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Potential new methods to analyze basal and total endogenous protein losses of host and bacterial origin in pigs.

Methods to analyze endogenous protein in pigs.

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Abbreviations: AA, amino acid; AID, apparent ileal digestibility; BB, black beans; BC, bovine collagen; bEPL, basal endogenous protein losses; CHP, chickpeas; CP, crude protein; DAPA, diaminopimelic acid; DM, dry matter; DMI, dry matter intake; EPL, endogenous protein losses; N, nitrogen; NF, nitrogen-free; PP, pigeon peas; RP, roasted peanuts; sEPL, specific endogenous protein losses; SID, standardized ileal digestibility; SO, sorghum; tEPL; total endogenous protein losses; TID, true ileal digestibility; TWB, toasted wheat bread; WB, wheat bran; WPI, whey protein isolate; ZE, zein.

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Abstract

Background: Current systems for assessing protein quality such as the Digestible Indispensable Amino Acid Score (DIAAS) correct apparent amino acid digestibility for basal endogenous protein losses (bEPL), ignoring the potential influence of the diet on these losses. However, the quantification of total endogenous protein losses (tEPL) poses a challenge.

Objective: To evaluate different methods for quantifying tEPL and bEPL, and to assess their potential in discriminating between tEPL originating from bacteria and host.

Methods: Using an incomplete Youden square design, twelve ileal cannulated pigs received ten different protein sources, and a nitrogen-free (NF) diet. Ileal digesta were collected on days 6 and 7 of each 1-week feeding period, to quantify endogenous protein losses (EPL) and analyze apparent ileal digestibility. Ileal EPL were estimated based on 1) 16S-+18S gene copy qPCR, 2) diaminopimelic acid (DAPA)+18S, 3) differential AA profiles in digesta, EPL and bacteria, equaling tEPL, and 4) an NF diet and 5) whey protein isolate (WPI), equaling bEPL.

Results: Ileal bEPL based on the NF and WPI method correlated moderately-highly (r=0.69, $P<0.05$), but the NF method probably underestimated bEPL. In pigs fed the WPI diet, EPL based on the WPI and AA profile method were highly correlated (r=0.88, $P<0.01$). Overall, tEPL based on the AA profile method were moderately correlated with the 16S+18S method (r=0.58, $P<0.001$), and DAPA+18S (r=0.57, $P<0.001$). Low correlations were observed between bacterial tEPL based on the AA profile method and 16S or DAPA. Host tEPL based on the AA profile method and 18S were weakly correlated (r=0.39, $P<0.001$).

Conclusions: The AA profile method seems the most appropriate method for tEPL quantification, while the WPI method is preferred for bEPL quantification. Despite challenges in distinguishing between bacterial and host EPL, it is evident that bacterial proteins substantially (on average 37-83%, depending on method) contribute to the EPL.

Keywords (5-10): basal endogenous protein losses, specific endogenous protein losses, total endogenous protein losses, pigs, 18S gene copy qPCR, 16S gene copy ddPCR, diaminopimelic acid (DAPA), digesta amino acid profile.

1. Introduction

To evaluate protein quality in human foods, the Food and Agriculture Organization recommends using the digestible indispensable amino acid score, i.e. DIAAS (1). This score uses the ileal amino acid (AA) digestibility, which is complex to measure in humans, and therefore FAO recommends the use of pigs as model animal. For the evaluation of the ileal digestibility of amino acids in pigs, the oro-ileal disappearance of amino acids is corrected for the non-dietary amino acids, so-called endogenous protein losses (EPL), present at the terminal ileum (2).

Endogenous protein losses include sloughed of intestinal epithelial cells, mucoproteins, digestive secretions, immunoglobulins, and, depending on the definition, bacterial proteins (3). Basal endogenous protein losses (bEPL) are defined as the protein losses at the terminal ileum that are not specifically induced by the test diet. These losses are commonly assumed to be proportional to the transit of food materials through the digestive tract. Basal EPL are often quantified by measuring the presence of nitrogen (N) in ileal digesta after feeding a N-free diet. However, this method underestimates the bEPL compared with normal physiological conditions, and results in increased proline and glycine contents (4-8). In contrast, alternative methods, such as feeding a highly digestible protein source or enzymatically-hydrolyzed proteins, may overestimate bEPL (9, 10). The linear regression method seems more reliable, but is laborious and requires measurements on a series of diets with incremental protein concentrations (9). Ileal protein digestibility corrected for bEPL is called ‘standardized ileal digestibility (SID)’ (11).

Endogenous protein losses that are induced by specific properties of investigational food ingredients are referred to as specific endogenous protein losses (sEPL, 4, 12). These specific properties of ingredients are protein characteristics, intrinsic fibers, and anti-nutritional factors,

94 which in turn can influence the secretion and reabsorption of digestive enzymes, production of
95 mucus, the shedding of epithelial cells, and bacterial mass (9, 13-16). For example, low
96 digestible protein sources seem to increase the quantity of digestive enzymes in the intestine,
97 by slowing down digestion and subsequent re-absorption of pancreatic secretions (14, 17, 18).
98 In addition, the presence of unabsorbed proteins likely increases bacterial mass in the ileum
99 (19). Fibers can affect the sloughing-off of epithelial cells, mucus production, protein digestion,
100 and bacterial mass, depending on the fiber source (20-23). Finally, anti-nutritional factors
101 (ANFs) are compounds in foods that can reduce nutrient availability, and also affect sEPL (14).
102 For example, trypsin inhibitors bind to the active sites of digestive enzymes, reducing protein
103 digestibility, leading to a compensatory increase of the pancreatic protein flow. Lectins, on the
104 other hand, bind to receptors on epithelial cells, leading to increased protein secretion and cell
105 turnover (4, 13, 24-26). Ileal protein digestibility corrected for both bEPL and sEPL is called
106 'true ileal digestibility (TID)' (11).

107 In the absence of a good methodology to measure sEPL, the DIAAS score currently corrects
108 the ileal protein digestibility only for bEPL (27). To quantify the total (basal + specific)
109 endogenous protein losses (tEPL), isotope dilution techniques, and the homoarginine technique
110 are well-known methods, but these methods require specialized facilities and personnel,
111 making them costly and labor-intensive (9, 28-31). Hence, other, less laborious methods are
112 required to correct the ileal protein digestibility for both bEPL and sEPL. Newly available
113 DNA-based techniques may offer potential for quantifying tEPL sources, such as bacteria and
114 host intestinal cells (32, 33). These techniques also have the potential to distinguish between
115 tEPL originating from bacterial and host sources, increasing our comprehension of the impact
116 of dietary composition on the different fractions of endogenous losses.

117 In this study, various methods are evaluated for the quantification of EPL, and their
118 effectiveness in distinguishing between EPL originating from bacterial and host origin.

Bacterial EPL are quantified using 16S gene copy ddPCR and diaminopimelic acid (DAPA). Host EPL are quantified using 18S gene copy qPCR. Additionally, bacterial and host EPL are estimated based on differential AA profiles in digesta, host EPL and bacterial EPL. By combining these analyses, we investigated the following methods to quantify EPL: 1) 16S + 18S gene copy PCR (16S+18S method), 2) diaminopimelic acid + 18S gene copy qPCR (DAPA+18S method), 3) estimation based on differential AA profiles in digesta, endogenous losses and bacteria (AA profile method), equaling tEPL, 4) feeding a nitrogen-free diet (NF method) and 5) feeding a highly digestible protein source (whey protein isolate; WPI method), equaling bEPL.

2. Methods

A project license (AVD104002015326) was granted by the Central Committee for Animal Experimentation (The Hague, the Netherlands). Experimental procedures were approved by the Animal Welfare Body of Wageningen University (Wageningen, The Netherlands).

2.1 Experimental design and housing

This experiment was part of a larger experiment in which standardized ileal amino acid digestibility coefficients were studied (2). A total of sixteen (12 + 4 spare) gilts (Topigs Norsvin TN70; Topigs, Helvoirt, the Netherlands) were obtained from a commercial farm in the Netherlands. The gilts weighed 27.6 ± 0.92 kg (mean ± SEM) at the start of the experiment and 80.0 ± 2.75 kg (mean ± SEM) six days after the end of period VII. Using an incomplete Youden square design (34), ten protein sources were tested for ileal EPL and apparent ileal digestibility in 7 consecutive periods of one week in twelve pigs. Four pigs were used as reserve animals and received a casein-based diet (basal diet) or one of test protein sources when replacing one of the experimental pigs (**Table 1**). To analyze the ileal bEPL, a nitrogen-free (NF) diet was fed for one period between period IV and V to all 16 pigs, followed by a wash-out period of one week where pigs received the basal diet.

In total this resulted in 6 observations for black beans, 7 for bovine collagen, 6 for chick peas, 13 for pigeon peas, 6 for roasted peanuts, 11 for sorghum, 6 for toasted wheat bread, 7 for wheat bran, 12 for whey protein isolate, 7 for zein, and 15 for the NF diet. The pigs were housed individually in metabolism pens (1.35 x 1.20 m). The first days after surgery the temperature was controlled between 21 and 23°C, thereafter the temperature was reduced to 19.5- 21.5°C. Pigs were exposed to 12h of light per 24h.

152 *2.2 Animal procedures*

153 After arrival at the research facilities of Wageningen University & Research, the pigs were
154 adapted to their new environment and received a casein-based diet (basal diet, **Table 1**). On
155 day 15-17, simple T-cannulas were surgically placed at the end of the small intestine (35). After
156 surgery, the pigs underwent a recovery time of 8-10 days, still receiving the basal diet.
157 Following recovery, pigs were assigned to the test diets, or the NF diet, as described above. On
158 days 6 and 7 of each measurement period ileal digesta was collected for 9h starting directly
159 after the first meal of each day. The collecting bags were changed every 30 minutes or when
160 full, and the digesta was immediately stored at −20°C. The pigs were fed twice a day (0700 h
161 and 1600 h), with a daily allowance of 8% of their metabolic body weight ($0.08 \times BW^{0.75}$). Pigs
162 had *ad libitum* access to water.

