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J.V. Nørgaard, E.A. Soumeh, M. Curtasu, E. Corrent, Jaap J. van Milgen, et al.. Use of metabolic profile in short-term studies for estimating optimum dietary isoleucine, leucine, and valine for pigs. *Animal Feed Science and Technology*, 2017, 228, pp.39-47. 10.1016/j.anifeedsci.2017.04.002 . hal-01607181

HAL Id: hal-01607181

<https://hal.science/hal-01607181v1>

Submitted on 25 May 2020

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Accepted Manuscript

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PII: S0377-8401(16)31090-2
DOI: <http://dx.doi.org/doi:10.1016/j.anifeedsci.2017.04.002>
Reference: ANIFEE 13754

To appear in: *Animal Feed Science and Technology*

Received date: 6-12-2016
Revised date: 31-3-2017
Accepted date: 3-4-2017

Please cite this article as: Nørgaard, J.V., Soumeh, E.A., Curtasu, M., Corrent, E., van Milgen, J., Hedemann, M.S., Use of metabolic profile in short-term studies for estimating optimum dietary isoleucine, leucine, and valine for pigs. *Animal Feed Science and Technology* <http://dx.doi.org/10.1016/j.anifeedsci.2017.04.002>

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Use of metabolic profile in short-term studies for estimating optimum dietary isoleucine, leucine, and valine for pigs

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Highlights

- Increasing levels of isoleucine, leucine or valine affected the metabolic profiles
- Modelling plasma metabolites could estimate optimum amino acid requirement in piglets
- Two days of feeding may be sufficient to mediate relevant biological changes in blood

ABSTRACT

Traditional AA dose-response studies utilize many animals for evaluation of growth performance, and it is hypothesized that a new experimental design based on modern analytical techniques can reduce the number of used animals. The objective was to evaluate a short-term approach with a low number of pigs based on plasma metabolites as a method to determine the dietary Ile, Leu, and Val requirements. Three separate 6 x 6 Latin square experiments having 6 replicates per treatment were conducted with 6 diets containing increasing concentrations of Ile, Leu, and Val which were fed to 6 pigs (BW 8 to 9 kg) for 2 days, each without a wash-out period for a period of 12 days. The diets were prepared and used in 3 previous traditional-design dose-response studies and had been stored at -20 °C. Blood samples were collected at the end of each 2-day

period, and plasma was analyzed for AA and other metabolites using a metabolomics approach. Out of the 18 analyzed plasma AA, 11, 16, and 3 AA for Ile, Leu, or Val, respectively, showed linear or quadratic responses ($P < 0.05$) which could be linked to animal growth. The same was found for 4 non-AA metabolites in the Ile, and for 7 non-AA metabolites in the Leu study. 3-Methyl-2-oxovaleric acid, ketohexanoic acid, and α -ketoisovaleric acid were discriminating metabolites for both Ile and Leu. It was possible to fit least squares means of 5, 14, and 2 metabolites in the Ile, Leu, and Val experiments to curvilinear-plateau, broken-line, or quadratic models and thereby estimate an optimum dietary BCAA level. The average optimum BCAA levels across metabolites and models were 0.54 standardized ileal digestible (SID) Ile:Lys, 1.04 SID Leu:Lys, and 0.68 SID Val:Lys which was close to optimums of 0.52, 0.93, and 0.70 found in the previous dose-response studies based on animal growth performance. In conclusion, certain plasma metabolites could be used to estimate Ile, Leu, and Val requirements, and 2 days of adaptation to a new diet was sufficient to reflect relevant biological changes in the blood to different levels of dietary AA in the current study.

Abbreviations: BCAA, branched chain amino acid; BL, broken-line; CL, curvilinear-plateau; PUN, plasma urea nitrogen; SID, standardized ileal digestible; Q, quadratic.

Keywords: Branched chain amino acid; growth performance; metabolomics; metabolic profile; pig1.

Introduction

Supplementation of crystalline AA allows reduction of the dietary CP level and supplies the animal with a more balanced AA profile covering the animal's requirements for maintenance and growth. This approach to improve nitrogen utilization and, consequently, to reduce nitrogen excretion and emissions to the environment without affecting animal performance is known to be effective (Hansen et al., 2014; Nørgaard

et al., 2014). To reduce dietary CP by supplementing crystalline AA, it is necessary to have a clear understanding of the AA requirement to supply an ideal dietary protein profile (Boisen et al., 2000).

A common approach to studying AA requirement is to run dose-response experiments where body weight gain and feed intake are evaluated for 2 to 4 weeks. In our previous 3 studies on Ile (Soumeh et al., 2014), Leu (Soumeh et al., 2015a), and Val (Soumeh et al., 2015b), the requirement of those AA was estimated for 8 to 18 kg pigs by evaluating feed intake and weight gain during 2 weeks, but also by quantifying AA and other metabolites in the blood and urine. In these studies, a general trend of a decrease in plasma AA concentrations with increasing provision of the limiting AA was observed which can be indicative of improved AA utilization for growth. Furthermore, blood plasma and urine samples were analyzed by a metabolomics approach identifying several metabolites which could be related to growth performance (Soumeh et al., 2016). The plasma concentrations of glycocholic acid and taurocholic acid could be used to estimate the optimum Ile level in the diet, and plasma creatine and urinary 2-aminoadipic acid, ascorbic acid, and choline could be used to estimate the optimum Leu level, whereas the metabolic response to the optimum dietary Val level was less pronounced in plasma, and only profiles of the other branched chain AA (BCAA) could be used to estimate the optimum BCAA level (Soumeh et al., 2016).

