gradually decline to the baseline again. Differences between experimental treatments can be reported as the time points at which the concentration of one treatment differs from another treatment, using a repeated-measures model (Bos et al., 2003). Also, the area under the curve (AUC) can be calculated, which is typically done by dividing the response curve in trapezoids between two successive observations. The AUC is then the sum of the areas of each trapezoid. The trapezoid method is frequently used in pharmacokinetics to study the fate of an administered drug, because the plasma concentration of the drug starts at zero (before the administration of the drug) and ends at zero (once the drug is fully metabolised). The interpretation of the AUC is more difficult for nutrients, which are not only digested, absorbed, and catabolised but may also be synthesised from other nutrients, and stored in and mobilised from body reserves. Alternatively, regression models can be used to describe the change in the nutrient concentration in the plasma as a function of time. McKnight et al. (2013) developed a framework

of conceptual models to analyse the change in nutrient concentration after ingestion of a meal or provision of a marked nutrient. The nutrients in these models were represented as compartments with different inflows (e.g., through nutrient absorption, *de novo* synthesis, or marker supply) and outflows (e.g., through uptake of the nutrient by tissues or catabolism). Gloaguen et al. (2012) used an Erlang function to describe the change in amino acid concentration in the plasma. The model was mainly used to express the plasma kinetics using a limited number of parameters but without a biological interpretation of the model parameters. The goal of this paper was to further develop the Erlang function to make the underlying biological assumptions more explicit.

Material and methods

The Erlang function

The Erlang function can be seen as a series of n compartments in which each compartment receives input from the previous compartment and where the output is described by first-order kinetics (Matis et al., 1989):

$$\frac{\partial X_n}{\partial t} = \lambda X_{n-1} - \lambda X_n$$

where X_n is the size of compartment n ($\mu\text{mol/L}$), $\partial X_n/\partial t$ the change in size of the compartment ($\mu\text{mol/L per h}$), t the time (h), and λ the fractional outflow rate (1/h). For this study, it was assumed that $n = 3$, but this has no consequence for the rationale developed further. The last compartment (i.e., X_3) may be seen as the nutrient concentration in the plasma (C , $\mu\text{mol/L}$; top panel of Fig. 1). The differential equations describing the system are as follows:

$$\frac{\partial X_1}{\partial t} = -\lambda X_1$$

$$\frac{\partial X_2}{\partial t} = \lambda X_1 - \lambda X_2$$

$$\frac{\partial X_3}{\partial t} = \frac{\partial C}{\partial t} = \lambda X_2 - \lambda X_3$$

with initial conditions when time = 0: $X_1(0) = Q$, $X_2(0) = 0$, and $X_3(0) = C(0) = 0$. The Q ($\mu\text{mol/L}$) is the apparent quantity of the dietary nutrient arriving in the plasma provoking the change in the nutrient concentration. The AUC for each of the three compartments equals Q/λ . Integrating the system of differential equations with Maple (version 5.1, Waterloo Maple Inc., Waterloo, Canada) results in the following equation for the last compartment $C(t)$:

$$C(t) = \frac{1}{2} AUC \lambda^3 t^2 \exp(-\lambda t) \quad (1)$$

where AUC $\mu\text{mol h/L}$ is the area under the curve, λ the fractional outflow rate from C (1/h), and t the time (h).

The modified Erlang function

In the model depicted in the top panel of Fig. 1 and Eq. (1), the concentration $C(t)$ will initially be zero, but will gradually increase and decrease again to zero due to the flow of Q (i.e., the initial value of X_1) through X_2 and C . This may be sufficient to describe the fate of a drug, but is not appropriate to describe the metabolism of a nutrient because it is unlikely that the nutrient concentration will equal zero, even during fasting. To include the concept of a basal nutrient concentration in the model, the Erlang function was modified as depicted in the bottom panel of Fig. 1, where a target nutrient concentration is included that the animal seeks to maintain (C_{target} , $\mu\text{mol/L}$). The control of C is bidirectional and a nutrient concentration greater than C_{target} results in a net outflow from C ,