163

164 *2.3 Diets*

165 The diets were designed as described by Hodgkinson et al. (35). Briefly, each experimental
166 diet contained one of the test ingredients, which was the only source of protein. Diets were
167 formulated to have a protein concentration of 100 g/kg DM (**Table 1**). The tested protein
168 sources were black beans (BB), bovine collagen (BC), chickpeas (CHP), pigeon peas (PP),
169 roasted peanuts (RP), sorghum (SO), toasted wheat bread (TWB), wheat bran (WB), whey
170 protein isolate (WPI), and zein (ZE). The chemical composition of the test ingredients is
171 provided in **Supplementary Table 1**.

172 To minimize any potential impact of amino acid irregularities, a maximum of two diets
173 containing amino acids below NRC recommendations (36) were fed consecutively. In the zein-
174 based diet, L-Lysine (12 g/kg DM) and L-Tryptophan (2 g/kg DM) were added on top during
175 days 1-5 of the feeding period, and only withdrawn on sampling days.

176

177 *2.4 Chemical analysis*

178 Immediately after each measurement period, digesta samples were thawed, homogenized, and
179 pooled for each pig per diet. From each of these samples a subsample was taken and stored at
180 −80°C. Subsequently, the digesta samples were freeze-dried, and ground through a 1 mm
181 screen using a centrifugal mill (Retsch ZM200, Haan, Germany) at 12000 rpm. Then, they
182 were analyzed for Titanium (Ti), dry matter (DM), and amino acids (AA) to determine the AA
183 profile and the AA digestibility according to ISO 1999 and 2005 (37-39). Reactive lysine was
184 determined according to Moughan and Rutherfurd (40). Titanium was analyzed after
185 hydrolysis with concentrated sulfuric acid in the presence of a copper catalyst at 420°C and the
186 subsequent addition of peroxide. The resulting orange/yellow colored complex was
187 spectroscopically determined at 408 nm (41, 42). Analyses were performed in duplicate.

188

189 *2.5 Methods to quantify EPL*

190 Ileal bEPL were analyzed in pigs (n=11) fed an NF diet (NF method) between period IV and
191 V, and in pigs fed a WPI diet (WPI method), one of the tested protein sources, assuming that
192 all nitrogen (N) from WPI was absorbed at the end of the small intestine (43). Ileal tEPL (basal
193 + specific) were analyzed based on the 16S+18S method (described in section 2.5.1),
194 DAPA+18S method (described in section 2.5.2) and the AA profile method (described in
195 section 2.5.3). In **Figure 1**, the experimental design in relation to the different measurements
196 is shown.

197

198 *2.5.1 16S+18S method*

199 Real-time qPCR was used to detect 18S gene copies in the ileal digesta . Droplet-digital PCR
200 (ddPCR) was used to detect 16S gene copies. Using the number of 16S rRNA gene copies,
201 which encodes for the highly-conserved RNA component of the 30S subunit of a prokaryotic
202 ribosome, an estimation can be made of the ileal bacterial mass (see 2.5.1.3, 43). Using the
203 number of 18S rRNA gene copies, which encodes eukaryotic RNA, an estimation can be made
204 of the amount of protein from pig epithelial cells (32). The sum of endogenous protein losses
205 of bacterial and host origin, represents the tEPL. The 18S copy number was based on reference
206 tissue values. For this, 30 mg pre-washed jejunal scrapings of three pigs from another
207 experiment (±8 kg) was mechanically homogenized in Dulbecco's phosphate-buffered saline
208 (D-PBS; Gibco^{TM}, US) using a Turrax disperser tool (T10, IKA), and DNA was extracted using
209 a DNeasy blood and tissue extraction kit (ID: 69504, Qiagen, the Netherlands) following
210 manufacturers' protocol. The resulting DNA was used for developing a standard curve in qPCR
211 for protein content estimation. For total protein, the same tissue homogenization process was
212 followed and protein content was measured using a protein quantification kit (Qubit Protein
213 Broad Range (BR) Assay, Thermo Fisher, US).

214

215 *2.5.1.1 DNA extraction for the 16S+18S method*

216 DNA was extracted from the ileal digesta subsamples as previously described (45). In total,
217 200 mg per sample were thawed on ice and added to bead-beating tubes containing 700 µL of
218 Stool Transport and Recovery (STAR)-solution (Roche), 0.5 g of 0.1 mm autoclaved zirconia
219 beads, and five 2.5 mm glass beads. Samples were homogenized by repeated bead-beating (5.5
220 ms, 3 x 1 min, FastPrep-24^{TM}; MP Biomedicals, LLC) at room temperature. Next, the samples
221 were incubated at 95°C for 15 min, while shaking at 300 rpm, followed by centrifugation at
222 4°C (15000 *x* g for 5 min). The supernatant was then transferred to 1.5 mL Eppendorf tubes on

ice. The sample pellet was resuspended in 300 µL of STAR buffer and reprocessed for bead-beating to generate an additional supernatant. Both resulting supernatants (in total 1000 µL) were pooled, and stored at −20 °C. Per sample, 250 µL pooled supernatant was used for DNA purification, using Maxwell 16® (Promega). The supernatant was added in a 0.5 mL elution tube, eluted in 50 µL of water (DNAse and RNAse-free). Total DNA concentrations were measured using 1 µL sample on a DS-11 spectrophotometer (DeNovix) and fluorometric quantification dsDNA (HS and BR, *Qubit*, Thermo-Fisher Scientific). DNA extractions were performed singularly.

2.5.1.2 Total bacterial load and host endogenous protein estimation by real time qPCR

Bacterial protein mass was quantified by droplet-digital PCR (ddPCR) using the BactQuant qPCR assay (33). All materials for the ddPCR analysis were ordered from Bio-Rad Laboratories (Carlsbad, CA, USA). Briefly, for 16S SSU rRNA gene amplification DNA samples were 25.000× diluted, whereafter 5 µL of diluted sample was mixed with 10 µl of 2x ddPCR Supermix for Probes, 1 µL forward (FW) primer 5′-CCTACGGGDGGCWGCA-3′ (18 µM), 1 µL reverse (RV) primer 5′-GGACTACHVGGGTMTCTAATC-3′ (18 µM), 1 µL probe (6FAM) 5'-CAGCAGCCGCGGTA-3' (MGBNFQ, 5 µM), and 2 µL RNAse- and DNAse free water (Promega). After droplet generation, PCR was initiated by a 95°C denaturation for 30 seconds, and 58°C annealing and elongation for 2 minutes. The PCR was finished with an additional extension at 98°C for 10 minutes, followed by 30 minutes at 4°C. Samples were then counted in a QX200 Droplet Reader, and analyzed using QuantaSoft software (Version 1.7.4.0917). A subset of samples (10 of 79) was analyzed in duplicate.

Host endogenous protein was quantified using quantitative PCR, following methodologies established previously (46-49). DNA was initially diluted to a concentration of 2 ng/µL. As

standard curve, 10-fold serial dilutions were made using DNA derived from epithelial jejunum tissue scrapings with known protein content. 18S amplification was performed using primers FW – AACTTTCGATGGTAGTCGCCGT - and RV – TCCTTGGATGTGGTAGCCGTTT. Master mix contained per reaction 10 µL SYBR green (SsoAdvanced universal SYBR green, Bio-Rad), 1.25 µL primer mix (10 µM), 6.75 µL RNAse- and DNAse free water, and 2 µL template DNA. PCR was initiated by a 95°C denaturation hot start for 5 minutes and followed by 35 repeated cycles of 95°C denaturation for 20 seconds, 60°C annealing for 30 seconds, and 72°C elongation for 30 seconds. The PCR was finished with an additional extension at 72°C for 30 seconds and amplicon specificity was assessed by melt curve from 60°C to 95°C at 0.5°C increments on a CFX-96 Touch Real-Time PCR detection system (Bio-Rad). Total endogenous protein content was calculated by standard curve in CFX Maestro software with quantified protein numbers as previously described (50).

2.5.1.3 Calculations for the 16S+18S method

Bacterial tEPL based on 16S gene copy numbers were calculated using the following equations:

1. Bacterial protein (g/kg dry matter; DM digesta) =

$$\frac{16S\ digesta\ (copies/kg\ DM)}{4.2\ (copies/bacterium)} \text{ x } \frac{12.5\ (g/100g)}{100} \text{ x } 10^{-12}\ \text{(g)},$$

where 16S digesta is the number of 16S copies in the digesta, 4.2 reflects the average number of 16S copies per bacterium (44), 12.5 reflects the average protein content of bacteria (g/100 g bacterial cells, 51), and 10^{-12} reflects the average mass of bacteria (52).

2. Bacterial protein (g/kg dry matter intake; DMI) =

bact. protein (g/kg DM digesta) x $\frac{Ti\ diet\ (g/kg\ DM)}{Ti\ digesta\ (g/kg\ DM)}$

where bact. protein is the bacterial protein concentration in digesta calculated in step 1, Ti diet is the titanium concentration in the diet, and Ti digesta is the titanium concentration in the digesta.

Host tEPL based on 18S gene copy numbers were calculated using the following equations:

3. EPL from porcine intestinal cells (EPL Int.C. ; g/kg DM digesta) =

$$\frac{protein\ (g/ng\ DNA)\ \text{x}\ DNA\ (ng/g\ ileal\ digesta)}{DM\ (g/kg)}$$

where EPL = endogenous protein losses, protein = g protein/ng DNA (based on the analysis of the protein content and DNA content of the ileal digesta), DNA = ng DNA/g ileal digesta, DM = dry matter content of the ileal digesta.

