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1 **Running title:** Feeding pigs proteins or free amino acids

2 **Apparent jejunal amino acid digestibility, gut morphology, and the expression of**
3 **intestinal amino acid transporters in pigs fed protein or free amino acids¹**

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9

LAY SUMMARY

10 The amino acids (AA) in the diet of pigs are usually supplied as a mix of proteins and free AA.
11 Proteins need to be digested first before their constituent AA become available to the animal.
12 Free AA, together with small peptides, are readily available without the need for digestion.
13 Moreover, the different forms by which AA are supplied may also have an effect on intestinal
14 physiology. In this experiment, pigs were fed different diets with the same AA profile but were
15 provided either as protein, as free AA and small peptides originating from hydrolyzed protein,
16 or as a mix of individual free AA. Results showed that feeding pigs free AA and/or small
17 peptides resulted in higher apparent jejunal digestibility of AA compared to feeding proteins.
18 Furthermore, the apparent jejunal digestibility of the AA of a hydrolyzed protein were higher
19 than that of free AA. Providing diets with readily absorbable AA resulted in a higher surface
20 area for absorption and an increase in the gene expression of AA transporters as opposed to
21 proteins.

22 **Teaser text**

23 Dietary amino acids supplied in the diet of pig as protein or free form have different effects on
24 the intestinal morphology and gene expression of amino acid transporters.

ABSTRACT

25
26 Dietary amino acids (**AA**) supplied as protein or in free form are digested and absorbed at
27 different rates, but can also induce differences in the intestinal physiology of pigs. We compared
28 the apparent jejunal AA digestibility, intestinal morphology, and gene expression of AA
29 transporters of pigs fed diets providing different forms of AA. Thirty growing pigs (33.7 ± 4.1
30 kg) were fed one of three experimental diets that provided AA either as protein from feather
31 meal (**INT**), as free AA and small peptides obtained by extensive acid hydrolysis of feathers
32 (**HYD**), or as a mix of individual purified AA with the same AA profile as HYD (**FAA**). Pigs
33 were fed the same quantity of feed, energy, and AA. After 14 days, pigs were slaughtered 3 h
34 after feeding a meal with indigestible markers. Digesta and tissue were collected from different
35 sections of the small intestine. Jejunal digesta was used to measure apparent jejunal digestibility
36 of AA. Samples of the duodenum, jejunum, and ileum were used to measure intestinal
37 morphology and the gene expression of intestinal AA transporters. The measured apparent
38 jejunal digestibility of AA of INT was lower compared to HYD and FAA ($P < 0.05$). The
39 apparent jejunal digestibility of Cys, Gly, His, Met, and Pro was lower for FAA compared to
40 HYD ($P < 0.05$). This may be due to the small peptides in HYD, which are absorbed faster than
41 individual AA. The villi area in the ileum of HYD fed pigs was the highest ($P < 0.05$) among
42 the treatments, which may be associated with the reabsorption of endogenous proteins, which
43 occurs mostly in the ileum. In the duodenum, HYD and FAA had lower expression of PepT1
44 ($P < 0.01$) probably due to the rapid transit time of digesta compared to INT fed pigs. Pigs fed
45 HYD expressed more ASCT2 ($P = 0.02$) and CAT-1 ($P = 0.04$) in the jejunum compared to the
46 pigs fed the other diets. The expression of these transporters along the intestine depended on
47 the relative abundance of readily absorbable dietary AA. Results showed that dietary AA form
48 can have an influence on the morphology and on the expression of different AA transporters
49 along the different sections of the small intestine.