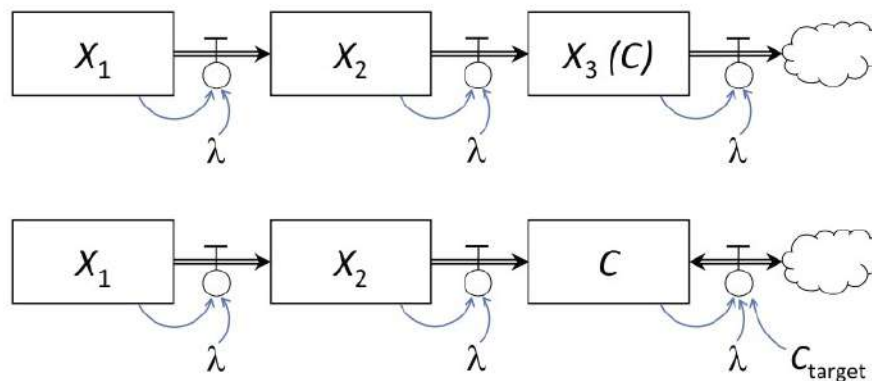


Fig. 1. Top panel: Forester diagram of the Erlang function described as a series of n sequential compartments ($n = 3$). The last compartment (C) is assumed to represent the nutrient concentration in the plasma ($\mu\text{mol/L}$). Ingestion of a meal provokes a change in C via two compartments (X_1 and X_2). The λ ($1/\text{h}$) is the fractional outflow rate. Bottom panel: Modified Erlang function where the nutrient concentration in the plasma is controlled by a target concentration (C_{target} ; $\mu\text{mol/L}$) that the animal seeks to maintain.

whereas a lower concentration results in a net inflow into C . The differential equation describing the change in C is as follows:

$$\frac{\partial C}{\partial t} = \lambda X_2 - \lambda(C - C_{\text{target}})$$

Integrating this equation with $C(0) = C_{\text{target}}$ results in:

$$C(t) = \frac{1}{2} AUC \lambda^3 t^2 \exp(-\lambda t) + C_{\text{target}} \quad (2)$$

Eq. (2) equals Eq. (1) with a constant term (i.e., C_{target}) added to it. The AUC is the area under the curve of $C(t)$ over and above C_{target} , and can be considered as the “metabolic exposure” due to ingestion of the meal. The top panel of Fig. 2 illustrates the nutrient concentration as described by Eq. (2). The maximum of $C(t)$ occurs at $t_{\text{max}} = 2/\lambda$ and equals $C_{\text{max}} = C_{\text{target}} + 2 AUC \lambda \exp(-2)$.

There are experimental situations in which the postprandial nutrient concentration does not return to the target concentration, at least not during the period of measurements. This can be represented by assuming that the target concentration changes due to ingestion of a meal to become $C_{\text{target}} + C_{\delta}$, so that:

$$\frac{\partial C}{\partial t} = \lambda X_2 - \lambda(C - (C_{\text{target}} + C_{\delta}))$$

Integrating the system of equations with $C(0) = C_{\text{target}}$ results in:

$$C(t) = \frac{1}{2} AUC \lambda^3 t^2 \exp(-\lambda t) + C_{\text{target}} + C_{\delta}(1 - \exp(-\lambda t)) \quad (3)$$

Eq. (3) equals Eq. (2) with an extra term (i.e., $C_{\delta}(1 - \exp(-\lambda t))$) that describes the dynamics of the change in the target concentration from C_{target} to $C_{\text{target}} + C_{\delta}$. An example of Eq. (3) is given in the bottom panel of Fig. 2, illustrating that the observed nutrient concentration in the plasma is the result of two modes of action provoked by the ingestion of a meal: a change in the nutrient concentration related to the supply and further metabolism of the nutrient from the meal, and a change in the target concentration during the measurement period. The dynamics of both modes of action are determined by λ .

Fitting the model to experimental data

The use of the equations is illustrated by fitting these to the histidine concentration in the plasma of a growing pig that received, after an overnight fast, a diet with a balanced or unbalanced supply of amino acids. The supply of some amino acids, including histidine, was deficient in the unbalanced diet. Fitting the model to the data was done using the nls procedure of R (version 4.1.2).

The example data and the R code are available from <https://doi.org/10.15454/GKFOEH>.