4. EPL from mucus (g/kg DM digesta) =

$$\frac{EPL\ Int.C\ (g/kg\ DM).}{6.7\ (\%\ of\ total\ protein)}\ \text{x}\ 14.1\ (\%\ \text{of total protein})$$

where 6.7 reflects the proportion of protein in intestinal cells and 14.1 in mucus observed in the study of Miner-Williams et al. (3).

5. Total host protein (g/kg DM digesta) =

EPL Int. Cells. (g/kg DM) + EPL Mucus (g/kg DM)

6. Host protein (g/kg DMI) =

host protein (g/kg DM) x $\frac{Ti\ diet\ (g/kg\ DM)}{Ti\ digesta\ (g/kg\ DM)}$

where host protein is the host protein concentration in digesta calculated in step 5, Ti diet is the titanium concentration in the diet, and Ti digesta is the titanium concentration in the digesta.

2.5.2 DAPA+18S method

Diaminopimelic acid (DAPA) was used as an alternative for quantifying bacterial mass (DAPA method). DAPA is a unique component of cell walls of many gram-negative bacteria and thus based on DAPA concentration in the sample an estimate of bacterial biomass can be made (53, 54). Total endogenous protein was quantified as the sum of bacterial protein, estimated by the DAPA method, and host protein, estimated by 18S gene copies (described in 2.5.1.)

2.5.2.1 DAPA analysis

DAPA was analyzed after oxidation overnight with performic acid/phenol at 0°C and neutralization with sodium disulphite, followed by hydrolysis with 6 M HCL during 23 hrs. The hydrolysate was adjusted to a pH of 2.2. DAPA was separated by ion exchange chromatography and determined by post column reaction with ninhydrin, using photometric detection at 570 nm (ISO 2005, 38).

2.5.2.2 Calculations for the DAPA+18S method

Bacterial tEPL based on DAPA were calculated using the following equations:

7. Bacterial protein (g/kg DM digesta) =

$$\frac{DAPA\ (mg/kg\ DM)}{26.4\ (mg/g)} \text{ x } 6.25$$

where DAPA is the concentration of DAPA in the digesta, 26.4 reflects the average DAPA : bacterial nitrogen ratio (55), and 6.25 is the N conversion factor used.

8. Bacterial protein (g/kg DMI) =

bacterial protein (g/kg DM digesta) x $\frac{Ti\ diet\ (g/kg\ DM)}{Ti\ digesta\ (g/kg\ DM)}$

where bacterial protein is the bacterial protein concentration in digesta calculated in step 7, Ti diet is the titanium concentration in the diet, and Ti digesta is the titanium concentration in the digesta.

For the calculations regarding 18S, see 2.5.1.3.

2.5.3 AA profile method

For this method, the AA profiles of the diet, endogenous protein, bacterial protein, and ileal digesta were used to calculate the ratio diet : endogenous protein in the ileum that best reflected the AA profile of the ileal digesta. An Excel file illustrating this method can be found in the supplementary file.

2.5.3.1 Calculations for the AA profile method

To quantify ileal tEPL based on the AA profile method, protein in the ileal digesta was separated into endogenous, bacterial, and dietary protein based on their distinctly different AA profiles. The ratio between endogenous, bacterial, and dietary protein was calculated using the solver function in Microsoft ® Excel ® for Microsoft 365. The AA profiles of host tEPL and bacterial tEPL (**Supplementary Table 2**) were based on data from Miner-Williams et al. (3). Because tryptophan (Trp) was not measured in that study, the concentration of host Trp was estimated at 14.2 g/kg protein. This estimation was based on the ratio of bacterial (60.9%) : host endogenous protein (39.1%; 3), Trp losses in pigs fed a NF diet in the current study (14.1 g/kg digesta), and Trp in microbial protein (14.0 g/kg; 56).

For these calculations, some adjustments were made. Firstly, proline and glycine were excluded from the AA pattern used for the calculation, because these amino acids may be overestimated

when using an NF diet (57-59). Secondly, for these calculations, digestibility of the various AA was assumed to be equal. In practice, however, this is not correct. Therefore, AA with an apparent ileal digestibility (AID) greater or lower than the mean AID plus or minus the SD were excluded in these calculations, determined per protein source (BB: Cys, BC: Trp, SO: Met, Leu, Thr, TWB: Lys, Glx, WB: Met, Glx, Lys, ZE: Lys). After entering the AA profiles, the solver function (GRG Nonlinear) was used to calculate the ratio diet : host endogenous protein : bacterial protein losses in the ileum that best reflected the AA profile of the ileal digesta.

2.5.4 Digestibility calculations

Apparent ileal protein digestibility was calculated using equation 9 (60):

9. Apparent ileal digestibility protein (AID) =

$$\left(1-\frac{Ti\ diet\ (g/kg\ DM)\ \text{x}\ protein\ digesta\ (g/kg\ DM)}{Ti\ digesta\ (g/kg\ DM)\ \text{x}\ protein\ diet\ (g/kg\ DM)}\right)\ \text{x}\ 100\%,$$

where Ti diet is the titanium concentration in the diet, protein digesta is the protein concentration in the digesta based on the sum of individual amino acid concentrations (instead of N concentrations), Ti digesta is the titanium concentration in the digesta, and protein diet is the protein concentration in the diet.

Standardized ileal protein digestibility based on the NF or WPI method was calculated using equation 10:

10. Standardized ileal digestibility protein (SID) =

$$\frac{protein\ in\ digesta\ (g/kg\ DMI)}{dietary\ protein\ (g/kg\ DM)}+\text{AID},$$

where protein in digesta = protein content in the digesta in pigs fed the NF diet or WPI-based diet; based on the sum of individual amino acid concentrations (bEPL), dietary protein is the protein concentration in the diet, and AID is the apparent ileal digestibility of the protein.

2.6 Statistical analysis

For all statistical analyses, R for Windows 3.6.0 was used (packages: car, 61; dplyr, 62; emmeans, 63; ggplot2, 64; glmmTMB, 65; LambertW, 66; moments, 67; multcomp, 68; tidyr, 69). Differences were considered significant if $P<0.05$ and differences at $P<0.10$ were considered a trend.

Spearman correlation coefficients and Bland-Altman Plots were used to evaluate relations among the different methods to quantify EPL. Correlations were considered strong when $r>0.70$, moderate when between $0.40> r > 0.70$, and weak when $r<0.40$ (70).

Because DNA extraction and subsequent qPCR analysis of 16S and 18S is a sensitive technique where small inaccuracies can have a major impact on the final values, outliers in 16S and 18S gene copy numbers/DM digesta were identified using a combination of two methods: potential outliers identified in boxplots using the interquartile range criterion, determined per protein source, and values that were higher or lower than the mean ± two standard deviations (the latter over all diets). The values highlighted by both methods were excluded from the data. For 16S, 5 pigs were excluded (BC, CHP, SO, 2x WPI) and for 18S, 3 pigs were excluded (RP, PP, SO, **Supplementary Figure 1**).

The normality of the model residuals of the response variables was checked visually using quantile-quantile plots and the Shapiro-Wilk test. If the residuals were not normally distributed, statistical analyses were performed on transformed data. For the differences in AA profiles, data from Thr, Pro and His were transformed using log transformation, data from Ala, Phe and

Lys were transformed using square root transformation, and data from Tyr were transformed using LambertW transformation. For the SID data, data from PP, TWB, and WPI were transformed using the Box Cox transformation. Means are reported as untransformed means ± SD. Pig was considered as the experimental unit.

For the differences in AA profiles, data were analyzed using t-tests. For apparent and standardized digestibility, data were analyzed using a generalized linear mixed model (gaussian family, link = identity), as follows:

$$Y_{ij} = \mu + M_i + P_j + e_{ijk}$$

where Y_{ij} = dependent variable, μ = overall mean, M_i = method, P_j = random pig effect and e_{ijk} = residual error.

3. Results

Due to canula problems and illness, two reserve pigs were used to replace experimental pigs. Furthermore, protein digestibility of roasted peanuts of one pig was excluded from the dataset because digestibility was close to 0, which is considered to be unlikely. For several pigs, whole pieces of peanuts were observed in the digesta, which in this pig might have caused this extremely low digestibility. In another pig, protein digestibility of sorghum was excluded from the dataset because Ti concentrations were very low, probably due to an analytical error. Finally, when fed the NF diet, one pig was identified as an outlier in the EPL estimates, possibly attributable to cannula problems, and was excluded from this data set.

In the quantification of endogenous losses, outliers were detected across the different methods. However, no identifiable causes for these outliers were found, and thus, they were included in the dataset. Excluding these data points from the analyses did not affect the main conclusions.

The average weight gain of pigs during the 88 day experimental period was 62 kg, close to the expected weight gain based on NRC (36).

3.1 Ileal bEPL

Ileal bEPL estimates based on the NF- and WPI method correlated moderately to highly (r=0.69, $P<0.05$), but using the WPI method resulted in about twice as much EPL compared to the NF method (**Figure 2a and 2b**).

The AA profile of the bEPL differed between both methods. In particular proline (8.3%-units) and glycine (2.9%-units) were lower, and threonine (4.6%-units), serine (2.6%-units), and glx (glutamic acid + glutamine; 2.8%-units) were higher in pigs fed the WPI diet compared to NF (**Figure 3**).