50 **Key words:** amino acid transporter, apparent jejunal digestibility, free amino acid, protein,
51 intestinal morphology, pig
52

- 53 **List of abbreviations**
- 54 AA – amino acids
- 55 TiO₂ – titanium dioxide
- 56 Co-EDTA – cobalt EDTA
- 57 RNA – ribonucleic acid
- 58 DNA - deoxyribonucleic acid
- 59 PPIA – peptidylprolyl isomerase A
- 60 HSPCB – heat shock 90kD protein 1 β
- 61 C_t – crossing threshold
- 62 PCR – polymerase chain reaction

INTRODUCTION

63

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

73 The dynamics of protein digestion can affect the availability of AA for the animal and
74 subsequently their metabolism. Pre-hydrolyzed dietary proteins have a higher digestibility than
75 proteins (Vente-Spreuwenberg et al., 2004; Grazziotin et al., 2006) and free AA and small
76 peptides do not need to be digested and can be rapidly absorbed after feeding (Weijzen et al.,
77 2022). Both the reduction and the lack of a need for digestion of ingested food can induce
78 differences in the digestive and absorptive capacities of the small intestine (Dai et al., 2013;
79 Morales et al., 2017). These differences can be seen through physiological changes in the
80 morphology and the expression of different AA and peptide transporters in the intestine of pigs
81 (Ma et al., 2019).

82 The objective of this experiment was to compare the apparent digestibility of AA, intestinal
83 morphology, and gene expression of AA transporters in the three sections of the small intestine
84 of pigs after feeding a diet that supplies AA either as proteins or as free AA and/or small
85 peptides from an extensively hydrolyzed protein or as purified AA. However, digesta samples
86 in the duodenum and ileum were insufficient for AA analysis so only jejunum samples were
87 analyzed. We hypothesized that the higher availability of absorbable AA in the proximal part

88 of the small intestine would result in an increased surface area of absorption and increased
89 expression of AA transporter genes in the proximal part compared to the latter part of the small
90 intestine.

91 **MATERIALS AND METHODS**

92 *Ethics approval for animal experimentation*

93 The experiment followed the ARRIVE animal experimentation guidelines and the EU directive
94 2010/63/EU for animal experiments. The protocol was approved by the regional ethical
95 committee and the experiment was granted authorisation (APAFiS #27196-2020091509061764
96 v2) by the Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation.

97 *Animal housing, diets, and experimental design*

98 Thirty male Piétrain x Large-White x Landrace growing pigs (33.7 ± 4.1 kg) were selected at
99 12 wk of age and were randomly assigned to one of three experimental diets during this 14-d
100 experiment. The animals used in the experiment were all fitted with jugular catheters because
101 they were also used in another experiment (Eugenio et al., 2022) involving a meal test followed
102 by serial blood sampling. This was done in the middle of this experiment (at 7 d). The
103 experiment was done in the facilities of UE3P (INRAE, Saint-Gilles, France
104 <https://doi.org/10.15454/1.5573932732039927E12>), where the pigs were housed in individual
105 pens (1.2 x 2.0 m) equipped with a nipple drinker and a feeding trough in an environmentally
106 controlled room (temperature: 26 °C; relative humidity: 60%; 12 h of light). The experimental
107 diets provided AA either as protein from feather meal (**INT**), as free AA and small peptides
108 from extensively acid hydrolyzed feathers (**HYD**), or as free AA with the same AA profile as
109 HYD using purified AA (**FAA**). The ingredient and nutrient composition of the experimental
110 diets are given in Table 1. Purified L-Trp (Sigma Aldrich Chimie, St. Quentin Fallavier, France)
111 was also added in HYD and FAA as the extensively hydrolyzed feather was completely devoid
112 of this essential AA. Apart from L-Cys and L-Tyr, which were provided by BCF Life Sciences

113 (Pleucadeuc, France), all other purified AA were obtained from Sigma Aldrich Chimie (St.
114 Quintin Fallavier, France). Experimental diets were offered as mash. A daily feed allowance of
115 1,200 g was distributed twice daily as meals (morning and afternoon). For each meal, the pigs
116 were offered a mixture of the experimental diet (at 90%) and a regular, pelleted starter diet (at
117 10%; 15.3% CP) as there were palatability issues when exclusively feeding the experimental
118 diets. The inclusion of the starter diet was marginal, and only a minor and similar impact on the
119 measured responses for the three diets was expected. On the 14th day of the experiment, pigs
120 were offered a small meal (300 g), the amount corresponding to the meal given during the meal
121 test used to measure the postprandial plasma AA kinetics (Eugenio et al 2022), of their
122 respective experimental diets mixed with indigestible markers titanium dioxide (**TiO₂**) and
123 cobalt-EDTA (**Co-EDTA**) that were each added at 4%.