As an alternative to evaluating animal performance to determine AA requirement, Pedersen and Boisen (2001) developed, and later validated (Pedersen et al., 2003), an approach which was based on measuring plasma urea nitrogen (PUN) as the sole response trait in dose-response studies. In brief, they fed pigs diets with different levels of Thr:Lys in a randomized order for 2 days with 3 daily feedings. Jugular vein blood samples were obtained 2, 4, and 6 hours after the sixth feeding whereafter the next experimental treatment was fed. Thus, by using this approach, a dose-response experiment was conducted with 4 AA levels for a duration of 8 days and with the use of 5 pigs per period in a cross-over design (Pedersen et al., 2003). In the present study, it was hypothesized that feeding increasing levels of Ile, Leu, or Val would result in appearance the metabolites previously identified in blood (Soumeh et al., 2016), which correlate

with animal performance, and that these metabolites could be used in short-term studies as response traits instead of feed intake and weight gain.

The objective of the study was to evaluate a short-term approach with a limited number of animals based on blood samples as a method to determine the optimal ratio for Ile, Leu, and Val in young piglets fed diets previously used in dose-response growth performance studies, thus having a documented effect on growth performance.

2. Materials and Methods

All animal experimental procedures were carried out in accordance with the Danish Ministry of Justice, Law no. 253 of March 8, 2013 concerning experiments with animals and care of experimental animals and license issued by the Danish Animal Experiments Inspectorate, Ministry of Food, Agriculture and Fisheries, the Danish Veterinary and Food Administration.

2.1. Animals and diets

A total of 18 cross-bred (Danish Landrace, Yorkshire × Duroc) gilts were weaned at 28 days of age and fed a commercial diet for 1 week before the experiment started. Pigs were weighed at the beginning and every second day of the experiment without preceding fasting. The average initial body weights of pigs (mean ± SD) were 7.6 ± 0.1 , 7.8 ± 0.1 , and 8.0 ± 0.2 kg in the Ile, Val, and Leu experiments, respectively. The average final body weights of pigs (mean ± SD) were 10.2 ± 1.0 , 10.2 ± 0.4 , and 10.3 ± 1.0 kg in the Ile, Val, and Leu experiments, respectively. Pigs were housed individually in 1×2.2 m pens with two-thirds heated concrete floor and one-third cast iron slatted floor. The temperature was reduced gradually from 24 °C to 22 °C, and humidity was kept around 60% during the experimental period.

Diets from the 3 previous studies (Soumeh et al., 2014; Soumeh et al., 2015a; Soumeh et al., 2015b) were stored at -20 °C and used in the 3 new experiments. The diets were based on wheat, barley, and

fermented soybean meal as the main ingredients and were formulated to provide the same nitrogen and NE content (Table 1). Estimation of NE and standardized ileal digestible (SID) AA was done by using a diet optimization software (WinOpti version. 1.127, AgroSoft A/S, DK-7160 Tørring, Denmark) based on the feed evaluation system and matrix values published by Boisen (2007). The diets were planned to contain 0.42, 0.46, 0.50, 0.54, 0.58, and 0.62 SID Ile:Lys; 0.58, 0.62, 0.66, 0.70, 0.74, and 0.78 SID Val:Lys; and 0.70, 0.80, 0.90, 1.00, 1.10, and 1.20 of SID Leu:Lys in the Ile, Val, and Leu dose-response studies, respectively. Dietary Lys was 12.2 g total Lys/kg (11.4 g SID Lys/kg) corresponding to 93% of the requirements (Tybirk et al., 2012) in the Ile dose-response experiment and 11.8 g total Lys/kg (10.95 g SID Lys/kg) corresponding to 90% of the Lys requirement (Tybirk et al., 2012) in the Val and Leu dose-response experiments. The other non-dispensable AA and calcium and phosphorus were supplied according to or slightly exceeding the Danish recommendations for pigs weighing 9 to 15 kg (Tybirk et al., 2012). The calculated compositions of the diets are presented in Table 2.

2.2. Protocol

The experiment was designed as 3 separate randomized 6 x 6 Latin squares designs (Fisher and Yates, 1934) where 6 pigs were fed 1 of the 6 diets for 2 days, i.e. 12 days in total for each experiment having 6 replicates per treatment. During the 2 days on each diet, pigs had free access to feed for 1.5 days and were fasted overnight before they were fed 25 g diet/kg BW^{0.75} at 0700 hour on day 2. Blood samples were collected 180 min after feeding the fixed ration. The meal size and feeding time before blood sampling were fixed to reduce variation. Blood samples were assumed to be collected during the absorptive phase (Nørgaard et al., 2016) and were collected by jugular vein puncture into 10-mL heparinized tubes (Greiner BioOne GmbH, Kremsmünster, Austria) and placed on ice. Blood samples were centrifuged at 1,050 × g at 4 °C for 10 min, and the plasma was immediately harvested and stored at -80 °C until the laboratory analyses.

2.3. Chemical Analysis

Representative samples (n = 3) of each diet were analyzed to confirm the dietary treatment and assure that storage did not change the AA composition. Samples were hydrolyzed for 23 hours at 110 °C with (for Cys and Met) or without (for all other AA) performic acid oxidation, and AA were separated by ion exchange chromatography and quantified by photometric detection after ninhydrin reaction (European Commission, 1998). Plasma free AA, taurine, urea, 1-methyl-L-histidine, L- α -amino adipic acid, and L- α -amino-n-butyric acid were analyzed using an AA analyzer fitted to a lithium high performance system for physiological AA (Biochrome 30+ Amino Acid Analyzer; Biochrome, Cambridge, England). The AA analyzer was calibrated using standards for acidic, neutral, and basic AA (Sigma Aldrich, St. Louis, MO).