414

415 *3.2 Ileal tEPL*

416 In pigs fed the WPI diet, ileal bEPL estimates based on the WPI method and ileal tEPL
417 estimates based on the AA profile method correlated highly (r=0.88, *P*<0.01, **Figure 4a and**
418 **d**). Moderate correlations were observed between bEPL estimates based on the WPI method
419 and tEPL based on the 16S+18S method (r=0.42, *P*=0.27, **Figure 4b and e**) or DAPA+18S
420 method (r=0.56, *P*=0.10, **Figure 4c and f**).

421 Based on these results, the AA profile method was selected as the reference for comparing ileal
422 tEPL. In pigs fed with different protein sources, ileal tEPL estimates based on the AA profile
423 method correlated moderately with the tEPL estimates based on the 16S+18S method (r=0.58,
424 *P*<0.001, **Figure 5a and c**), and the DAPA+18S method (r=0.57, *P*<0.001, **Figure 5b and d**).

425

426 *3.3 Ileal bacterial and host EPL*

427 Poor correlations were observed between bacterial EPL estimates based on the AA profile
428 method and 16S method (r=0.004, *P*=0.98, **Figure 6a and 6c**), and the DAPA method (r=0.10,
429 *P*=0.40; **Figure 6b and 6d**).

430 A weak correlation was observed between host EPL estimates based on the AA profile method
431 and host EPL estimates based on the 18S method (r=0.39, *P*<0.001; **Figure 7a and 7b**).

4. Discussion

The main objectives of this experiment were to explore and compare different methods to quantify total (basal + specific) EPL, to compare two methods to quantify bEPL, and to explore the ability to discriminate between EPL originating from bacterial biomass and the host. The findings show that the AA profile method is most promising for quantifying tEPL, while the WPI method is preferred over the NF method for quantifying bEPL.

4.1 Ileal bEPL

Ileal bEPL estimates ranged between 4-24 g/kg DMI for the NF method and 8-27 g/kg DMI for the WPI method, in accordance with previous findings (4, 7, 71). The strong correlation between ileal bEPL estimates based on the NF- and the WPI method indicates that both methods may be suitable for quantifying bEPL. Nonetheless, bEPL estimates based on the NF method were lower, which may be due to a lower rate of protein synthesis and a lower secretion of gastric and pancreatic enzymes in pigs fed the NF diet (4-8). Contrary, as the WPI method relies on the assumption that WPI-proteins are fully digested and absorbed, any inadvertent undigested WPI in the ileum leads to an overestimation of bEPL. In rats, the true caecal protein digestibility (used as a proxy of ileal digestibility) of ^{15}N and ^{2}H labelled goat WPI was reported as 98% (72, 73), but the use of labelled dietary proteins also may give an underestimation of digestibility due to isotope recycling (74). Apart from comparing to literature, the assumption of full WPI digestion can be confirmed with calculations of the AA profile method. In our pigs fed the WPI diet on average 7.6% of the proteins in the ileal digesta were calculated to be of dietary origin. Correcting the WPI digestibility based on the presence of these estimated unabsorbed dietary proteins, the true ileal digestibility would be 99.8%. Based on these

455 calculations, the assumption of WPI being fully digested and absorbed is confirmed and
456 therefore the WPI method is considered to be accurate in quantifying bEPL.

457 In line with previous literature, feeding the NF diet resulted in relatively high ileal levels of
458 proline and glycine (7, 57-59). Feeding an NF diet increases muscle catabolism, resulting in
459 increased blood glutamine levels, which can be converted via glutamate to proline in the
460 intestine (75, 76). The reason for the relatively high ileal levels of glycine is unknown, but
461 decreased enzyme levels may lead to impaired re-absorption of bile, in which glycine is a major
462 component (30). Bile acids are mainly actively absorbed via specific transport proteins in the
463 distal ileum (77). In addition, Gibson et al. (78) observed that low dietary protein intake
464 decreased protein turnover in the human body, but increased glycine synthesis. Since glycine
465 is a precursor for the formation of purines, porphyrins, creatinine, glutathione, phospholipids,
466 and cysteine, endogenous glycine synthesis is increased at low protein levels to maintain blood
467 glycine levels as much as possible. In particular, threonine, serine, and glutamine/glutamate
468 (Glx) levels were lower in pigs fed the NF diet compared to pigs fed the WPI diet. Consistent
469 with the results for Glx, Jansman et al. (7) showed relatively low Glx contents in pigs fed an
470 NF diet, compared to pigs fed a diet based on highly digestible protein sources such as casein
471 and wheat gluten. The relatively high glutamate content in WPI (19% of crude protein; CP,
472 used in our study), casein (22% of CP, used by Jansman et al. (7)), and wheat gluten (34% of
473 CP, used by Jansman et al. (7)) possibly explains the higher glx content in the EPL estimates
474 for these diets (79). The reason for the relatively low levels of threonine and serine in pigs fed
475 the NF diet is unknown. Based on the results on amino acid profile, the WPI method is preferred
476 over the NF method.

477

478 *4.2 Ileal tEPL*

Ileal EPL estimates based on the NF and WPI method were moderately to highly correlated, but since feeding the NF diet may underestimate endogenous losses, the WPI method was used as a reference to compare EPL with EPL estimates based on the AA profile, the 16S+18S, and DAPA+18S methods. Nevertheless, comparable results were found when using the NF method as reference. The strong correlation between EPL estimates based on the WPI method and EPL estimates based on the AA profile method in pigs fed the WPI diet indicates that quantifying tEPL with the AA profile method has the greatest potential compared to the 16S+18S, and the DAPA+18S method.

Using the AA profile method as reference, tEPL estimates based on the 16S+18S and DAPA+18S method were evaluated across different test diets. The tEPL estimates based on the 16S+18S method and DAPA+18S methods were both moderately correlated with the AA profile method. Since the 16S+18S and DAPA+18S methods are still explorative, the moderate correlations indicate that both methods have the potential to quantify tEPL in the future, after refining the methods (see 4.3).

The fraction of bacterial protein in EPL was estimated to range between ~37 – 83%, depending on the method used. This is in line with findings of Miner-Williams et al. (3), who estimated that ileal EPL consisted for 54% of bacterial protein. Based on the AA profile method, the bacterial protein fraction in ileal tEPL were highest (>50% of tEPL) in pigs fed with bovine collagen, wheat bran, zein, and sorghum. These are all well fermentable protein sources, which may enhance the bacterial growth in the ileum (80-82).

4.3 Methodology

This study introduces new methods to quantify tEPL. Based on the current results, the AA profile method is most promising. Nevertheless, the 16S+18S- and DAPA+18S methods also

503 have potential, after refinement of the methods, and may be less laborious in the future. Current
504 assumptions, limitations and suggested adjustments are discussed below.

505

506 *4.3.1 Endogenous losses calculated by AA profile*

507 Bacterial and host EPL were estimated using the AA profiles of the diet, endogenous protein,
508 bacterial protein, and ileal digesta. For this method, it was assumed that all dietary amino acids
509 had identical digestibility coefficients. In reality, the digestibility differs among the different
510 amino acids, depending on, among other things, the position in the chain and the presence of
511 S- or H linkages (83, 84). This results in a different AA profile of the undigested feed compared
512 to that of the ingested feed, affecting the estimates of EPL (**Figure 8**). By excluding the amino
513 acids with an AID greater or lower than the average AID for all amino acids combined plus or
514 minus the SD, we attempted to minimize the impact of digestibility differences. Furthermore,
515 for this calculation, AA profiles of the host and bacterial EPL were based on literature.
516 Although AA profiles of EPL analyzed in various studies were comparable (3, 7, this study),
517 even small differences in AA profiles, for example caused by the different protein sources fed,
518 can lead to different results.

519

520 *4.3.2 Ileal bacterial tEPL analyzed by 16S and DAPA*

521 In this investigation, we used droplet-digital PCR to quantify the ileal bacterial mass by the
522 number of 16S RNA gene copies. While implementing this technique, we acknowledged that
523 variations in cell lysis efficiency and technical nuances in DNA extraction could influence the
524 total biomass estimations (85). Given the anticipated high bacterial density in our ileal samples,
525 the impact of contamination, commonly a concern in low biomass samples, was deemed to be
526 minimal (86). This assumption was based on the rationale that high bacterial counts would

significantly overshadow any minor contribution of variations in cell lysis efficiency and DNA extraction.

In the calculations, several assumptions have been made which could potentially influence the outcomes. Nevertheless, considering the substantial quantity of bacterial mass present in the colon of pigs ($1 \times 10^{10} - 1 \times 10^{11}$ CFU per gram digesta; 87), we assumed that these assumptions would not fundamentally change the conclusion. First, bacteria were assumed to contain an average of 4.2 16S copies. The number of 16S copies varies by bacterial genome and ranges between 1 and 21, but the averages and medians over different studies ranged between 4 and 6 (44, 88). In future studies, it may be useful to combine the quantification of 16S RNA gene copy number with 16S rRNA gene sequencing (89). However, the accuracy of interfering absolute species concentrations using 16S rRNA gene sequencing can in turn be influenced by different factors, such as the method of microbial qualification, sample biomass, and the relative abundance of the species of interest (85, 89-91). Secondly, we assumed that the bacterial protein content was 12.5g/100 gram bacterial cells, which also varies between and within bacterial species (51, 92). Finally, the average mass of bacteria, which was assumed to be 1×10^{-12} g in this study, also varies in reality (52). In order to assess the effect of the assumptions made, the EPL estimates were recalculated with a deviation of plus and minus 25% from the initial assumptions (**Figure 8**). The effect was consistent across all assumptions, indicating that no particular assumption dominates the final estimates.

For the DAPA method, the quantification of bacterial tEPL was based on the mean DAPA : bacterial nitrogen ratio of 26.4 mg/g, but like 16S copies, DAPA : bacterial nitrogen ratio differs among bacterial species (53, 93). Nevertheless, mixed bacterial populations are believed to be relatively constant in mean DAPA : bacterial nitrogen ratios. In addition, DAPA is also present in certain common feedstuffs and in protozoa, however in small amounts (53).