124 *Slaughter and tissue sample collection*

125 Three hours after feeding the diet with indigestible markers, pigs were sedated with general
126 anesthesia by injecting 10 mL per kg body weight of ketamine (Imalgene 1000, Merial, Lyon,
127 France), then euthanized by injection of 5 mL per 50 kg body weight of a cocktail of
128 embutramide, mebezonium, and tetracaine (T61, MSD Santé Animale, Beaucozé, France) via
129 the catheter followed by exsanguination. Immediately after opening the abdominal cavity, the
130 stomach until the distal ileum was removed, and the cardiac, pyloric, and ileocecal sphincters
131 were immediately clamped. The small intestine was divided in three parts corresponding to the
132 duodenum, jejunum, and ileum. The duodenum was defined as the first 70 cm of the small
133 intestine, the ileum as the last 70 cm (Al-Masri et al., 2015), and the jejunum as the segment in-
134 between. The contents of each intestinal segment were collected, weighed, and frozen. The
135 frozen intestinal contents were freeze-dried later, ground, and stored in a cold room for future
136 analysis. For the tissue samples, the middle part of each segment was collected and rinsed with
137 saline water and placed on a cold plate rinsed with RNase-away (Invitrogen Corp, Carlsbad,

138 USA). This sample was then divided into two parts. One sample of around 3 to 4 cm was
139 immediately placed in 10% neutral buffered formalin and kept in a cold room. The other sample
140 was finely cut and around 2 g of tissue was collected in microtubes and immediately placed in
141 liquid nitrogen before being stored at -80 °C for future analysis.

142 *Chemical analyses of feed and intestinal content samples*

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

153 *Marker quantification and apparent jejunal digestibility calculations*

154 It was intended to measure the AA digestibility in the duodenum, jejunum, and ileum. However,
155 as previously mentioned, the collected quantity of digesta from the duodenum and ileum was
156 not enough to perform the AA analysis. Hence, only samples collected from the jejunum were
157 used. The apparent jejunal digestibility was used as an indicator of the difference in AA
158 digestibility between diets as most of the AA absorption happens in the jejunum (Bröer and
159 Fairweather, 2018).

160 The indigestible markers were quantified in the feed and jejunal contents using a modified
161 technique by Tsanaktsidou and Zachariadis (2020). Briefly, the TiO₂ and Co-EDTA in the
162 samples were determined using wet acid digestion and mineralization by first boiling the

163 samples in 30 mL of concentrated nitric acid for 2 min followed by addition of concentrated
164 sulfuric acid while heating for 4 min or until the solution becomes clear to white yellowish. The
165 mineralized samples were then allowed to cool by placing the samples in an ice bath and diluted
166 to 100 mL using deionized water (Milli-Q system, Millipore, USA). For each marker, a
167 calibration curve was established using 6-point curve (concentrations: 0.0, 0.1, 0.2, 0.5, 0.8,
168 1.0, and 2.0) by preparing standard solutions with TiO₂ and Co-EDTA. Using an inductively
169 coupled plasma-atomic emission spectrometer (SpectrAA 220FS, Varian, Grenoble, France),
170 the concentrations (as mg·L⁻¹) of titanium and cobalt were determined at 336.112 nm and
171 231.160 nm, respectively. These concentrations were then used to compute the quantities (as
172 g·kg⁻¹) of TiO₂ and Co-EDTA in the samples.