Results

Fig. 3 illustrates the fit of Eq. (2) to the histidine concentration in the plasma of a pig that received diet balanced in the amino acid supply. The estimated parameter values were $C_{\text{target}} = 44.4 \mu\text{mol/L}$, $AUC = 116 \mu\text{mol}\cdot\text{h/L}$, and $\lambda = 1.83/\text{h}$, with a residual standard deviation of $6.4 \mu\text{mol/L}$. The maximum concentration (C_{max}) of $102 \mu\text{mol/L}$ was attained at 1.09 h .

Fig. 3 also gives the fit of Eq. (3) to the experimental data in which the same pig was given a diet unbalanced in different amino acids and deficient in histidine. The estimated parameter values were $C_{\text{target}} = 48.3 \mu\text{mol/L}$, $C_{\delta} = -17.7 \mu\text{mol/L}$, $AUC = 34.1 \mu\text{mol}\cdot\text{h/L}$, and $\lambda = 2.47/\text{h}$, with a residual standard deviation of $2.7 \mu\text{mol/L}$. The C_{δ} differed significantly from zero ($P < 0.001$).

The histidine concentrations were very different when the pig was given a diet balanced or unbalanced in amino acids, which is reflected by the estimated parameters. The C_{target} after an overnight fast was similar in both situations, but the deficient histidine supply in the unbalanced diet resulted in a lower target histidine concentration (i.e., $C_{\text{target}} + C_{\delta} = 30.6 \mu\text{mol/L}$) compared to the balanced diet ($C_{\text{target}} = 44.4 \mu\text{mol/L}$). Also, the maximum histidine concentration was achieved earlier and attained a lower maximum value when the pig received the unbalanced diet compared to the balanced diet. The histidine concentration in the unbalanced diet was approximately 70% lower than in the balanced diet (1.00 vs 3.43 g/kg diet), which also resulted in a 70% reduction in the AUC (i.e., 34.1 vs $116 \mu\text{mol}\cdot\text{h/L}$).

Authors' points of view

The models and parameters

The models used here allow to summarise a series of observed nutrient concentrations in three or four model parameters. The parameters C_{target} , AUC , λ , and C_{δ} were chosen to parameterise the models, because of their biological interpretation and because initial values are easy to obtain “visually” from the observed data (i.e., $\lambda = 2/t_{\text{max}}$ and $AUC = (t_{\text{max}}(C_{\text{max}} - C_{\text{target}}))/(4 \exp(-2))$ for the 3-parameter model), ensuring rapid convergence in the non-linear parameter estimation procedure.

The models were derived from the Erlang function, to which several modifications were made in relation to the control of C . However, the same fractional rate (λ) was used to determine the