551

552 *4.3.3 Ileal host tEPL analyzed by 18S*

553 To our knowledge, this is the first study to quantify tEPL in the ileum by using qPCR to detect
554 18S copies in the ileal digesta. Based on the 18S copy numbers, endogenous protein from
555 porcine epithelial cells was quantified, and subsequently, total host EPL were estimated. To
556 estimate the amount of mucin proteins, a fixed ratio of porcine epithelial cells to mucus was
557 used, based on the study of Miner-Williams et al. (3), but presumably, this ratio is not fixed
558 and differs among diets (94). In future research, we therefore recommend also estimating the
559 mucus content in the digesta, for example by measuring the amino sugars glucosamine and
560 galactosamine, or by the quantification of MUC2 using enzyme-linked immune sorbent assay
561 (ELISA; 3, 95, 96). In addition, digestive secretions and immunoglobulins were not included
562 in the calculations, potentially leading to an underestimation of the ileal host tEPL (3).

563

564 *5. Implications for true digestibility*

565 Correcting the AID by bEPL analyzed in pigs fed with the WPI diet resulted in an 2-7% unit
566 increased SID compared to the NF diet (**Table 2,** 11). Following the lower amount of EPL
567 estimates based on the NF method compared to WPI, using the NF diet for estimating bEPL
568 led to lower SID estimates. As anticipated, the TID of chickpeas, toasted wheat bread, and
569 wheat bran was greater than the SID based on the WPI method, but for the other protein sources
570 no significant differences were observed. The differences between SID and TID may be
571 important for protein evaluation and diet formulation, emphasizing the relevance to more
572 accurately quantify tEPL.

5. Conclusions

In light of our current findings in pigs, the amino acid profile method emerges as the most promising approach for quantifying total endogenous protein losses (tEPL), encompassing both basal and specific losses, and may serve as a reference for future developments for quantifying host and bacterial proteins in ileal digesta. The 16S+18S and DAPA+18S methods have potential to quantify tEPL after refinement of the techniques.

Despite the challenges in distinguishing between bacterial and host EPL in this study, it is evident that bacterial proteins substantially (on average 37-83%, depending on method) contribute to the tEPL. This contribution is likely modulated by the digestibility and fermentability of the dietary ingredients.

To quantify basal EPL (bEPL), the whey protein isolate (WPI) method is preferred over the NF method, as the NF method may result in an underestimation of bEPL.

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Author contributions

LN, NW, SV, WJJG designed research, BH, KCMJ, LN conducted research, AKK, BH, GJEJH, LN, NW analyzed data, AKK, BH, KCMJ, LN, NW, SV, WJJG wrote paper, LN, SV, WJJG had primary responsibility for the final content. All authors read and approved the final manuscript.

Data sharing plan

Data described in the manuscript, code book, and analytic code will be made available upon request pending application.

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602

603 **Figure 1.** Experimental design of the experiment in which different methods were evaluated
604 to quantify total endogenous protein losses and basal endogenous protein losses, and assessed
605 for their potential in discriminating between total endogenous protein losses from bacteria and
606 host. bEPL = basal endogenous protein losses, sEPL = specific endogenous protein losses,
607 tEPL = total endogenous protein losses.

608 **Figure 2.** a) Relations between ileal basal endogenous protein losses analyzed after feeding a
609 nitrogen-free diet (NF method), or a diet based on whey protein isolate (WPI method) in ileal
610 cannulated pigs (n=11). b) Bland-Altman plot; the solid line represents the average difference
611 between basal endogenous protein losses estimated based on the NF and WPI method (g/kg
612 DMI) and the dashed lines represent the 95% confidence interval limits. bEPL = basal
613 endogenous protein losses

614 **Figure 3.** Amino acid profile of ileal basal endogenous protein losses (mean ± SD) measured
615 after feeding a nitrogen-free diet, or a diet based on whey protein isolate to pigs (n=16 for
616 nitrogen-free; n=12 for whey protein isolate). * = $P<0.05$. ALA = alanine, ARG = arginine,
617 ASP= aspartic acid, CYS = cysteine, GLX = glutamic acid + glutamine, GLY = glycine, HIS
618 = histidine, ILE = isoleucine, LEU = leucine, LYS = lysine, MET = methionine, NF = nitrogen-
619 free, PHE = phenylalanine, PRO = proline, SER = serine, THR = threonine, TRP = tryptophan,
620 TYR = tyrosine, VAL = valine, WPI = whey protein isolate.

621 **Figure 4.** a-c) Relations between basal and total endogenous protein losses in the ileum,
622 analyzed after feeding a diet based on whey protein isolate (WPI method) to ileal cannulated
623 pigs (n=9). Ileal bEPL were quantified by the digesta protein content (WPI method), and ileal
624 tEPL were quantified by the AA profile method, 16S+18S method, and DAPA+18S method.
625 d-f) Bland-Altman plots; the solid line represents the average difference between basal
626 endogenous protein losses and total endogenous protein losses (g/kg DMI) and the dashed lines
627 represent the 95% confidence interval limits. bEPL = basal endogenous protein losses, EPL =
628 endogenous protein losses, tEPL = total endogenous protein losses.

629 **Figure 5.** a-b) Relations between total endogenous protein losses in the ileum, analyzed after
630 feeding different protein sources to pigs. Ileal total endogenous protein losses were qualified
631 by the AA profile method, compared to the 16S+18S method, and the DAPA+18S method. c-
632 d) Bland-Altman plots; the solid line represents the average difference in total endogenous
633 protein losses between the different methods (g/kg DMI) and the dashed lines represent the
634 95% confidence interval limits. BB = black beans (n=6), BC = bovine collagen (n=5), CHP =

chickpeas (n=5), NF = nitrogen free (n=11), PP = pigeon peas (n=10), RP = roasted peanuts (n=5), SO = sorghum (n=10), tEPL = total endogenous protein losses, TWB = toasted wheat bread (n=4), WB = wheat bran (n=5), WPI = whey protein isolate (n=12), ZE = zein (n=6).

Figure 6. a-b) Relations between bacterial endogenous protein losses in the ileum, analyzed after feeding different protein sources to pigs. Bacterial endogenous protein losses were quantified by the AA profile method, compared to the 16S method and DAPA method. c-d) Bland-Altman plots; the solid line represents the average difference in bacterial endogenous protein losses between the different methods (g/kg DMI) and the dashed lines represent the 95% confidence interval limits. BB = black beans (n=6), BC = bovine collagen (n=5), CHP = chickpeas (n=5), EPL = endogenous protein losses, NF = nitrogen free (n=11), PP = pigeon peas (n=10), RP = roasted peanuts (n=5), SO = sorghum (n=10), TWB = toasted wheat bread (n=4), WB = wheat bran (n=5), WPI = whey protein isolate (n=12), ZE = zein (n=6).

Figure 7. a) Relations between host endogenous protein losses in the ileum, analyzed after feeding different protein sources to pigs. Host ileal endogenous protein losses were qualified by the AA profile method, and compared with the 18S method. b) Bland-Altman plot; the solid line represents the average difference in host endogenous protein losses between the different methods (g/kg DMI) and the dashed lines represent the 95% confidence interval limits. BB=black beans (n=6), BC = bovine collagen (n=5), CHP = chickpeas (n=5), EPL = endogenous protein losses, NF = nitrogen free (n=11), PP = pigeon peas (n=10), RP = roasted peanuts (n=5), SO = sorghum (n=10), TWB = toasted wheat bread (n=4), WB = wheat bran (n=5), WPI = whey protein isolate (n=12), ZE = zein (n=6).

Figure 8. Impact of assumptions made in the calculations for bacterial, host, and total endogenous protein losses. For each method, the mean, minimal, and maximal endogenous protein losses estimations were used and the impact of a 25% change in each assumed value in the equations on the endogenous protein losses estimates was evaluated. The white bars represent the estimated endogenous protein losses calculated using the default assumption as described in section 2.5, the black bars represent endogenous protein losses estimates based on the default assumption plus or minus 25%. [1]Assumptions 16S method: 1) Bacteria contain 4.2 16S-copies, 2) Bacterial protein content is 12.5%, 3) Bacterial mass is 1 x 10^{-12} g. [2]Assumptions DAPA method: diaminopimelic acid content is 26.4 mg/g bacterial nitrogen. Assumptions 18S method: ratio intestinal cells : mucus in endogenous protein losses is 6.7 : 14.1. Assumptions AA profile method: amino acid profile used, based on literature, is correct;

667 error bars represent total endogenous protein losses calculated with the average digesta amino
668 acid profile in pigs fed NF and WPI. [3]Assumptions AA profile method: digestibility of all
669 amino acids is equal. Impact of digestibility was checked for two selected amino acids,
670 glutamic acid + glutamine with a high concentration in endogenous protein losses, and
671 methionine with a low concentration in endogenous protein losses. For both amino acids,
672 endogenous protein losses were calculated when the amino acids had a 25% lower or 25%
673 higher digestibility compared to the other amino acids. AA = amino acid, DAPA =
674 diaminopimelic acid, EPL = endogenous protein losses, Glx = glutamic acid + glutamine, lit.
675 = literature, Met = methionine, N = nitrogen.