Plasma was analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) for the Ile and Leu experiments. Based on our previous study (Soumei et al., 2016), in which only 1 metabolite (hippuric acid) was found by liquid chromatography–mass spectrometry (LC-MS) analysis of blood from pigs fed increasing levels of SID Val:Lys, it was decided not to carry out LC-MS analysis in the current Val experiment. The analysis was done as described in detail by Soumei et al. (2016). In brief, metabolites were extracted from 200 μ l plasma mixed with 600 μ l ice-cold acetonitrile (ACN) containing glycocholic acid-(glycyl-1- 13 C) (Sigma-Aldrich, St. Louis, MO, US) and p-chlorophenylalanine (Sigma-Aldrich, St. Louis, MO, US) as internal standards (IS). After centrifugation, the supernatant was evaporated to complete dryness and re-suspended in 200 μ l water/ACN/formic acid (94.9 : 5:0.1, v/v/v) followed by transferring to vials for LC-MS/MS analysis. Chromatographic separation was performed on an UltiMate 3000 System (Thermo Scientific Dionex, Sunnyvale, CA, US) equipped with a Fortis 1.7 μ m C18 column 100 \times 2.1 mm and a Fortis UHPLC filter (Fortis Technologies, Ltd., Chesire, England). The HPLC system was connected to an Impact HD Quadrupole Time-of-Flight (QTOF) mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany).

2.4. Data processing and statistical analysis

In the study by Soumeh et al. (2016) 9, 7, and 7, molecular features were reported as discriminating metabolites which correlate with the optimum dietary Ile, Leu, and Val, respectively. Fragments and unidentified molecular features were excluded resulting in 8 compounds (i.e., 3-methyl-2-oxovaleric acid, hypoxanthine, tryptophan, indoxylsulfuric acid, glycocholic acid, tauroursodeoxycholic acid, taurocholic acid, and [M+CH₃COOH] PC (14:0/0-1:0)) which were further analyzed in the Ile dose-response and 5 compounds (i.e. α -ketoisovaleric acid, creatine, isoleucine, tryptophan, and 3-methyl-2-oxovaleric acid) in the Leu dose-response. Although 2-ketohexanoic acid was not reported in the initial study, it showed good correlations to the dietary effects of Ile and Leu in plasma and was included in the analysis. Information about compound mass and retention times was used to target and extract data from the analyzed samples using QuantAnalysis (version 2.2, Bruker Daltonics GmbH) and imported to SAS for statistical analysis as described by Soumeh et al. (2016).

The AA and metabolomics data were analyzed by the MIXED procedure of SAS (version 9.3; SAS Inst. Inc., Cary, NC, US). The experimental unit was the individual pig. The data were analyzed as a Latin square design with AA level as fixed effect and pig and sample day as random effects. Orthogonal polynomial contrast coefficients were used to determine linear and quadratic effects of increasing the AA level (Littell et al., 2002). The PROC NLIN and NLMIXED procedures of SAS were used to estimate the optimum AA level by subjecting the least squares means of the individual response metabolite to curvilinear-plateau (CLP) and broken-line (BL) models (Robbins et al., 2006). The PROC GLM was used for fitting quadratic (Q) models. The breakpoint or optimum of the curve in each model (where the first derivative could be defined) was considered the minimum AA requirement, and this estimate is presented in the Results section following the R² (Q models) or the parameter estimate standard error (SE) (BL and CLP models). Statistical significance was accepted at $P < 0.05$ and tendencies at $P < 0.10$. Presented data are least squares means and standard error of the mean (SEM).

3. Results

The plasma concentrations of AA and other metabolites are shown in Tables 3 to 5 for the Ile, Leu, and Val studies, respectively, and the results of fitting descriptive models to the metabolites are presented in Table 6 for all studies.

3.1. Isoleucine study

Eight discriminating metabolites other than AA were evaluated using the metabolomics approach, and plasma concentrations of 11 out of 18 analyzed AA and 4 out of the 8 other metabolites showed linear or quadratic responses ($P < 0.05$) to the SID Ile:Lys level (Table 3). The concentration of urea was lowest at 0.54 SID Ile:Lys but showed neither a linear ($P = 0.55$) nor a quadratic ($P = 0.61$) response. Increasing SID Ile:Lys increased Ile concentration both linearly ($P < 0.001$) and quadratically ($P < 0.001$) by a steeper increase for the 2 highest SID Ile:Lys levels, and corresponding keto-acid for Ile, 3-methyl-2-oxovaleric acid, showed a similar pattern. The other 2 BCAA were linearly ($P < 0.05$) increased by increasing SID Ile:Lys, and Leu also displayed a quadratic ($P = 0.005$) pattern with highest concentrations at 0.42 and 0.62 SID Ile:Lys. The Val keto-acid α -ketoisovaleric acid showed a quadratic response ($P = 0.01$) with lowest concentrations at 0.54 SID Ile:Lys. A common pattern of the 5 AA and other metabolites which could be modelled was a decreasing concentration until the optimum and an increased concentration at the highest SID Ile:Lys level. Optimum dietary SID Ile:Lys was found by fitting His (0.54; SE 0.09), Ser (0.52; SE 0.03), and indoxylsulfuric acid (0.54; SE 0.09) to BL models (mean of 0.53); His (0.58; SE 0.18), Ser (0.55; SE 0.05), and indoxylsulfuric acid (0.55; SE 0.12) to CLP models (mean of 0.56); and His (0.54; R^2 0.59), Ser (0.56; R^2 0.96), ketohexanoic acid (0.52; R^2 0.77), indoxylsulfuric acid (0.54; R^2 0.78), and α -ketoisovaleric acid (0.53; R^2 0.87) to Q models (mean of 0.54) with an overall mean of 0.54 SID Ile:Lys as optimum across the models (Table 6).