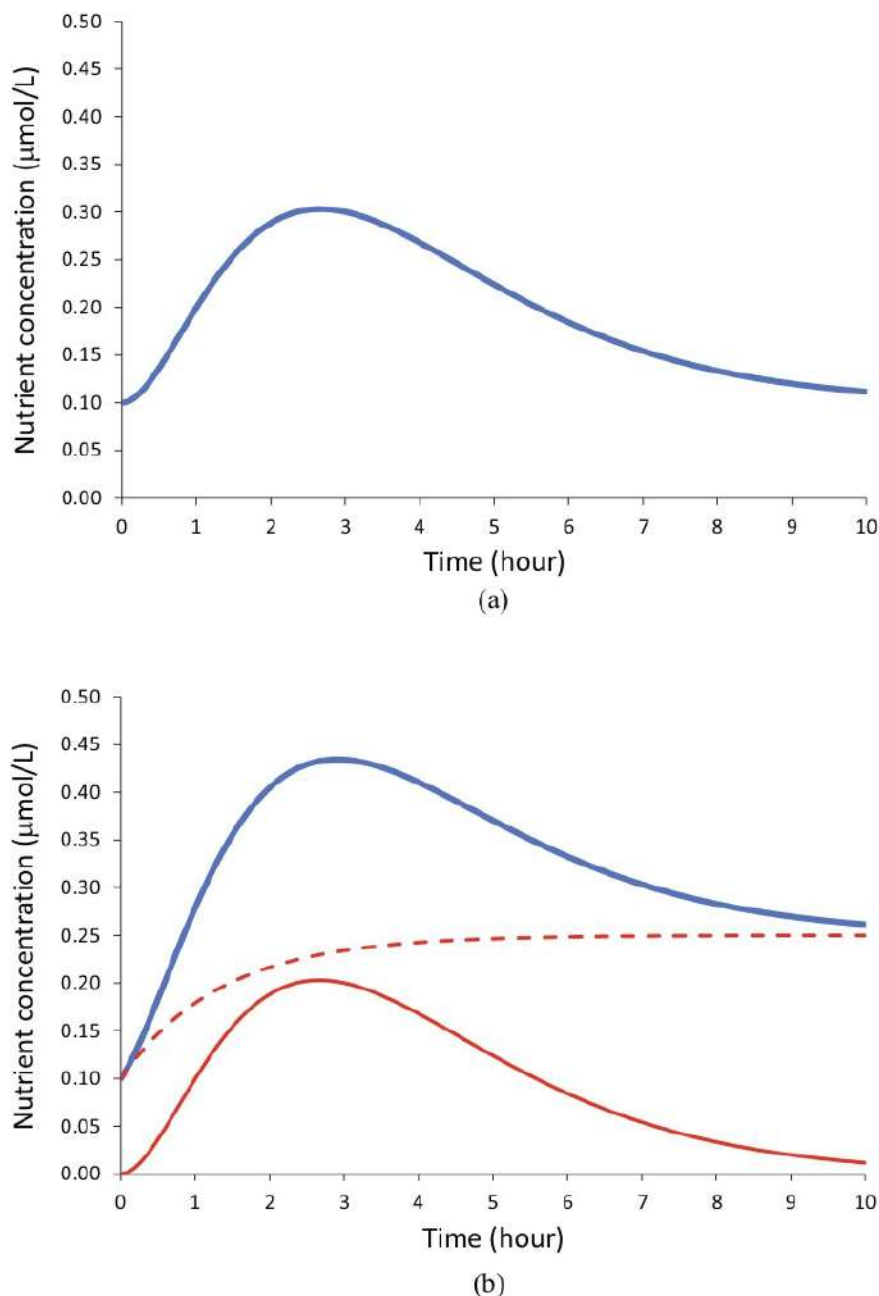


Fig. 2. Top panel: Postprandial nutrient concentration in the plasma of the modified Erlang function in which concentration is controlled by a constant target concentration (Eq. (2)). Bottom panel: Postprandial nutrient concentration in the plasma of the modified Erlang function in which the concentration is controlled by a changing target concentration due to the ingestion of a meal. The solid red line represents the change in the nutrient concentration provoked by ingestion of a meal (i.e., the first term of Eq. (3)) and the dashed red line the change in the target concentration from C_{target} to $C_{\text{target}} + C_0$ (i.e., the last two terms of Eq. (3)). The blue line is the sum of the two red lines and describes the nutrient concentration in the plasma. The $C_{\text{target}} = 0.1 \mu\text{mol/L}$, $C_0 = 0.15 \mu\text{mol/L}$, $AUC = 1 \mu\text{mol}\cdot\text{h/L}$, and $\lambda = 0.75$ per hour (see text for details).

inflow into C from the diet (through X_1 and X_2) and to control C (through C_{target} and C_0). An alternative approach would be to differentiate these fractional flow rates, but such an approach would require an additional parameter to be estimated.

Nutrient concentrations and nutrient flows

Changes in nutrient concentration are related to changes in nutrient flows but should not be interpreted as absolute values of flows. In steady-state conditions, the net nutrient flow through

C equals zero but this does not mean that the actual flow through nutrient mobilisation and further metabolism is also zero. Likewise, Q is an “apparent” quantity and cannot be compared directly with the histidine supply by the meal.