173 For each marker, the apparent jejunal digestibility (%) was calculated as:

$$174 \quad \text{Digestibility} = \left[1 - \left(\frac{\text{Marker}_{\text{Diet}}}{\text{Nutrient}_{\text{Diet}}} \right) * \left(\frac{\text{Marker}_{\text{Digesta}}}{\text{Nutrient}_{\text{Digesta}}} \right) \right] * 100$$

175 *Intestinal morphology*

176 The tissue samples from the duodenum, jejunum, and ileum that were fixed in formalin were
177 transferred to 70% ethanol in less than 48 h. Two small samples from each tissue sample were
178 cut and embedded in paraffin. Small bands of the paraffin embedded tissue were finely cut (5
179 μM) using a microtome (HistoCore Multicut, Leica Biosystems, Illinois, USA) and mounted
180 on glass slides. The samples were then stained with hematoxylin and eosin and examined under
181 a light microscope (Eclipse E400, Nikon, Champigny-sur-Marne, France) using an image
182 analysis software (NIS-Elements AR 3.0; Nikon Instruments). The measurements on villi
183 (height and area) and crypts (depth) were done on at least 20 villi and 20 crypts that were well
184 oriented for each sample with x 40 magnification using the imageJ software
185 (<https://imagej.net/software/imagej/>). These were then averaged per pig for all further analysis.
186 From these measurements, the villi height to crypt depth ratio was calculated.

187 *Gene expression of amino acid and peptide transporters*

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

199 Complementary deoxyribonucleic acid (**DNA**) was synthesized from 2 μ g of total RNA in a
200 reverse transcription reaction using a commercial high-capacity RNA-to-DNA kit (Applied
201 Biosystems, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) and incubated in a
202 thermocycler (PTC-100 Thermal Cycler, MJ Research, Massachusetts, USA) for 60 min at 37
203 $^{\circ}$ C. The reaction was stopped by elevating the temperature to 95 $^{\circ}$ C for 5 min. The primers for
204 the peptide transporter, PepT1 (SLC15A1), and the AA transporters ASCT2 (SLC1A5), b⁰⁺AT
205 (SLC7A9), and y⁺LAT1 (SLC7A7) have been described previously (Sun et al., 2015; Morales
206 et al., 2017) while for CAT-1 (SLC7A1), a specific primer was designed using their published
207 sequences at the Genbank. Two reference genes, peptidylprolyl isomerase A (**PPIA**) and heat
208 shock 90kD protein 1 β (**HSPCB**), were used to normalize the variations on messenger RNA
209 levels (Park et al., 2015). The primers and their sequences are summarized in Table 2.

210 A calibration curve for each primer, including that of reference genes, was generated using a
211 pooled complementary DNA sample (calibrator) with an equal representation of each diet and

212 each tissue that was diluted seven times (16, 4, 1, 0.25, 0.06, 0.02, and 0.003 ng·μL⁻¹) and
213 plotting its crossing threshold (C_t) against the log₁₀ value of each dilution after a polymerase
214 chain reaction (**PCR**; Applied Biosystems, California, USA) run. The arbitrary efficacy of each
215 primer was then calculated using the log₁₀ of the slope (linear regression) for each gene.

216 The expression of the target genes in the duodenum, jejunum, and ileum was estimated using a
217 quantitative PCR assay using the Fast SYBR Green Master Mix (Applied Biosystems). Briefly,
218 a mixture consisting of 2.5 ng of the complementary DNA samples, 0.5 μM of each forward
219 and reverse primer for each target gene, 6.25 μL of Fast SYBR Green Master Mix, and RNA
220 free water to complete a final volume of 12.5 μL was prepared in PCR plates (Thermo
221 Scientific) and centrifuged (2,250 g for 30 s at room temperature). In each PCR plate, three
222 calibrators and two negative controls (pooled RNA sample per tissue without any reverse
223 transcription step) and one RNA-free water were also placed. The StepOnePlus software
224 (Applied Biosystems) was programmed to run an amplification protocol consisting of 40 cycles
225 of 3 s at 95 °C for complementary DNA denaturation, followed by 30 s at 60 °C for annealing
226 and extension reactions to synthesize messenger RNAs. The dissociation protocol consisted of
227 a linear increase of 1 °C per min and was performed from 60 to 95 °C. Throughout this protocol,
228 a continuous fluorescence acquisition was performed to obtain a melting curve. The melting
229 curve of each specific PCR product was analyzed to ensure that no primer dimers or nonspecific
230 DNA products were amplified.