Table 1. Ingredient composition and chemical composition of the experimental diets fed to pigs

			Test Diets[1]									
	BD	NF	BB	BC	CHP	PP	RP	SO	TWB[2]	WB	WPI	ZE[3]
Ingredient (g/kg)												
Bread meal	688											
Maize starch		785.4	155.8	687.5	128.2	141.4	432.5				676.1	668.1
Purified cellulose		27.3	13.0	27.6	13.8	13.6	28.1			71.3	27.6	27.6
Sucrose		91.0	43.5	91.9	45.9	45.2	93.8				91.9	92.1
Rapeseed oil	50	45.3	21.6	45.7	22.9	22.5	46.7			71.0	45.7	45.8
Premix[4]	10	1.4	0.7	1.4	0.7	0.7	1.4	1.3	1.4	1.5	1.4	1.4
Dicalcium phosphate	17	22.7	10.9	23.0	11.5	11.3	23.5	22.4	22.8	24.6	23.0	23.0
Magnesium oxide		0.9		1.0							1.0	1.0
Calcium carbonate	5	2.7	1.3	2.8	1.4	1.4	2.8	2.7	2.7	3.0	2.8	2.8
Potassium carbonate	3	6.4	3.0	6.4	3.2	3.2	6.6	6.3	6.4	6.9	6.4	9.2
Sodium hydrogen carbonate	5	2.7	1.3	2.8	1.4	1.4	2.8	2.7	2.7	3.0	2.8	4.6
Salt		3.8	1.8	3.8		1.9	3.9	3.7		1.0	3.8	3.8
L-Lysine HCl	2											
Casein	70											
Wheat gluten meal	50											
Whey powder	50											
Potato protein	30											
Skimmed milk powder	20											
Tested protein source[5]			742.0	95.7	765.8	752.5	347.1	950.6	954	806.5	107.0	110
Titanium dioxide		3.6	1.7	3.7	1.8	1.8	3.8	3.6	3.5	3.9	3.7	3.7
Celite		6.8	3.3	6.9	3.4	3.4	7.0	6.7	6.5	7.4	6.9	6.9
Chemical components (g/kg DM)												
Protein[6]	243		100	104	107	120	87	99	110	93	112	121
Total dietary fiber[7]	96[9]	53[9]	58[8,9]	50[9]	136[8,9]	120[8,9]	72[8,9]	74[8,9]	93[8,9]	456[9]	50[9]	49[9]

[1]Test diets are abbreviated as follows: BD=basal diet, NF =nitrogen free diet, BB=black beans, BC=bovine collagen, CHP=chickpeas, PP=pigeon peas, RP=roasted peanuts, SO=sorghum, TWB=toasted wheat bread, WB=wheat bran, WPI=whey protein isolate, ZE=zein. [2]Titanium dioxide and celite were

added to the bread dough for the toasted wheat bread. [3]L-Lysine (12 g/kg DM) and L-Tryptophan (2 g/kg DM) were added to the diet on top at days 1-5 of the feeding period. [4]The premix offers the following per kilogram of diet: Cu 10 ppm, I 1.3 ppm, Fe 125 ppm, Mn 60 ppm, Se 0.3 ppm, Zn 100 ppm, niacin 44 mg, cobalamin 0.03 µg, pantothenic acid 24 mg, riboflavin 6.6 mg, phytonadione 1.4 mg, biotin 0.44 mg, retinol 11 IU, cholecalciferol 2.2 IU, d,l-α-tocopherol 66 IU, pyridoxine 0.24 mg, folate 1.6 mg, thiamin 0.24 mg. [5]For suppliers of the test protein sources, see Hodgkinson et al. 2022. Roasted peanuts were obtained from a local supplier in Argentina, chickpeas were obtained from Sofia Foods in Italy, and sorghum was obtained from a local supplier in the USA. [6]Analyzed for test diets (based on the sum of individual amino acid concentrations), calculated for basal diet. [7]Calculated ([8]USDA; FoodData Central, [9]CVB 2018; calculated as organic matter – crude protein – crude fat – starch – sugar).

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Table 2. Apparent total amino acid ileal digestibility (AID), standardized total amino acid ileal digestibility (SID), and true total amino acid ileal digestibility (TID) of various protein sources in pigs.[1]

	No. of pigs[2]	AID (%)	SID (%) NF method	SID (%) WPI method	TID (%) AA profile method	*P*-Value[3]
Protein Source						
Black beans	6	67 (5.8)	76 (5.0)	78 (6.0)	78 (6.9)	0.82
Bovine collagen	7	77 (11.9)	84 (14.1)^{a}	89 (15.6)^{ab}	97 (2.5)^{b}	0.010
Chickpeas	6	72 (4.0)	78 (3.6)^{a}	82 (4.4)^{a}	93 (5.8)^{b}	<0.001
Pigeon peas	13	80 (3.9)	87 (5.4)^{a}	90 (5.4)^{ab}	94 (3.2)^{b}	<0.001
Roasted peanuts	6	57 (20.1)	65 (11.2)^{a}	69 (11.3)^{b}	73 (7.2)^{b}	<0.001
Sorghum	11	75 (15.1)	89 (8.6)	91 (5.5)	93 (4.2)	0.15
Toasted wheat bread	6	74 (5.5)	81 (7.3)^{a}	88 (5.3)^{b}	99 (1.1)^{c}	<0.001
Wheat bran	7	59 (7.4)	69 (6.9)^{a}	70 (5.5)^{a}	84 (9.9)^{b}	<0.001
Whey protein isolate	12	91 (3.8)	97 (5.2)^{a}	100^{b}	99 (0.9)^{ab}	0.016
Zein	7	70 (17.8)	77 (18.9)	79 (18.9)	82 (13.5)	0.50

[1]Data are presented as calculated means of untransformed data (standard deviation). [2]All animals were individually housed. [3]Model established *P*-values for the fixed effect of method and the random effects of pig. Values within each row not sharing a common letter (a, b, c) differ significantly ($P<0.05$).

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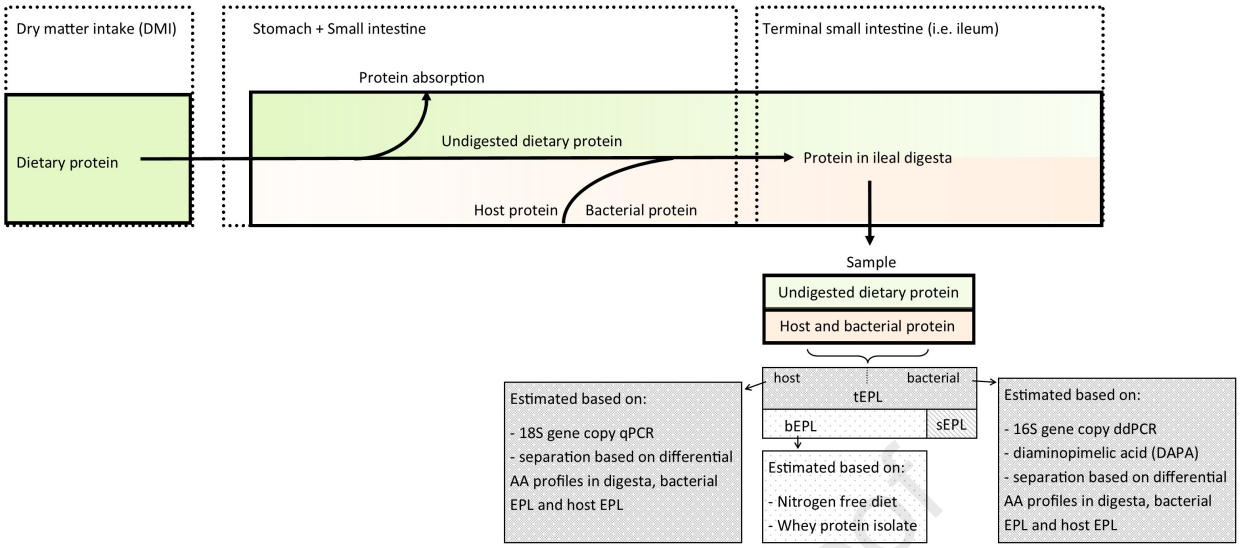