The assumption that the nutrient concentration is controlled by nutrient homeostasis in the plasma is a rather strong assumption, because nutrient homeostasis can also be the result of the difference between inflow and outflow, each of which may be controlled independently without an explicit control of C . This has consequences on the interpretation of certain model concepts

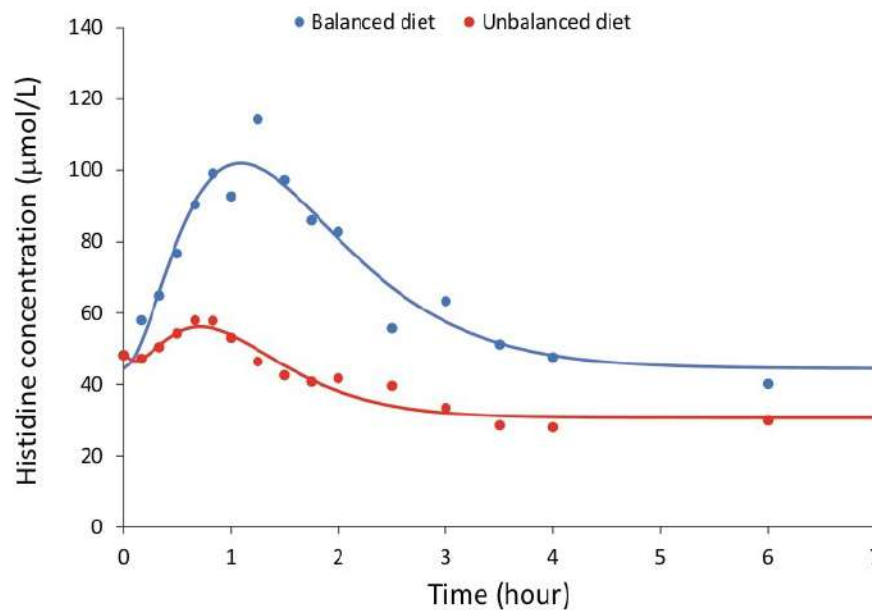


Fig. 3. Observed (dots) and estimated (line) histidine concentration in the plasma of a pig given either a diet balanced or unbalanced in the amino acid supply after an overnight fast.

and derived model parameters. There are several instances where the unknown nutrient inflow in C equals the unknown outflow (i.e., at C_{target} , $C_{\text{target}} + C_{\delta}$, and C_{max}), but they result from different metabolic conditions. For example, the increase in concentration from C_{target} to C_{max} likely resulted from an increased inflow of nutrients provided by the meal, followed by an increased outflow, to become equal again at t_{max} . With this sequence of events, the nutrient flow is thus greater at C_{max} than at C_{target} , and the ratio between C_{max} and C_{target} is indicative for the nutrient flow at t_{max} relative to that at t_0 . (i.e., for the balanced amino acid supply data, the histidine flow is $102/44.4 = 2.30$ times greater at t_{max} than at t_0). However, such a reasoning must be used with caution, because opposing attributions of cause and effect can result in the same plasma concentration. For example, for the unbalanced amino acid supply, ingestion of the meal resulted in a lower target nutrient concentration (i.e., $C_{\delta} < 0$). This could be the result of an increased outflow followed by an increased inflow, but also from a decreased inflow followed by a decreased outflow. The ratio between $C_{\text{target}} + C_{\delta}$ and C_{target} of (i.e., $(48.3 - 17.7)/48.3 = 0.63$) may be indicative of an increased postprandial histidine utilisation resulting in an increased histidine mobilisation (compared to the situation during fasting), because the histidine concentration in the meal was limiting. However, the same response could also be the result of a reduced postprandial histidine mobilisation, followed by reduced histidine utilisation. Opposing mechanisms of cause and effect may thus result in the same response in nutrient concentration.

Conclusion

Modelling approaches can be used to analyse and interpret the postprandial kinetics of nutrients. These approaches allow to summarise the observed data in a limited number of parameters. These parameters can be interpreted biologically as target nutrient concentrations that the animal seeks to maintain (during fasting and after ingestion of a meal), metabolic exposure, and the dynamics of the response of the animal due to ingestion of a meal. The ratios between concentrations where the nutrient inflow equals the outflow (i.e., at minimum, maximum, and steady-state concentrations) are indicative of ratios in nutrient flows. These changes

cannot be assigned to an explicit cause or effect, even though certain interpretations are more plausible than others.