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

$$235 \quad \textit{Normalized relative expression} = \frac{\textit{Efficacy}^{-(C_t \textit{ Sample} - C_t \textit{ Calibrator})}}{\textit{Normalization factor}}$$

236 *Statistical analysis*

237 All statistical analyses were done using R version 3.6.2 (R Core Team, 2019). The pig was the
238 experimental unit. Except for the apparent jejunal digestibility, statistical analysis was done per
239 intestinal segment. Data were analyzed with a linear mixed model using the lmer function of R
240 in the lmerTest package (Kuznetsova et al., 2017). The model included the main effect of diet
241 with pig as the random effect. Least square means were computed using the emmeans function
242 of R under the EMMEANS package (Lenth, 2020). A *P*-value < 0.05 was considered a
243 statistically significant difference.

244 **RESULTS**

245 *General observations*

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

250 *Apparent jejunal digestibility*

251 The apparent jejunal digestibilities measured with the two markers did not differ (*P* > 0.10).
252 Therefore, values we report here are the average between the two markers. The apparent jejunal
253 digestibility of all AA was considerably lower for INT than both HYD and FAA (Table 3). For
254 INT, the apparent jejunal digestibility of His, Val, and Trp were less than 70% and the highest
255 value was for Ile at 89.1%. For HYD and FAA, most of the measured apparent jejunal
256 digestibility were higher than 90% with the exception of His for HYD and Cys, Gly, His, Lys,
257 and Pro for FAA. The apparent jejunal digestibility of all AA were numerically higher for HYD
258 compared to FAA, with that of Cys, Gly, His, Met, and Pro being statistically higher.

259 *Intestinal morphology of duodenum, jejunum, and ileum*

260 The results of the intestinal morphology of the duodenum, jejunum, and ileum of the pigs are
261 given in Fig. 1. The surface area of the villi was the largest for HYD pigs and the smallest for
262 INT pigs in the ileum. Crypt depth was lower in the jejunum for HYD pigs compared to INT
263 and FAA pigs. The ratio of the villi length over crypt depth in the jejunum was lower for INT
264 pigs compared to both HYD and FAA pigs.

265 *Relative expression of amino acid and peptide transporters along the small intestine*

266 The relative expression of the gene coding for the intestinal peptide transporter PepT1 was
267 higher in the duodenum of INT pigs compared to both HYD and FAA pigs (Fig. 2). In the
268 jejunum, the expression of the genes coding for the sodium-neutral AA exchanger, ASCT2, and
269 the high affinity cationic amino acid transporter, CAT-1, was the highest in HYD pigs and the
270 lowest in FAA pigs. Regardless of the diet, the expression of PepT1, y⁺LAT1, and b⁰⁺AT was
271 generally higher in the jejunum compared to that in both duodenum and ileum. The inverse held
272 for ASCT2 and CAT-1, which were the least expressed in the jejunum.

273 **DISCUSSION**

274 This experiment aimed to study the changes in the intestinal physiology of pigs that can be
275 induced when feeding AA either as proteins and/or free AA and small peptides. The
276 digestibility of free AA in the diet is much higher than that of proteins (Chung and Baker, 1992;
277 Ma et al., 2019). This important difference may explain the morphological changes observed in
278 the small intestine (Morales et al., 2017). In the proximal part of the intestine, other studies
279 found that changing the form of dietary AA supplied to weanling pigs increased the villi height
280 to crypt depth ratio (Ma et al., 2019). Furthermore, the provision of increasing levels of free
281 AA in the diet has been shown to increase the expression of intestinal AA transporters in
282 different sections of the intestine (Zhang et al., 2013; Morales et al., 2017).