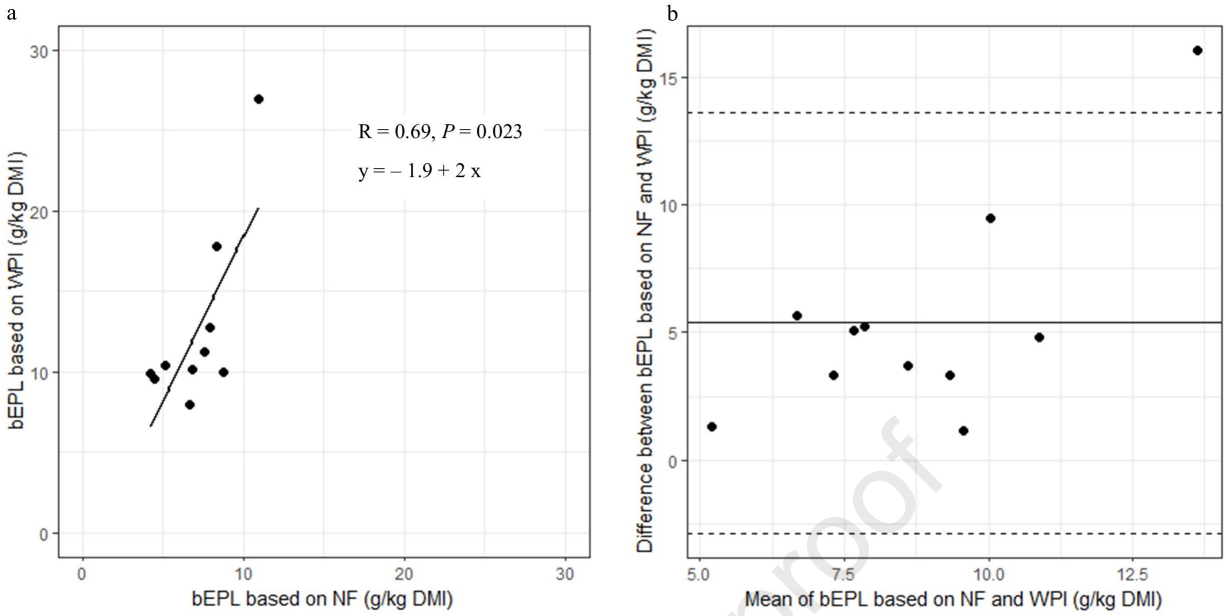

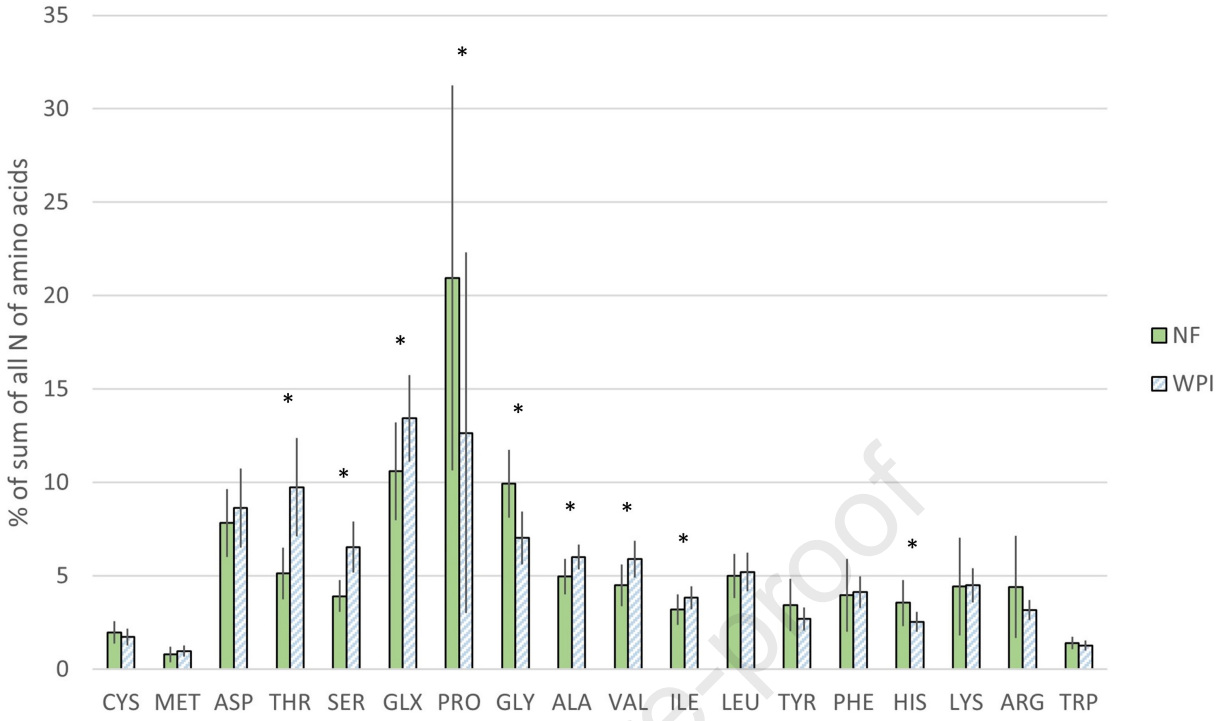

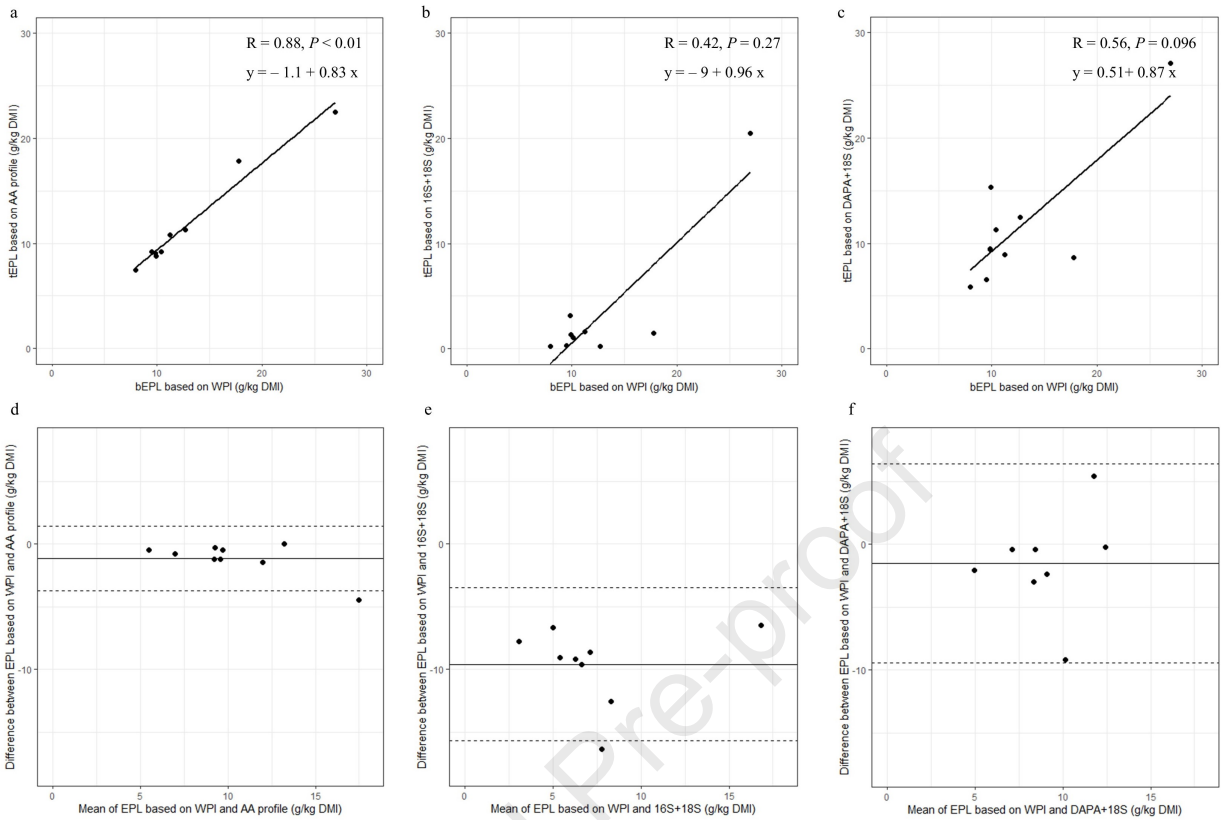

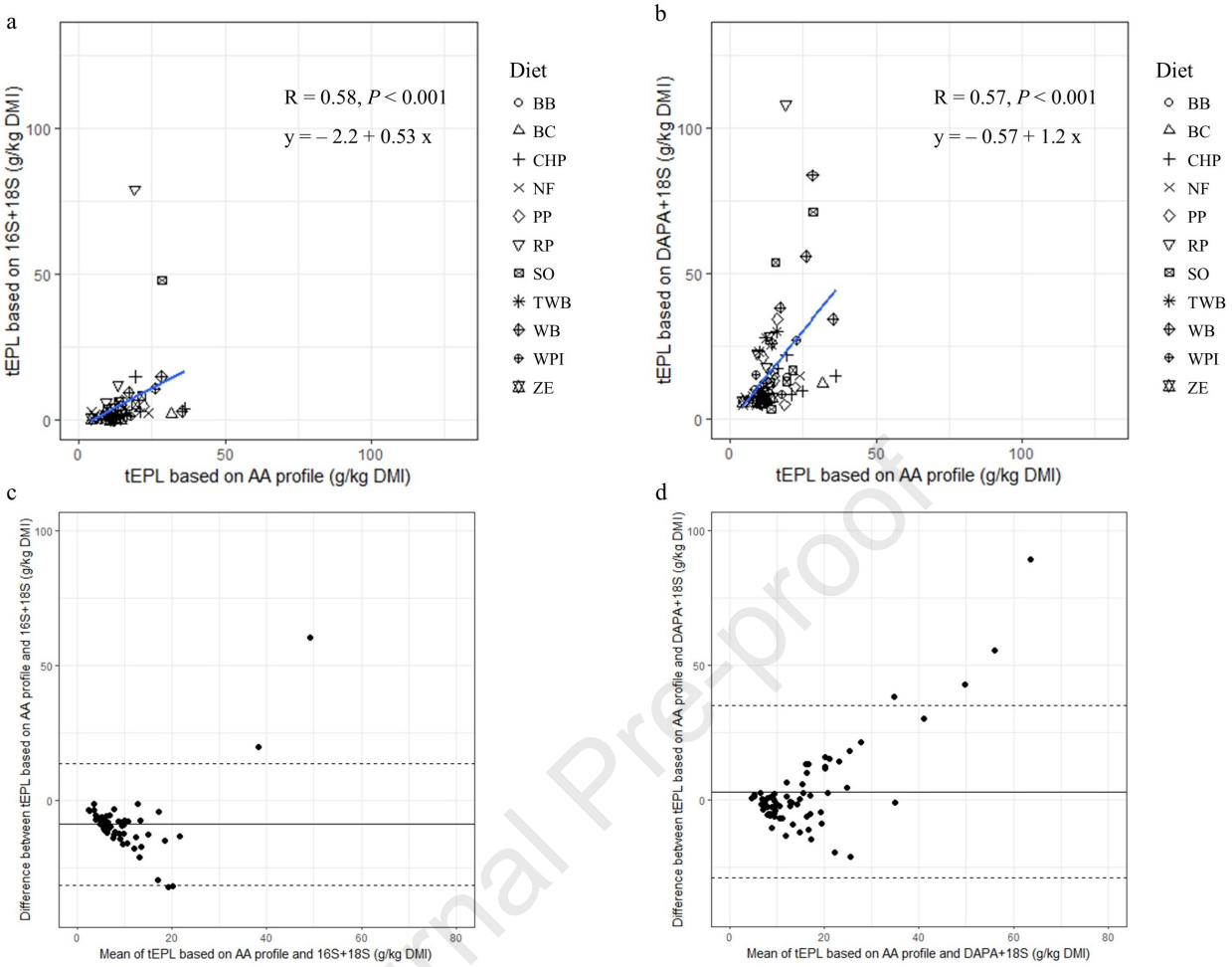

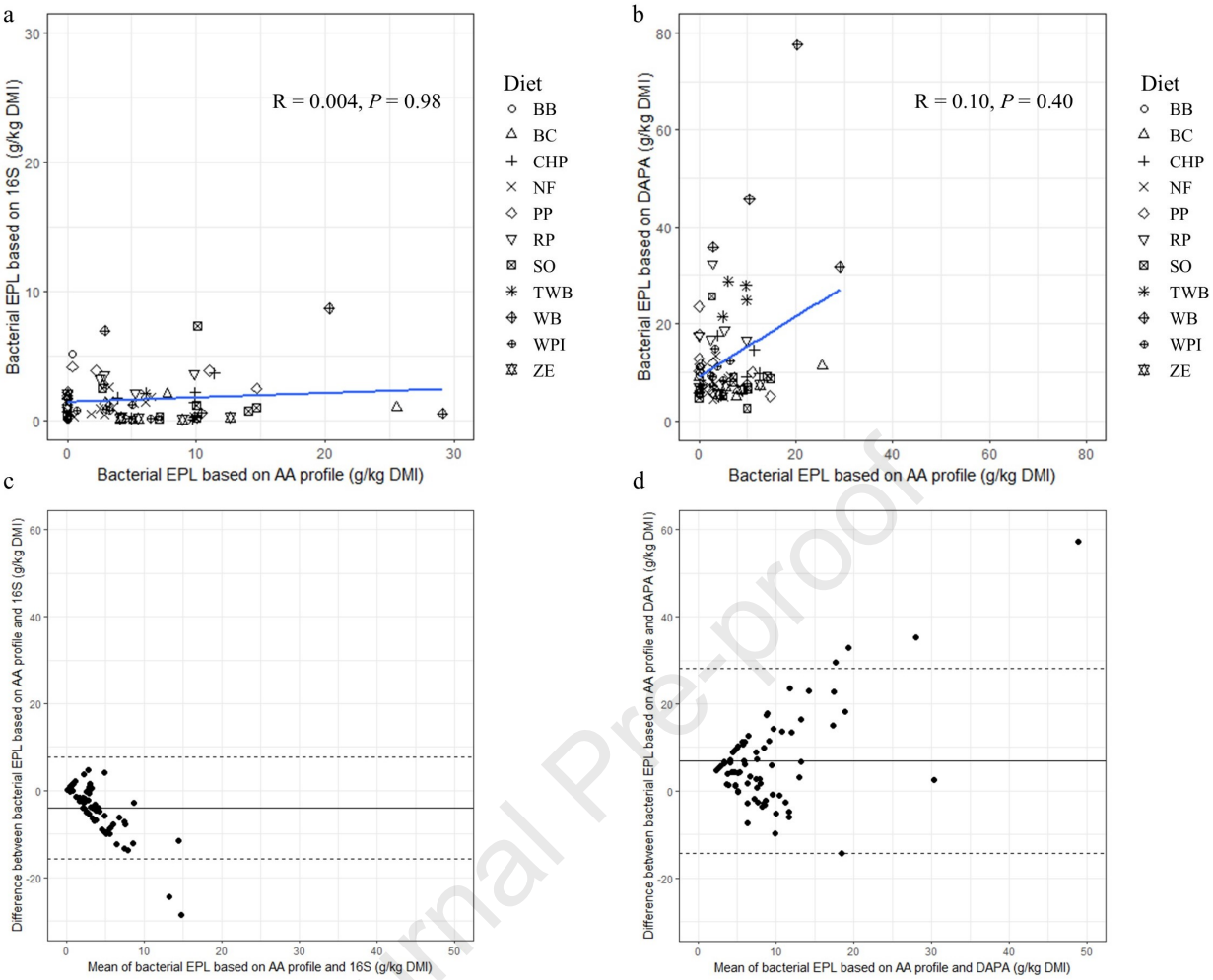

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