3.2. Leucine study

Nine non-AA metabolites and 16 out of the 18 analyzed AA in plasma showed linear or quadratic responses ($P < 0.05$) to an increasing SID Leu:Lys level (Table 4). Plasma urea showed a quadratic response ($P = 0.001$) by being reduced until 1.10 SID Leu:Lys and then an increase. Keto-hexanoic acid showed an increase until 1.10 SID Leu:Lys and then a decrease in concentration. The other 13 AA and other metabolites which could be modelled commonly showed a steep decrease from 0.70 to 0.90 SID Leu:Lys and then a plateau or a slight decrease. The concentration of Leu itself increased until 1.00 SID Leu:Lys and was then slightly reduced. The concentration of Ile and Val, and their corresponding keto-acids 3-methyl-2-oxovaleric acid and α -ketoisovaleric acid, respectively, showed a similar linear and quadratic pattern ($P < 0.05$) with a 3-5 fold decrease until 1.10 SID Leu:Lys and then a plateau. Optimum dietary SID Leu:Lys was defined by fitting Arg (0.86; SE 0.14), Glu (0.90; SE 0.12), Ile (0.93; SE 0.01), Met (0.90; SE 0.04), Phe (1.03; SE 0.10), Ser (0.95; SE 0.02), Tyr (0.90; SE 0.04), Val (0.93; SE 0.02), Tau (0.90; SE 0.04), L- α -aminoadipic acid (1.04; SE 0.07), L- α -amino-n-butyric acid (0.96; SE 0.02), 3-methyl-oxovaleric acid (1.03; SE 0.01), keto-hexanoic acid (1.04; SE 0.04), and α -ketoisovaleric acid (1.02; SE 0.02) to BL models (mean of 0.96 SID Leu:Lys). Fitting CLP models estimated the optimum dietary SID Leu:Lys by Arg (0.91; SE 0.24), Glu (0.96; SE 0.15), Ile (1.06; SE 0.04), Met (0.99; SE 0.12), Ser (1.11; SE 0.04), Tyr (1.00; SE 0.11), Val (1.08; SE 0.04), Tau (1.01; SE 0.14), L- α -aminoadipic acid (1.14; SE 0.17), L- α -amino-n-butyric acid (1.10; SE 0.06), 3-methyl-oxovaleric acid (1.21; SE 0.05), and α -ketoisovaleric acid (1.17; SE 0.05) (mean of all metabolites: 1.06 SID Leu:Lys). Quadratic modelling found optimums for Tau (1.03; R^2 0.92), L- α -aminoadipic acid (1.10; R^2 0.89), L- α -amino-n-butyric acid (1.10; R^2 0.98), and α -ketoisovaleric acid (1.17; R^2 0.99) (mean of 1.10 SID Leu:Lys). The overall mean across models and metabolites was 1.04 SID Leu:Lys as the optimum (Table 6).

3.3. Valine study

In the Val experiment, besides Val, only Cys and Gly showed a linear decreasing response ($P < 0.05$) to increasing SID Val:Lys (Table 5). The pattern of plasma urea tended to be quadratic ($P = 0.07$) with the lowest concentration at 0.70 SID Val:Lys. Broken-line models could be fitted to Lys (0.63; SE 0.04) and Tyr

(0.69; SE 0.14) with an mean of 0.66 SID Val:Lys as optimum, and CLP models could be fitted to Lys (0.65; SE 0.10) and Tyr (0.71; SE 0.22) with a mean of 0.68 as the optimum dietary SID Val:Lys (Table 6).

4. Discussion

The pattern of plasma AA and other metabolites allowed regression model fitting only to a few of them (Table 6). In previous dose-response studies in our lab using the same feed batches as the current study, it was demonstrated that 0.52 SID Ile:Lys, 0.93 SID Leu:Lys, and 0.70 SID Val:Lys were the minimum Ile, Leu, and Val requirements, respectively, to support the best growth performance of weaned piglets (Soumeh et al., 2014; Soumeh et al., 2015a; Soumeh et al., 2015b). Thus, the average of optimums of 0.54 SID Ile:Lys, 1.04 SID Leu:Lys, and 0.68 SID Val:Lys found across the 3 types of regression models reflected well the optimums found in the previous standard-design dose-response studies.

Previous work on PUN as a response variable in AA studies has led to conclusions that 2 (Pedersen and Boisen, 2001) and 3 (Coma et al., 1995) days of feeding a new diet was sufficient to re-equilibrate PUN concentrations after a change in the dietary AA concentration. The duration of feeding the experimental diets before blood sampling was 2 days, and it was hypothesized that this would be long enough to reflect dietary BCAA effects on plasma concentrations of metabolites. Plasma Ile, Leu, and Val concentrations showed close to identical relations to dietary AA levels compared to our previous 3 studies. This indicates that 2 days of adaptation may be sufficient and thus supports the conclusions by Pedersen and Boisen (2001). In their later experiment where they evaluated the Thr requirement by using a short-term plasma urea based method, a growth performance or a nitrogen balance approach, they found Thr requirement estimates close to previously reported values regardless of the methodological approach. It was not possible to fit mathematical models to the plasma urea concentrations of the current experiments, and in several other studies on estimating optimum AA requirements, plasma urea has not been proven a good biomarker (Nørgaard et al., 2015; Soumeh et al., 2015b). Pedersen and Boisen (2001) argued for taking blood samples at 3 time points after feeding an 800 g meal to reduce variation, but in the current study, variation was reduced by feeding a

small and readily edible portion (120 to 145 g) according to metabolic body weight, because variation in feed intake is a main source of variation in plasma urea concentrations (Dean et al., 2005). The inconsistency in results on plasma urea as a biomarker for protein utilization was one of the driving forces for studying the use of other plasma biomarkers as response traits in dose-response studies.

The BCAA, Ile, Leu, and Val, besides being indispensable AA, are of interest to study, because they share the same enzymes in their catabolic pathways which causes interactions among them (Harper et al., 1984), and the excess of one BCAA could increase the catabolism of the others and change the nutritional requirements (van Milgen et al., 2012). The regulatory role of especially Leu on Val and Ile degradation is reflected in the concentrations of Ile, Val, and their corresponding α -keto acids in both plasma and urine (Soumeh et al., 2016).

For the Ile experiment, the concentrations of indoxylsulfuric acid, ketohexanoic acid, and α -ketoisovaleric acid allowed to estimate an Ile requirement. In our previous study on blood samples obtained after feeding diets with increasing SID Ile:Lys for 8 and 15 days (Soumeh et al., 2016), plasma glycocholic acid and taurocholic acid were also well linked to the animal performance. The concentrations of these metabolites did not differ significantly among the dietary treatments in the present study and could not be used to estimate the Ile requirement. The current study cannot state if plasma glycocholic acid and taurocholic acid are appropriate biomarkers of different SID Ile:Lys levels because of the different study design, and it cannot be ignored that the number of days of feeding (i.e. 2 vs. 8 and 15 days) may affect the metabolic profile. In addition, to claim that a specific metabolite is a good and general biomarker, the metabolite should be validated in another experiment with different diets.

In plasma, only concentrations of creatine and 3-methyl-2-oxovaleric acid could discriminate the optimum dietary Leu to support animal growth performance (Soumeh et al., 2016), whereas in the current experiment, several AA and other metabolites could be used to estimate the Leu requirement. 3-Methyl-2-oxovaleric acid is the corresponding α -keto acid for Ile, and α -ketoisovaleric acid is the corresponding α -keto

acid for Val. The concentrations of these metabolites as well as Ile and Val were relatively lower in plasma with increasing SID Leu:Lys. The results underline the important roles of Leu in the regulation of protein synthesis and activity of enzymes involved in oxidative decarboxylation of BCAA.

Only metabolites showing biologically meaningful patterns are reasonable to model, and thus it may be difficult to model response curves for AA where the growth performance response is unknown. There is a variation among studies in which AA and other metabolites are reflecting the changes in growth performance. Thus, it may not be possible to choose a distinct panel of biomarkers beforehand, and relevant biomarkers need to be identified from study to study by their metabolic relevance and consistency of their expression patterns. Although this way to evaluate an optimal AA requirement is based on a somewhat subjective evaluation, the strength in the methodological approach will lie in a greater number of evaluated and modelled metabolites as response traits. However, the choice of whether to perform an intensive short-term experiment based on blood samples or carry out a traditional longer-term experiment based on performance traits from numerous animals may also be affected by the greater costs of laboratory analysis associated with evaluating blood biomarkers. Thus, the blood biomarkers analyzed by LC-MS/MS can be useful in evaluating AA requirements in dose-response experiments – at least as supplements to the traditional growth performance traits. In the long term, i.e. when a sufficient validation of consistency of the metabolic profile has occurred, cheaper analytical methods such as ELISA (Enzyme-linked immunosorbent assay) may be developed for efficient analysis.

5. Conclusions

These results indicated that the response of plasma metabolites, associated with a short-term feeding to pigs, were suitable to fit to regression models to obtain an optimum SID Ile, Leu, and Val ratios close to those obtained in previous conventional studies using the same diets. Furthermore, the results indicated that 2 days of adaptation may be sufficient to reflect relevant biological changes in blood to different levels of dietary AA.

6. Implications

The current experiment evaluated a new approach to study Ile, Leu, and Val requirements in pigs with a considerably lower number of experimental animals compared to traditional animal performance experiments. The results indicated that modelling plasma metabolites in AA dose-response studies can be used as the sole response trait in estimating the AA requirement of pigs. Until this approach is fully validated, metabolites found by metabolomics can complement the traditional gain and feed intake response traits. The use of resources for laboratory analysis using LC-MS/MS is much greater than a performance-based approach, and more efficient assays should be developed. However, in addition to estimating a value for optimum dietary AA level, a metabolite-based approach may provide interesting knowledge on physiological mechanisms.

Conflict of interest

The authors have no conflict of interests regarding this work

Acknowledgements

The authors greatly acknowledge the financial support by the Danish Council for Independent Research – Technology and Production Science, Ajinomoto Eurolysine S.A.S., and Aarhus University.

References

- Boisen, S., 2007. A new concept for practical feed evaluation systems. DJF Animal Science No. 79. Aarhus University, Denmark. Retrieved from <http://web.agrsci.dk/djfpublikation/djfpdf/djfhus79.pdf> (21.03.2017).
- Boisen, S., Hvelplund, T., Weisbjerg, M.R., 2000. Ideal amino acid profiles as a basis for feed protein evaluation. *Livest. Prod. Sci.* 64, 239-251.
- Coma, J., Carrion, D., Zimmerman, D.R., 1995. Use of plasma urea nitrogen as a rapid response criterion to determine the lysine requirement of pigs. *J. Anim. Sci.* 73, 472-481.

- Dean, D.W., Southern, L.L., Kerr, B.J., Bidner, T.D., 2005. Isoleucine requirement of 80- to 120-kilogram barrows fed corn-soybean meal or corn-blood cell diets. *J. Anim. Sci.* 83, 2543-2553.
- European Commission, 1998. Commission Directive 98/64/EC of 3 September 1998 establishing Community methods of analysis for the determination of amino-acids, crude oils and fats, and olaquinox in feedingstuffs and amending Directive 71/393/EEC. *Official Journal of the European Communities Eur-Lex* - 31998L0064.
- Fisher, R.A., Yates, F., 1934. The 6 x 6 Latin squares. *Proc. Camb. Philos. Soc.* 30, 492-507.
- Hansen, M.J., Nørgaard, J.V., Adamsen, A.P.S., Poulsen, H.D., 2014. Effect of reduced crude protein on ammonia, methane, and chemical odorants emitted from pig houses. *Livest. Sci.* 169, 118-124.
- Harper, A.E., Miller, R.H., Block, K.P., 1984. Branched-chain amino acid metabolism. *Annu. Rev. Nutr.* 4, 409-454.
- Littell, R.C., Stroup, W.W., Freund, R.J., 2002. *SAS for linear models*. 4th ed. SAS Inst., Cary, NC, USA.
- Nørgaard, J.V., Canibe, N., Soumeh, E.A., Jensen, B.B., Nielsen, B., Derkx, P., Cantor, M.D., Blaabjerg, K., Poulsen, H.D., 2016. Evaluation of in situ valine production by *Bacillus subtilis* in young pigs. *Animal* 10, 1796-1802.
- Nørgaard, J.V., Hansen, M.J., Soumeh, E.A., Adamsen, A.P.S., Poulsen, H.D., 2014. Effect of protein level on performance, nitrogen utilisation and carcass composition in finisher pigs. *Acta Agr. Scand. A-AS.* 64, 123-129.
- Nørgaard, J.V., Pedersen, T.F., Soumeh, E.A., Blaabjerg, K., Canibe, N., Jensen, B.B., Poulsen, H.D., 2015. Optimum standardized ileal digestible tryptophan to lysine ratio for pigs weighing 7–14 kg. *Livest. Sci.* 175, 90-95.

- Pedersen, C., Boisen, S., 2001. Studies on the response time for plasma urea nitrogen as a rapid measure for dietary protein quality in pigs. *Acta Agr. Scand. A – AS*. 51, 209-216.
- Pedersen, C., Lindberg, J.E., Boisen, S., 2003. Determination of the optimal dietary threonine:lysine ratio for finishing pigs using three different methods. *Livest. Prod. Sci.* 82, 233-243.
- Robbins, K., Saxton, A., Southern, L., 2006. Estimation of nutrient requirements using broken-line regression analysis. *J. Anim. Sci.* 84, E155-E164.
- Soumeh, E., M. S. Hedemann, H. D. Poulsen, E. Corrent, J. van Milgen, J. V. Nørgaard. 2016. Non-targeted LC-MS metabolomics approach for metabolic profiling of plasma and urine from pigs fed branched chain amino acids for maximum growth performance. *J. Proteome Res.* 15, 4195-4207.
- Soumeh, E.A., Van Milgen, J., Sloth, N.M., Corrent, E., Poulsen, H.D., 2015a. The optimum ratio of standardized ileal digestible leucine to lysine for 8 to 12 kg female pigs. *J. Anim. Sci.* 93, 2218-2224.
- Soumeh, E.A., van Milgen, J., Sloth, N.M., Corrent, E., Poulsen, H.D., Nørgaard, J.V., 2014. The optimum ratio of standardized ileal digestible isoleucine to lysine for 8–15 kg pigs. *Anim. Feed Sci. Technol.* 198, 158-165.
- Soumeh, E.A., van Milgen, J., Sloth, N.M., Corrent, E., Poulsen, H.D., Nørgaard, J.V., 2015b. Requirement of standardized ileal digestible valine to lysine ratio for 8- to 14-kg pigs. *ANIMAL* 9, 1312-1318.
- Tybirk, P., Sloth, N.M., Jørgensen, L., 2012. Nutrient requirement standards. In: 17th Edition of the Danish Nutrient Standards. Pig Research Centre, Denmark. Retrieved from <http://www.pigresearchcentre.dk/About%20us/Nutrient%20standards.aspx> (02.12.2013).

van Milgen, J., Gloaguen, M., Le Floc'h, N., Brossard, L., Primot, Y., Corrent, E., 2012. Meta-analysis of the response of growing pigs to the isoleucine concentration in the diet. *Animal* 6, 1601-1608.

Table 1

Composition of experimental diets (g/kg, as-fed).

Item	Study		
	Isoleucine ¹	Valine ²	Leucine ³
Wheat	705.6	672.0	726.1
Barley	100.0	100.0	100.0
Fermented soybean meal ⁴	106.5	151.0	78.9
Animal fat	20.0	20.0	20.0
Calcium carbonate	16.6	17.0	16.3
Monocalcium phosphate	13.3	11.3	14.8
NaCl	4.4	4.3	4.4
Vitamin-mineral premix ⁵	4.0	4.0	4.0
L-Lys HCl (78%)	7.9	5.6	8.5
DL-Met (99%)	2.5	2.0	2.9
L-Thr (98%)	3.5	2.6	4.0
L-Trp (98%)	1.1	0.8	1.3
L-Val (99%)	3.2	-	3.8
L-Ile (98%)	-	0.7	2.4
L-His (98%)	1.0	0.5	1.4
L-Leu (98%)	3.8	2.1	-

L-Phe (98%)	1.7	0.6	3.3
L-Tyr (98%)	1.4	0.6	1.0
Phytase ⁶	0.2	0.2	0.2
Microgrits ⁷	0.7	0.7	0.7

¹ L-Ile (98%) was added to the basal diet at graded levels of 0, 0.5, 0.9, 1.4, 1.9, and 2.3 g/kg to provide 0.42, 0.46, 0.50, 0.54, 0.58, and 0.62 SID Ile:Lys levels. L-Glu (98%) was included at graded levels of 2.5, 2.0, 1.6, 1.1, 0.6, and 0.2 g/kg to compose isonitrogenic diets.

² L-Val (99%) was added to the basal diet at graded levels of 0, 0.4, 0.9, 1.3, 1.8, and 2.2 g/kg to provide 0.58, 0.62, 0.66, 0.70, and 0.78 SID Val:Lys levels. L-Glu (98%) was included at graded levels of 4.0, 3.6, 3.8, 3.1, 2.7, and 1.8 g/kg to compose isonitrogenic diets.

³ L-Leu (98%) was added to the basal diet at graded levels of 0, 1.1, 2.2, 3.3, 4.5, and 5.6 g/kg to provide 0.70, 0.80, 0.90, 1.00, 1.10, and 1.20 SID Leu:Lys levels. L-Glu (98%) was included at graded levels of 6.0, 4.9, 3.8, 2.7, 1.5, and 0.4 g/kg to compose isonitrogenic diets.

⁴ HP300 (Hamlet Protein, Horsens, Denmark).

⁵ Provided the following per kg of diet: 10,000 IU vitamin A, 2,000 IU vitamin D₃, 94 IU vitamin E, 2.4 mg vitamin K₃, 2.4 mg vitamin B₁, 4.8 mg vitamin B₂, 2.4 mg vitamin B₆, 0.02 mg vitamin B₁₂, 12 mg D-panthotenic acid, 26 mg niacin, 0.2 mg biotin, 200 mg Fe (Fe(II) sulphate), 165 mg Cu (Cu(II) sulphate), 200 mg Zn (Zn(II) oxide), 56 mg Mn (Mn(II) oxide), 0.3 mg KI, 0.3 mg Se (Se-selenite).

⁶ Natuphos 5000, 5641 FTU/g (BASF, Ludwigshafen, Germany).

⁷ Corn bran in various colors to identify diets.

Table 2

Chemical composition of the experimental diets (g/kg, as-fed).

Item	Study		
	Isoleucine ¹	Valine ²	Leucine ³
Net energy MJ/kg	10.5	10.4	10.4
Crude protein	163.0	177.0	154.0
Lys	12.2	11.8	11.8
Met	4.5	4.0	4.7
Met + Cys	7.2	6.6	7.3
Thr	8.2	7.4	8.2
Val	9.5	-	9.5
Ile	-	6.4	7.1
Leu	13.7	12.4	-
Phe	7.6	6.9	8.5
His	4.4	3.9	4.3

¹ Ile content was 5.4, 5.9, 6.3, 6.8, 7.2, and 7.8 g/kg in 0.42, 0.46, 0.50, 0.54, 0.58, and 0.62 SID Ile:Lys diets.

² Val content was 7.4, 7.8, 8.2, 8.7, 9.1, and 9.5 g/kg in 0.58, 0.62, 0.66, 0.70, 0.74, and 0.78 SID Val:Lys diets.

³ Leu content was 9.0, 10.1, 11.2, 12.2, 13.3, and 14.4 g/kg in 0.70, 0.80, 0.90, 1.00, 1.10, and 1.20 SID Leu:Lys diets.

Table 3

Effect of increasing dietary standardized ileal digestible (SID) Ile:Lys for pigs fed 1 of 6 diets for 2 days in a Latin square design on plasma concentrations of urea (mmol/L) amino acids (mmol/L) and non-amino acid metabolites (arb units).

	SID Ile:Lys						SEM ¹	P-value ²	
	0.42	0.46	0.50	0.54	0.58	0.62		Lin	Quad
Urea	1.625	1.471	1.706	1.054	1.508	1.454	0.437	0.55	0.61
Ala	0.925	0.817	0.842	0.922	0.799	0.820	0.069	0.19	0.90
Arg	0.182	0.133	0.176	0.159	0.139	0.193	0.017	0.46	0.002
Cys	0.020	0.019	0.022	0.022	0.022	0.022	0.005	0.11	0.99
Glu	0.304	0.256	0.241	0.314	0.230	0.262	0.036	0.27	0.54
Gly	1.151	1.117	1.069	1.081	0.952	0.993	0.128	0.02	0.93
His	0.018	0.017	0.015	0.015	0.012	0.017	0.003	0.25	0.24
Ile	0.079	0.061	0.113	0.110	0.144	0.197	0.014	<0.001	<0.001
Leu	0.241	0.181	0.226	0.233	0.224	0.271	0.017	0.01	0.005
Lys	0.521	0.373	0.422	0.505	0.442	0.506	0.053	0.36	0.03
Met	0.109	0.104	0.109	0.100	0.112	0.123	0.009	0.10	0.05
Orn	0.162	0.118	0.137	0.116	0.106	0.134	0.014	0.003	0.001
Phe	0.126	0.106	0.127	0.113	0.118	0.139	0.006	0.03	0.003
Pro	0.416	0.408	0.409	0.388	0.356	0.401	0.036	0.18	0.53
Ser	0.258	0.237	0.228	0.211	0.216	0.226	0.021	0.05	0.12
Tau	0.061	0.098	0.076	0.053	0.069	0.064	0.015	0.20	0.49
Thr	0.501	0.634	0.537	0.480	0.437	0.533	0.065	0.06	0.92
Trp	0.095	0.087	0.094	0.087	0.087	0.094	0.067	0.62	0.09

Tyr	0.161	0.135	0.159	0.138	0.138	0.179	0.017	0.48	0.05
Val	0.422	0.418	0.437	0.442	0.423	0.491	0.027	0.05	0.29
[M+CH ₃ COOH] PC ³	1980	1930	2133	1989	1996	2013	125	0.77	0.58
3-m-2-oxoval. ⁴	557	446	711	666	817	1176	124	<0.001	0.003
Glycocholic acid	349	242	203	303	294	237	63	0.37	0.31
Hypoxanthine	1153	1498	1023	888	1122	937	103	0.01	0.82
Indoxylsulfuric acid	2308	2129	2068	1744	1908	2136	430	0.45	0.289
Ketohexanoic acid	1643	1307	1334	1253	1175	1668	136	0.53	<0.001
Tauro.cholic acid ⁵	73	99	76	97	121	78	18	0.38	0.27
α-ketoisovaleric acid	290	266	250	237	238	287	22	0.43	0.01

¹SEM = standard error of the mean

² Orthogonal polynomial contrast coefficients were used to determine linear (Lin) and quadratic (Quad) effects of increasing SID Ile:Lys.

³ [M+CH₃COOH] PC = [M+CH₃COOH] PC(14:0/0-1:0).

⁴ 3-m-2-oxoval. = 3-methyl-2-oxovaleric acid.

⁵ Tauro.cholic acid = Tauroursodeoxycholic acid.

Table 4

Effect of increasing dietary standardized ileal digestible (SID) Leu:Lys for pigs fed 1 of 6 diets for 2 days in a Latin square design on plasma concentrations of urea (mmol/L) amino acids (mmol/L) and non-amino acid metabolites (arb units).