283 The content of AA in the diet affect the total and individual digestibilities of AA (Fan et al.,
284 1994). Free AA and small peptides are often considered to be 100% digestible (Chung and
285 Baker, 1992; Weijzen et al., 2022). In our experiment, there were some differences in the AA
286 content of the experimental diets. The lower content of Cys and Trp in diet INT compared to
287 the other diets may have affected their apparent jejunal digestibility, due to a greater
288 contribution of endogenous secretions which may also contain these AA (Miner-Williams et
289 al., 2014). However, the apparent jejunal digestibilities of these two AA were in line with those
290 of the other AA in diet INT and still much lower than the digestibilities of the other two diets,
291 suggesting that, in our experimental conditions, the form of the AA had a much bigger impact
292 on the apparent jejunal digestibility than the content of the AA in the diet. In the HYD and FAA
293 diets, the apparent jejunal digestibility of His was lower compared to that of the other AA. This
294 could again possibly be due to higher proportion of His from endogenous secretions in the
295 digesta or to a delayed absorption of His. Furthermore, digestibility can also be affected by
296 transit time. For pigs, the transit time of digesta from the stomach to the end of the small
297 intestine is comprised between 5 to 6 h for the solid fraction and between 3 to 4 h for the liquid
298 fraction (Schop et al., 2019). In our experiment, we assumed that no AA from the last meal
299 remained in the intestine after an overnight fast for any of the experimental diets when pigs
300 were slaughtered to collect digesta. The apparent jejunal digestibility reported here was
301 calculated from digesta samples taken 3 h after the meal, but only little digesta was found in
302 the duodenum or ileum. However, this does not preclude that digesta was still present in the
303 stomach to arrive in the duodenum later on, or that digesta would appear later in the ileum.
304 Hence, the values reported here are probably affected by the time between the meal and
305 slaughter. If the animals were slaughtered earlier, the apparent jejunal digestibility values would
306 probably have been lower. Consequently, the values reported in Table 3 are to be interpreted as
307 relative values to be compared between diets. Compared to proteins that need to be digested to

308 release AA, free AA and small peptides are immediately absorbed. As all the AA in HYD and
309 FAA were small peptides and/or free AA, we expected their higher apparent jejunal AA
310 digestibility compared to INT and this was confirmed in the study. The apparent jejunal
311 digestibility of AA of HYD was systemically higher than that of FAA with, on average, a 4%
312 difference. This is consistent with some studies that found that the absorption of small peptides
313 to be faster compared to individual free AA in the diet (Shiraga et al., 1999; Bröer and
314 Fairweather, 2018). In HYD, 16% of the total AA were supplied as small peptides which may
315 explain the higher AA digestibility. The pigs used in this experiment were also used to measure
316 the postprandial appearance of AA in the plasma (Eugenio et al., 2022). Interestingly, the
317 plasma appearance of AA was faster for pigs fed FAA diet compared to HYD diet. This suggests
318 that, even though HYD generally had higher apparent jejunal digestibilities and, by extension,
319 a faster disappearance from the intestinal lumen, the AA may have been retained or metabolized
320 more by the splanchnic tissues with diet HYD than with FAA diet.

321 As mentioned earlier, AA digestibility can affect intestinal morphology. The measured traits to
322 assess the morphology of the three sections of the small intestine in this experiment are in line
323 with those reported by other experiments (Park et al., 2020). Both villi length and crypt depth
324 can be used as indicators of digestion and absorption capacity of the small intestine (Xia et al.,
325 2005). Part of this is because the villi have a structural function and increase the surface area of
326 the small intestine (Xia et al., 2005; Guay et al., 2006). Another part is because the villi is the
327 main unit of AA absorption in the small intestine with the AA transporters located on its surface
328 (Gao et al., 2000; Salgado et al., 2002). A higher villi height thus reflects an increased total
329 intestinal surface area for AA absorption. Contrary to villi, crypts have very little absorptive
330 functions for dietary AA as it has virtually no AA transporters (O'Loughlin et al., 1996; Umar,
331 2010). Hence, an increased villi size and villi height to crypt depth ratio was interpreted as a
332 higher capacity for intestinal absorption of AA (Zhang et al., 2017). Our results confirm the

333 observations of Yun et al. (2005) and Park et al. (2020) who found that feeding pigs with highly
334 digestible diets resulted in higher villi height and villi height to crypt depth ratio as these indices
335 were higher in the jejunum of HYD and FAA compared to INT. Conversely, the lower villi
336 length to crypt depth ratio in the jejunum of pigs fed INT could be associated with the presence
337 of proteins that are still being digested. Indeed, a deeper crypt may be the outcome of increased
338 total endogenous secretions (Schulze, 2006; Stein et al., 2007). Some studies found more
339 endogenous secretions when feeding diets with protein compared to diets where the supplied
340 AA are immediately absorbable (Chung and Baker, 1992). However, it was unclear why the
341 villi area of HYD pigs was found to be the highest among the experimental diets in the ileum,
342 followed by FAA.

343 The expression of PepT1 was the highest in the duodenum of INT fed pigs. This was unexpected
344 because there should be less absorbable peptides in the duodenum of INT than in HYD pigs,
345 especially given their apparent jejunal digestibility. Another surprising finding was that the
346 expression of PepT1 was not different from HYD and FAA. We initially hypothesized that the
347 release of the gastric acid in the stomach may have hydrolyzed the small peptides in HYD into
348 their constituent AA, meaning there were virtually no dietary peptides arriving in the small
349 intestine for both HYD and FAA. This hypothesis was not confirmed as the small peptides that
350 remained intact after the process of extensive acid hydrolysis in the production of HYD are
351 probably not hydrolyzed in the stomach either. Otherwise, as the transit time of the soluble part
352 of the digesta containing free AA and peptides is very quick in the proximal part of the small
353 intestine (Schop et al., 2019), this may have not given enough time for peptides from HYD to
354 be absorbed in the duodenum compared to those in INT. This might also be the reason why we
355 saw higher expression of PepT1 in the jejunum compared to the duodenum across all the
356 experimental diets, but it may be more evident for HYD, whose PepT1 expression in the
357 jejunum was around twice to that of the duodenum. Furthermore, because digestive enzymes

358 were mostly released in the duodenum, their eventual breakdown (to be re-absorbed as they
359 were not used) may have caused an “infusion” of peptides which may be why the expression of
360 PepT1 in the jejunum of FAA and HYD fed pigs was higher than in the duodenum. In humans,
361 the infusion of hydrolyzed casein resulted in around 20% nitrogen secretion in the small
362 intestine compared to feeding its equivalent free AA solution (Miner-Williams et al., 2014),
363 which may explain our findings. Meanwhile, the increased expression of PepT1 in the jejunum
364 compared to the duodenum of INT fed pigs was expected as this was the result of normal protein
365 digestion, and therefore increased abundance of small peptides. We found that the expression
366 of ASCT2 and CAT-1 in the jejunum was the highest for pigs fed HYD. The ASCT2 transports
367 most proteinogenic AA except for Asp, Arg, Glu, His, and Lys (Bröer and Fairweather, 2018)
368 whereas CAT-1 transports the cationic AA Arg, His, and Lys (Yin et al., 2014; Bröer and
369 Fairweather, 2018). These two AA transporters are able to transport most proteinogenic AA in
370 the diet, except for Asp and Glu, and their higher expression in pigs fed HYD might explain
371 why the apparent jejunal digestibility of AA was marginally higher for HYD compared to FAA.

372 In conclusion, the results of this experiment showed that feeding AA as free AA and small
373 peptides instead of proteins results in changes in the intestinal physiology of pigs. Substrate
374 availability is probably the main determinant of these changes, which may have been caused by
375 the inherently different dynamics of digestion. However, even though free AA and small
376 peptides in the diet are readily available and do not need hydrolysis, the jejunal digestibility of
377 certain AA was still different between HYD and FAA, most likely due to the presence of small
378 peptides in HYD that were absorbed faster than individual free AA.