Ethics approval

The experiment from which data were derived was authorised by the French Ministry of Agriculture (APAFiS #2019041610073508).

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NLF: Writing – review & editing.

Declaration of interest

None.

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Titre : Équilibre et forme de l'apport en acides aminés dans l'alimentation : Conséquences physiologiques et métaboliques chez le porc

Mots clés : acide aminé libre, protéines, concentrations plasmatiques, transporteurs, digestibilité, hydrolysats de protéines de plumes

Résumé : L'aliment apporte aux porcs des nutriments indispensables à leurs fonctions biologiques comme les acides aminés (AA). Dans l'aliment, les AA peuvent être apportés sous forme de protéines, de peptides ou bien sous forme libre directement assimilables. Les réponses métabolique et physiologique des porcs à l'apport en AA par l'aliment peuvent être influencées par l'équilibre des AA (profil) ainsi que par la forme sous laquelle ces AA sont apportés (protéines, peptides, libres). L'objectif de la thèse était d'évaluer les réponses métaboliques et physiologiques des porcs nourris avec un profil équilibré ou non en AA apportés sous forme de protéines, de peptides, ou sous forme d'AA libres. La réponse métabolique des porcs a été évaluée en étudiant la cinétique plasmatique postprandiale des AA et de différents métabolites. Nous avons comparé les cinétiques postprandiales de porcs adultes et en croissance pour étudier l'influence de l'équilibre des AA alimentaires. Les effets métaboliques de la forme de l'apport en AA a été étudié chez des porcs en croissance. Chez ces mêmes animaux, les adaptations physiologiques au niveau de l'intestin ont été

évaluées par la mesure de l'expression des transporteurs d'AA et de peptides et de la morphologie de la muqueuse. Nous avons montré que l'alimentation des porcs avec un régime avec un profil équilibré en AA entraîne une utilisation plus rapide des AA alimentaires par rapport à un régime avec un profil déséquilibré en AA. L'utilisation des AA par les tissus périphériques des porcs adultes est plus lente que celle des porcs en croissance. Par contre, l'utilisation des AA par le tube digestif et le foie au "premier passage" est plus importante chez les porcs adultes que chez les porcs en croissance. La digestibilité des AA des protéines est plus faible que celle des l'AA libres et des petits peptides, car ces deux derniers sont immédiatement disponibles sans avoir besoin d'être digérés. Cela entraîne une différence dans leur biodisponibilité pour les tissus périphériques et le statut métabolique des porcs. En outre, la forme de l'AA alimentaire influence la physiologie intestinale, comme le montrent la morphologie et l'abondance de divers transporteurs intestinaux d'AA et de peptides dans les différentes sections de l'intestin grêle.

Title : Balance and form of amino acid intake in the diet: physiological and metabolic consequences in pigs

Keywords : free amino acid, proteins, plasma concentrations, transporters, digestibility, hydrolysed feathers

Abstract : Feeding animals is crucial for their life because they need nutrients like amino acids (AA). They use these AA for various biological processes like the synthesis of proteins and other biomolecules. Although, the metabolic and physiological response of pigs to feeding AA depends both on the dietary profile and form. The metabolic response of pigs was assessed by studying the dynamic changes in the postprandial plasma concentrations of different metabolites. Furthermore, as mature and growing animals have different metabolic needs and use of dietary AA, we used adult and growing pigs to study dietary AA balance. Meanwhile, as the form of AA has a direct impact on the rate of absorption of AA in the intestinal lumen, physiological adaptations like changes in morphology and gene expression of AA and peptide transporters were measured.

We found that feeding pigs with a diet with a balanced AA profile results in quicker utilization of dietary AA compared to a diet with an unbalanced AA profile. The use of AA in the peripheral tissues of adult pigs is slower compared to growing pigs, conversely, more AA are utilized in the first-pass for adult pigs compared to growing pigs. There is a big difference in the AA digestibility of intact proteins compared to free AA and small peptides as the latter two are immediately available without the need for digestion. This causes a difference in their bioavailability to peripheral tissues and the metabolic status of pigs. Furthermore, the form of dietary AA influences intestinal physiology as seen in the morphology and the abundance of various intestinal AA and peptide transporters in the different sections of the small intestine of pigs.