	SID Leu:Lys						SEM ¹	P-value ²	
	0.70	0.80	0.90	1.00	1.10	1.20		Lin	Quad
Urea	1.179	0.798	0.767	0.972	0.560	1.673	0.213	0.19	0.001
Ala	1.373	1.044	0.767	0.750	0.614	0.576	0.082	<0.001	<0.001
Arg	0.164	0.138	0.096	0.138	0.114	0.142	0.019	0.05	<0.001
Cys	0.024	0.022	0.022	0.023	0.022	0.019	0.003	0.09	0.55
Glu	0.411	0.338	0.237	0.315	0.221	0.251	0.040	<0.001	0.01
Gly	1.078	0.987	0.803	0.866	0.742	0.711	0.083	<0.001	0.21
His	bd	bd	bd	bd	bd	bd	bd		
Ile	0.593	0.380	0.183	0.157	0.115	0.109	0.023	<0.001	<0.001
Leu	0.079	0.114	0.135	0.177	0.171	0.146	0.019	<0.001	<0.001
Lys	0.460	0.448	0.324	0.424	0.379	0.406	0.056	0.11	0.05
Met	0.122	0.111	0.091	0.100	0.094	0.087	0.011	0.002	0.25
Orn	0.116	0.110	0.082	0.100	0.086	0.089	0.011	0.002	0.09
Phe	0.161	0.173	0.144	0.128	0.124	0.134	0.016	0.003	0.36
Pro	0.433	0.404	0.307	0.387	0.301	0.267	0.033	<0.001	0.94
Ser	0.238	0.212	0.181	0.172	0.166	0.165	0.019	<0.001	0.02
Tau	0.076	0.070	0.053	0.052	0.052	0.060	0.009	0.010	0.02
Thr	0.630	0.551	0.450	0.517	0.447	0.424	0.053	0.01	0.38
Trp	0.093	0.091	0.072	0.085	0.073	0.074	0.011	0.004	0.40

Tyr	0.164	0.147	0.111	0.125	0.113	0.108	0.013	<0.001	0.06
Val	1.565	1.036	0.585	0.517	0.380	0.358	0.053	<0.001	<0.001
1-Methyl-L-histidine	0.012	0.012	0.014	0.012	0.014	0.014	0.001	0.004	0.36
3-m-2-oxoval. ³	1237	997	725	498	422	402	92	<0.001	0.01
Creatine	176	139	147	139	89	202	42	0.89	0.03
Ketohexanoic acid	161	229	368	527	603	520	68	<0.001	0.010
L- α -aminoadipic acid	0.117	0.110	0.099	0.093	0.083	0.095	0.010	<0.001	0.01
L- α -amino-butyrac ⁴	0.095	0.079	0.055	0.046	0.043	0.047	0.007	<0.001	<0.001
α -ketoisovaleric acid	339	277	191	150	135	126	21	<0.001	0.003

¹SEM = standard error of the mean

² Orthogonal polynomial contrast coefficients were used to determine linear (Lin) and quadratic (Quad) effects of increasing SID Leu:Lys.

³ 3-m-2-oxoval. = 3-methyl-2-oxovaleric acid.

⁴ L- α -amino-butyrac = L- α -amino-n-butyrac acid.

Table 5

Effect of increasing dietary standardized ileal digestible (SID) Val:Lys for pigs fed 1 of 6 diets for 2 days in a Latin square design on plasma concentrations of urea (mmol/L) and amino acids (mmol/L).

	SID Val:Lys						SEM ¹	P-value ²	
	0.58	0.62	0.66	0.70	0.74	0.78		Lin	Quad
Urea	3.135	2.841	2.929	2.806	3.192	3.370	0.301	0.22	0.07
Ala	0.666	0.840	0.755	0.721	0.702	0.750	0.060	0.93	0.25
Arg	0.232	0.228	0.226	0.220	0.194	0.257	0.025	0.92	0.12
Cys	0.025	0.023	0.021	0.020	0.017	0.020	0.003	0.01	0.23
Glu	0.295	0.306	0.294	0.291	0.252	0.300	0.041	0.53	0.74
Gly	0.903	1.124	0.853	0.871	0.857	0.883	0.092	0.05	0.91
His	0.015	0.015	0.014	0.015	0.015	0.016	0.001	0.89	0.45
Ile	0.128	0.142	0.137	0.122	0.128	0.145	0.010	0.64	0.34
Leu	0.205	0.224	0.211	0.210	0.188	0.219	0.016	0.79	0.89
Lys	0.450	0.406	0.389	0.407	0.349	0.434	0.048	0.52	0.22
Met	0.088	0.088	0.089	0.084	0.074	0.089	0.008	0.51	0.63
Orn	0.150	0.144	0.143	0.140	0.119	0.147	0.013	0.31	0.37
Phe	0.135	0.131	0.120	0.128	0.117	0.126	0.008	0.13	0.27
Pro	0.395	0.444	0.425	0.398	0.397	0.428	0.052	0.99	0.97
Ser	0.232	0.261	0.252	0.219	0.210	0.243	0.022	0.10	0.81
Tau	0.045	0.052	0.056	0.044	0.051	0.050	0.061	0.63	0.31
Thr	0.400	0.450	0.442	0.372	0.306	0.440	0.051	0.13	0.36
Trp	0.092	0.102	0.097	0.084	0.084	0.096	0.008	0.36	0.54
Tyr	0.148	0.141	0.136	0.122	0.123	0.151	0.015	0.35	0.13

Val	0.191	0.232	0.288	0.306	0.325	0.446	0.018	<0.001	0.13
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¹SEM = standard error of the mean

² Orthogonal polynomial contrast coefficients were used to determine linear (Lin) and quadratic (Quad) effects of increasing SID Val:Lys.

Table 6

Optimum SID Ile:Lys, Leu:Lys and Val:Lys of broken-line (BL), curvilinear-plateau (CLP), and quadratic (Q) models fitted to blood metabolites and the number of metabolites.

	Fitting model				Former study	
				Optimum or n		
	BL	CLP	Q	metab. ¹	Reference	Conclusion
Ile:Lys	0.53	0.56	0.54	0.54	Soumeh et al., 2014	0.52
Leu:Lys	0.96	1.06	1.10	1.04	Soumeh et al., 2015a	0.93
Val:Lys	0.66	0.68	0.69	0.68	Soumeh et al., 2015b	0.70
Ile:Lys, n metab. ²	3	3	5	5		
Leu:Lys, n metab.	14	12	4	14		
Val:Lys, n metab.	2	2	1	2		

¹ Mean of the 3 models or number of unique amino acids and other metabolites used for modelling.

² Number of amino acids and other metabolites used for modelling.