379

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385 **Conflicts of interest/Competing interests**

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

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508

510 **Table 1.** Composition and nutrient analysis (as fed) of the three experimental diets fed to
 511 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 hydrolyzed feather		17.0	
Feather meal	17.0		
Vegetable oil	4.00	4.00	4.00
Corn starch	67.8	67.7	69.7
Synthetic fiber	5.00	5.00	5.00
L-Trp		0.10	0.10
Amino acid mix ³			15.0
Analyzed content			
Moisture, %	10.3	11.1	9.66
Total nitrogen, %	2.58	2.28	2.10
Gross energy, MJ·kg ⁻¹	16.6	15.8	16.4
Total amino acids, g·kg ⁻¹	143	150	136
Amino acids, g·kg ⁻¹			
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

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

514 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
 515 mg; vitamin B2, 800 mg; calcium pantothenate, 2,170 mg; niacin, 3,000 mg; vitamin B12, 4 mg; vitamin B6, 200 mg; vitamin
 516 K3, 400 mg; folic acid, 200 mg; biotin, 40 mg; choline chloride, 100,000 mg; iron (sulfate), 11,200 mg; iron (carbonate), 4,800
 517 mg; copper (sulfate), 2,000 mg; zinc (oxide), 20,000 mg; manganese (oxide), 8,000 mg; iodine (iodate), 40 mg; cobalt
 518 (carbonate), 20 mg; and selenium (selenite), 30 mg.

519 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,
 520 L-Cys, L-Phe, L-Pro, and L-Ser.

521 **Table 2.** Primers for the real-time quantitative PCR analyses of cDNA derived from mRNA of different target genes of intestinal amino acid and
 522 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		144	NM_214347.1
		Reverse:	ACCCGATGCACTTGACGA			
SLC1A5	ASCT2	Forward:	GCCAGCAAGATTGTGGAGAT	14	206	DQ231578
		Reverse:	GAGCTGGATGAGGTTCCAAA			
SLC7A9	b ⁰⁺ AT	Forward:	CCTTTCAGAAACCTGCCCCCT	16	144	NM_001110171
		Reverse:	GAAGGCCACGAAGAGTGGAA			
SLC7A7	y ⁺ LAT1	Forward:	TCAAGTGGGGAACCCTGGTA	5	259	NM_001110421
		Reverse:	ATGGAGAGGGGCAGATTCCT			
SLC7A1	CAT-1	Forward:	ATCTCTGCCTGAACAATGCCA	3	138	NM_001012613
		Reverse:	ATGCAGTCAAAGCCCACGAA			
Reference genes						
HSPCB	Heat shock 90kD protein 1, β	Forward:	GGCAGAAGACAAGGAGAAC		131	Q9GLE9_PIG
		Reverse:	CAGACTGGGAGGTATGGTAG			
PPIA	Peptidylpropyl isomerase A	Forward:	AGCACTGGGGAGAAAGGATT		220	NM_214353
		Reverse:	AAAAC TGGGAACCGTTTGTG			

523 ¹PepT1 – intestinal peptide transporter; ASCT2 – sodium-neutral amino acid exchanger; b⁰⁺AT – cationic amino acid plus cysteine transporter; y+LAT1 – cationic and neutral amino acid
 524 transporter; CAT-1 – high affinity cationic amino acid transporter.

525 **Table 3.** Apparent jejunal digestibility (%) of dietary amino acids of pigs fed a diet whose
 526 amino acids originated from intact protein (INT), extensively hydrolyzed protein (HYD), or
 527 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

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

530

FIGURE CAPTIONS

531 **Figure 1.** Intestinal morphology of the duodenum, jejunum, and ileum (mean \pm SD) of pigs fed a diet
532 whose amino acids originated from intact protein (INT), extensively hydrolysed protein (HYD), or free
533 amino acids (FAA). Different letters within an intestinal segment differ ($P < 0.05$).

534

535 **Figure 2.** The normalized, relative gene expression (mean \pm SD) of different amino acid transporters and
536 the intestinal peptide transporter of different segments of the small intestine of pigs fed a diet whose amino
537 acids originated from intact protein (INT), extensively hydrolysed protein (HYD), or free amino acids
538 (FAA). HSPCB and PPIA were used as reference genes. Different letters within a gene differ ($P < 0.05$).

539