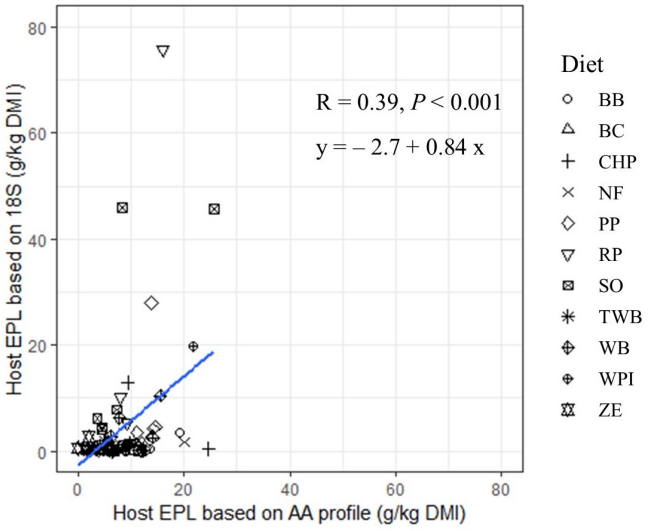

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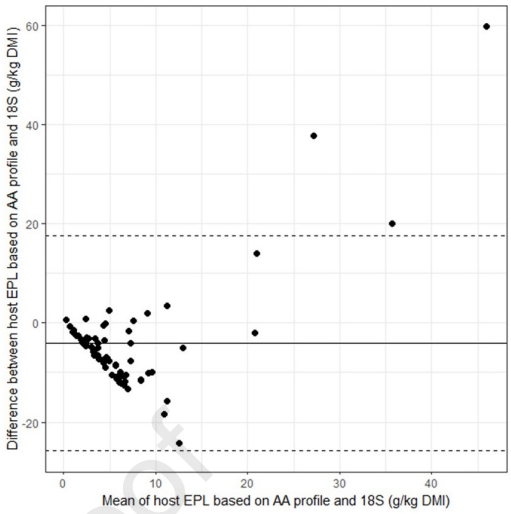

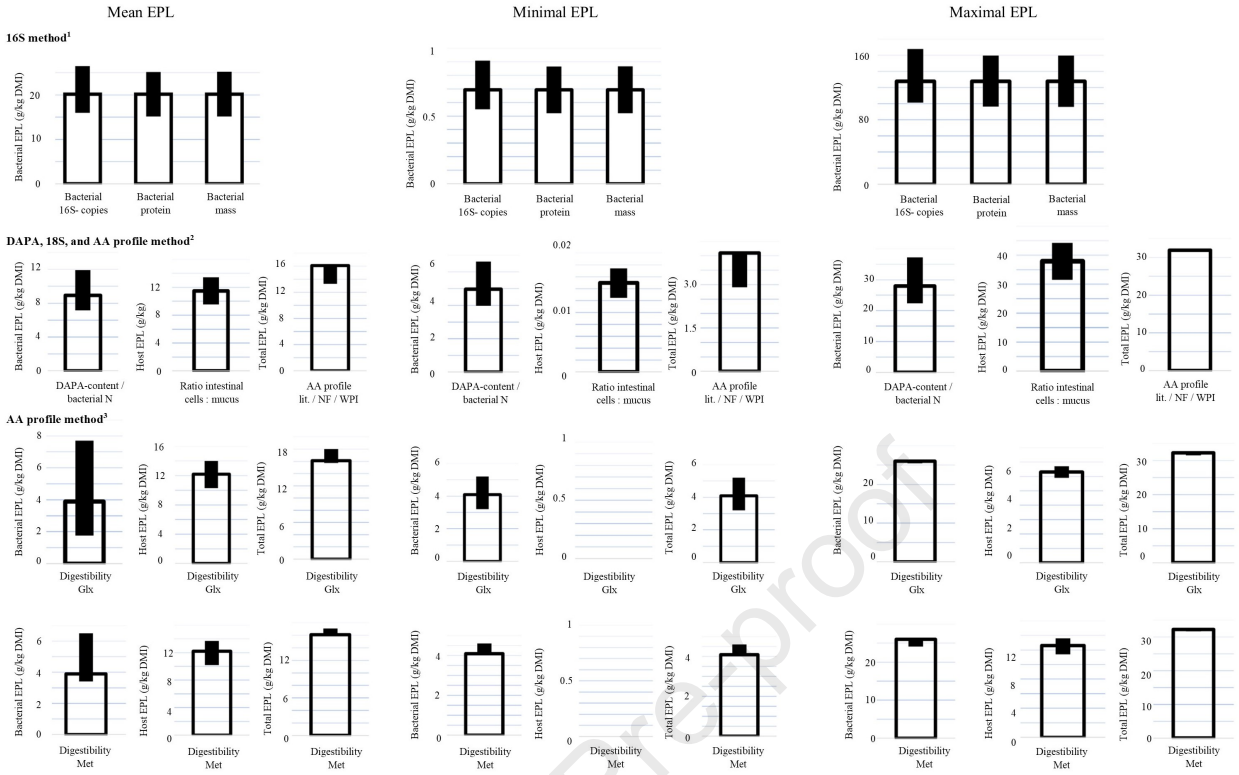

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: