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Potential New Methods to Analyze Basal and Total Endogenous Protein Losses of Host and Bacterial Origin in Pigs

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A B S T R A C T

Background: Current systems for assessing protein quality such as the Digestible Indispensable Amino Acid Score correct apparent amino acid (AA) 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.

Objectives: 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, 12 ileal cannulated pigs received 10 different protein sources, and a nitrogen-free (NF) diet. Ileal digesta were collected on days 6 and 7 of each 1-wk feeding period, to quantify endogenous protein losses (EPL) and analyze apparent ileal digestibility. Ileal EPL were estimated based on *1*) 16S-+18S gene copy quantitative polymerase chain reaction, *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 to 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, whereas 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: 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

Abbreviations: AA, amino acid; AID, apparent ileal digestibility; ANF, antinutritional factor; BB, black beans; BC, bovine collagen; bEPL, basal endogenous protein losses; CHP, chickpeas; CP, crude protein; DAPA, diaminopimelic acid; ddPCR, droplet-digital PCR; DIAAS, Digestible Indispensable Amino Acid Score; DM, dry matter; DMI, dry matter intake; EPL, endogenous protein losses; N, nitrogen; NF, nitrogen-free; NRC, National Research Council; PP, pigeon peas; RP, roasted peanuts; sEPL, specific endogenous protein losses; SID, standardized ileal digestibility; SO, sorghum; STAR, Stool Transport and Recovery; 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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Introduction

To evaluate protein quality in human foods, the FAO recommends using the Digestible Indispensable Amino Acid Score, that is, 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 AAs in pigs, the oro-ileal disappearance of AAs is corrected for the non-dietary AAs, so-called endogenous protein losses (EPL), present at the terminal ileum [2].

EPL 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 (NF) 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].

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

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

In this study, various methods are evaluated for the quantification of EPL, and their effectiveness in distinguishing between EPL originating from bacterial and host origin. Bacterial EPL are quantified using 16S gene copy droplet-digital PCR (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*) DAPA + 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 an NF diet (NF method), and *5*) feeding a highly digestible protein source (whey protein isolate; WPI method), equaling bEPL.

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).

Experimental design and housing

This experiment was part of a larger experiment in which standardized ileal AA digestibility coefficients were studied [2]. A total of 16 (12 + 4 spare) gilts (Topigs Norsvin TN70; Topigs) 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) 6 d after the end of period VII. Using an incomplete Youden square design [34], 10 protein sources were tested for ileal EPL and apparent ileal digestibility (AID) in 7 consecutive periods of 1 wk in 12 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, an NF diet was fed for one period between period IV and V to all 16 pigs, followed by a wash-out period of 1 wk where pigs received the basal diet.

In total, this resulted in 6 observations for black beans (BB), 7 for bovine collagen (BC), 6 for chickpeas (CHP), 13 for pigeon peas (PP), 6 for roasted peanuts (RP), 11 for sorghum (SO), 6 for toasted wheat bread (TWB), 7 for wheat bran (WB), 12 for WPI, 7 for zein (ZE), and 15 for the NF diet. The pigs were housed individually in metabolism pens (1.35 × 1.20 m). The first days after surgery the temperature was controlled between 21°C and 23°C, thereafter the temperature was reduced to 19.5°C–21.5°C. Pigs were exposed to 12 h of light per 24 h.

Animal procedures

After arrival at the research facilities of Wageningen University & Research, the pigs were adapted to their new environment

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[3]	WPI	ZE[4]
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[5]	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[6]			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[7]	243		100	104	107	120	87	99	110	93	112	121
Total dietary fiber[8]	96[10]	53[10]	58[9,10]	50[10]	136[9,10]	120[9,10]	72[9,10]	74[9,10]	93[9,10]	456[10]	50[10]	49[10]

[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] Kellogg's All Bran (Aust.) Pty. Ltd.
[4] 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.
[5] The premix offers the following per kg 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.
[6] For suppliers of the test protein sources, see Hodgkinson et al. [2]. 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 United States.
[7] Analyzed for test diets (based on the sum of individual amino acid concentrations), calculated for basal diet.
[8] Calculated
[9] USDA; FoodData Central
[10] CVB 2018; calculated as organic matter – crude protein – crude fat – starch – sugar.

and received a casein-based diet (basal diet, Table 1). On day 15–17, simple T-cannulas were surgically placed at the end of the small intestine [35]. After surgery, the pigs underwent a recovery time of 8–10 d, still receiving the basal diet. After recovery, pigs were assigned to the test diets, or the NF diet, as described above. On days 6 and 7 of each measurement period ileal digesta was collected for 9 h starting directly after the first meal of each day. The collecting bags were changed every 30 min or when full, and the digesta was immediately stored at −20°C. The pigs were fed twice a day (0700 h and 1600 h), with a daily allowance of 8% of their metabolic body weight ($0.08 \times BW^{0.75}$). Pigs had ad libitum access to water.

Diets

The diets were designed as described by Hodgkinson et al. [35]. Briefly, each experimental diet contained one of the test ingredients, which was the only source of protein. Diets were formulated to have a protein concentration of 100 g/kg DM (Table 1). The tested protein sources were BB, BC, CHP, PP, RP, SO, TWB, WB, WPI, and ZE. The chemical composition of the test ingredients is provided in Supplemental Table 1.

To minimize any potential impact of AA irregularities, a maximum of 2 diets containing AAs below National Research Council (NRC) recommendations [36] were fed consecutively. In the ZE-based diet, L-Lysine (12 g/kg DM) and L-Tryptophan (2 g/kg DM) were added on top during days 1–5 of the feeding period, and only withdrawn on sampling days.

Chemical analysis

Immediately after each measurement period, digesta samples were thawed, homogenized, and pooled for each pig per diet. From each of these samples a subsample was taken and stored at −80°C. Subsequently, the digesta samples were freeze-dried, and ground through a 1 mm screen using a centrifugal mill (Retsch ZM200) at 12,000 rpm. Then, they were analyzed for titanium (Ti), dry matter (DM), and AAs to determine the AA profile and the AA digestibility according to ISO 1999 and 2005 [37–39]. Reactive lysine was determined according to Moughan and Rutherfurd [40]. Titanium was analyzed after hydrolysis with concentrated sulfuric acid in the presence of a copper catalyst at 420°C and the subsequent addition of peroxide. The resulting

orange/yellow colored complex was spectroscopically determined at 408 nm [41,42]. Analyses were performed in duplicate.

Methods to quantify EPL

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

16S+18S method

Real-time qPCR was used to detect 18S gene copies in the ileal digesta. ddPCR was used to detect 16S gene copies. Using the number of 16S rRNA gene copies, which encodes for the highly-conserved RNA component of the 30S subunit of a prokaryotic ribosome, an estimation can be made of the ileal bacterial mass (see section "Calculations for the 16S+18S method") [44]. Using the number of 18S rRNA gene copies, which encodes eukaryotic RNA, an estimation can be made of the amount of protein from pig epithelial cells [32]. The sum of EPL of bacterial and host origin represents the tEPL. The 18S copy number was based on reference tissue values. For this, 30 mg prewashed jejunal scrapings of 3 pigs from another experiment (±8 kg) was mechanically homogenized in Dulbecco's phosphate-buffered saline (Gibco) using a Turrax disperser tool (T10, IKA), and DNA was extracted using a DNeasy blood and tissue extraction kit (ID: 69504, Qiagen) following manufacturers' protocol. The resulting DNA was used for developing a standard curve in qPCR for protein content estimation. For total protein, the same tissue homogenization process was followed and protein content was measured using a protein quantification kit [Qubit Protein Broad Range (BR) Assay, Thermo Fisher].

DNA extraction for the 16S+18S method. DNA was extracted from the ileal digesta subsamples as previously described [45]. In total, 200 mg per sample were thawed on ice and added to bead-beating tubes containing 700 µL of Stool Transport and Recovery (STAR)-solution (Roche), 0.5 g of 0.1 mm autoclaved zirconia beads, and five 2.5 mm glass beads. Samples were homogenized by repeated bead-beating (5.5 ms, 3 × 1 min, FastPrep-24; MP Biomedicals, LLC) at room temperature. Next, the samples were incubated at 95°C for 15 min, while shaking at 300 rpm, followed by centrifugation at 4°C (15,000 × *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.

Total bacterial load and host endogenous protein estimation by real-time qPCR. Bacterial protein mass was quantified by ddPCR using the BactQuant qPCR assay [33]. All materials for the ddPCR analysis were ordered from Bio-Rad Laboratories. 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 2× 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 s, and 58°C annealing and elongation for 2 min. The PCR was finished with an additional extension at 98°C for 10 min, followed by 30 min 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].

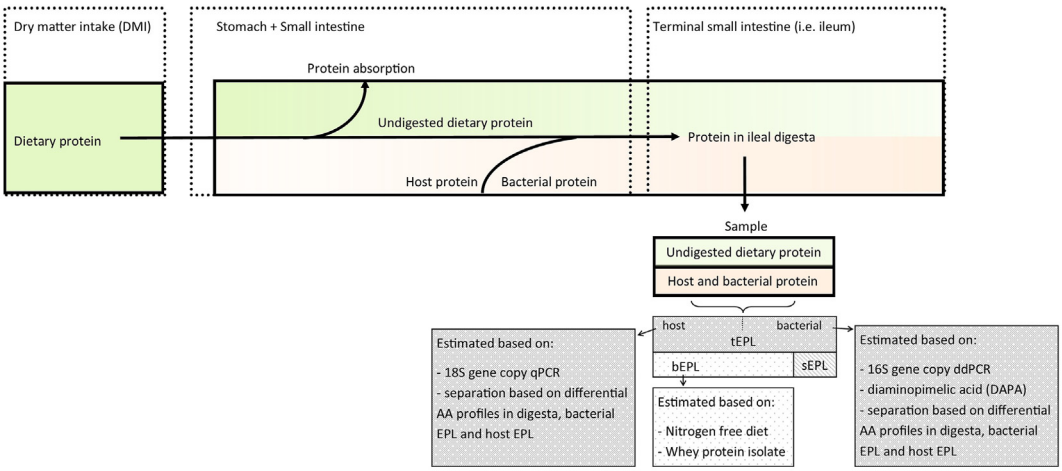

FIGURE 1. Experimental design of the experiment in which different methods were evaluated to quantify total endogenous protein losses and basal endogenous protein losses, and assessed for their potential in discriminating between total endogenous protein losses from bacteria and host. bEPL, basal endogenous protein loss; sEPL, specific endogenous protein loss; tEPL, total endogenous protein loss.

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—TCCTTGGAT GTGGTAGCCGTTT. 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 min and followed by 35 repeated cycles of 95°C denaturation for 20 s, 60°C annealing for 30 s, and 72°C elongation for 30 s. The PCR was finished with an additional extension at 72°C for 30 s 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].

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; DM digesta) =

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

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) =

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

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)

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

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) =

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

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) =

$$\text{EPL Int. Cells. (g / kg DM)} + \text{EPL Mucus (g / kg DM)}$$

6. Host protein (g/kg DMI) =

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

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.

DAPA+18S method

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 section "16S+18S method").

DAPA analysis. DAPA was analyzed after oxidation overnight with performic acid/phenol at 0°C and neutralization with sodium disulfite, followed by hydrolysis with 6 M HCL during 23 h. 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).

Calculations for the DAPA+18S method. Bacterial tEPL based on DAPA were calculated using the following equations:

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

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

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) =

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

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 section "Calculations for the 16S+18S method."

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 supplemental file.

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 (Supplemental 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. First, proline and glycine were excluded from the AA pattern used for the calculation, because these AAs may be overestimated when using an NF diet [57–59]. Second, for these calculations, digestibility of the various AA was assumed to be equal. In practice, however, this is not correct. Therefore, AA with an 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.

Digestibility calculations

Apparent ileal protein digestibility was calculated using equation 9 [60]:

9. AID protein =

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

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 AA 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. SID protein =

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

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 AA concentrations (bEPL), dietary protein is the protein concentration in the diet, and AID is the apparent ileal digestibility of the protein.

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 2 methods: potential outliers identified in boxplots using the IQR criterion, determined per protein source, and values that were higher or lower than the mean ± 2 SDs (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, 2× WPI) and for 18S, 3 pigs were excluded (RP, PP, SO, Supplemental 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.

Results

Because of canula problems and illness, 2 reserve pigs were used to replace experimental pigs. Furthermore, protein digestibility of RP of 1 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 SO was excluded from the dataset because Ti concentrations were very low, probably due to an analytical error. Finally, when fed the NF diet, 1 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 d experimental period was 62 kg, close to the expected weight gain based on NRC [36].

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 with the NF method (Figure 2A and B).

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 with NF (Figure 3).

Ileal tEPL

In pigs fed the WPI diet, ileal bEPL estimates based on the WPI method and ileal tEPL estimates based on the AA profile method correlated highly ($r = 0.88$, $P < 0.01$, Figure 4A and D). Moderate correlations were observed between bEPL estimates based on the WPI method and tEPL based on the 16S+18S method ($r = 0.42$, $P = 0.27$, Figure 4B and E) or DAPA+18S method ($r = 0.56$, $P = 0.10$, Figure 4C and F).

On the basis of these results, the AA profile method was selected as the reference for comparing ileal tEPL. In pigs fed with different protein sources, ileal tEPL estimates based on the AA profile method correlated moderately with the tEPL estimates based on the 16S+18S method ($r = 0.58$, $P < 0.001$, Figure 5A and C), and the DAPA+18S method ($r = 0.57$, $P < 0.001$, Figure 5B and D).

Ileal bacterial and host EPL

Poor correlations were observed between bacterial EPL estimates based on the AA profile method and 16S method ($r = 0.004$, $P = 0.98$, Figure 6A and C), and the DAPA method ($r = 0.10$, $P = 0.40$; Figure 6B and D).

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

Discussion

The main objectives of this experiment were to explore and compare different methods to quantify total (basal + specific) EPL, to compare 2 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, whereas the WPI method is preferred over the NF method for quantifying bEPL.

Ileal bEPL

Ileal bEPL estimates ranged between 4 and 24 g/kg DMI for the NF method and 8 and 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-labeled goat WPI was reported as 98% [72,73], but the use of labeled dietary proteins also may give an underestimation of digestibility due to isotope recycling [74]. Apart from comparing with 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 TID would be 99.8%. On the basis

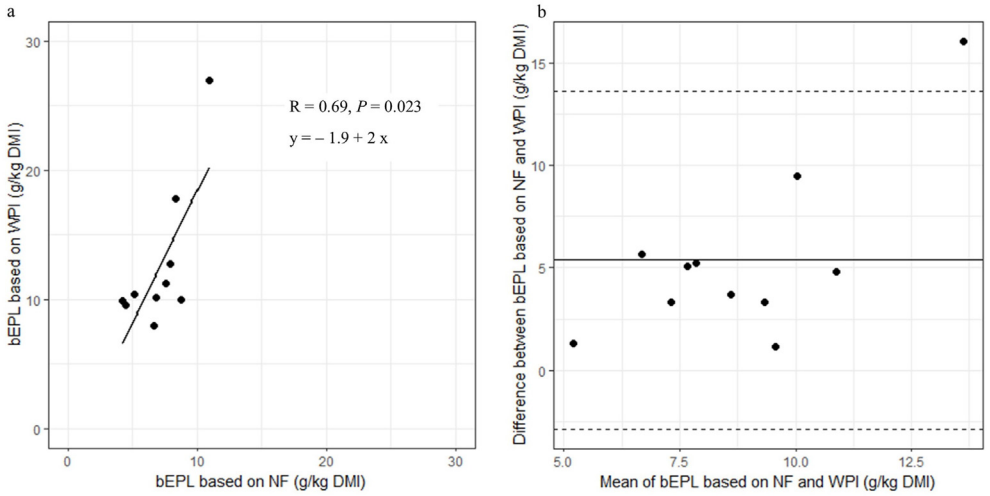

FIGURE 2. (A) Relations between ileal basal endogenous protein losses analyzed after feeding a nitrogen-free diet (NF method), or a diet based on whey protein isolate (WPI method) in ileal cannulated pigs ($n = 11$). (B) Bland-Altman plot; the solid line represents the average difference between basal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

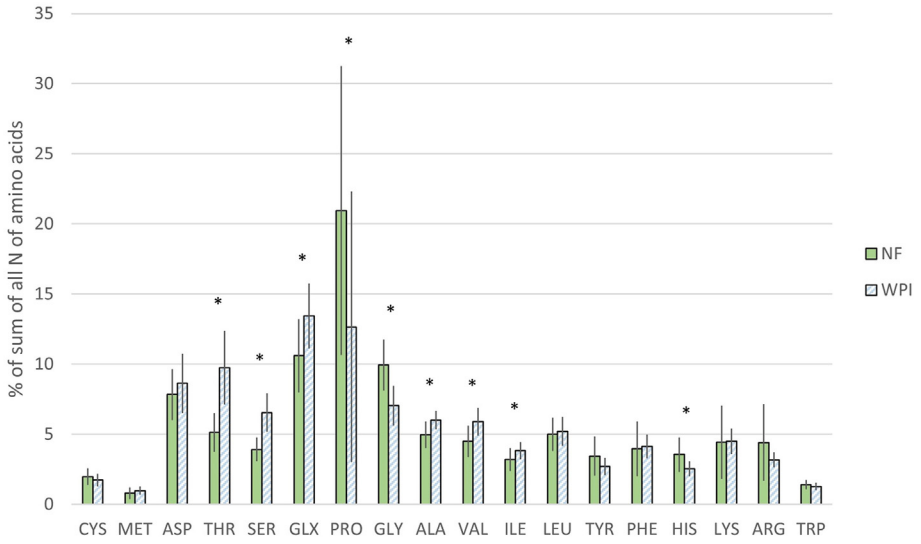

FIGURE 3. Amino acid profile of ileal basal endogenous protein losses (mean ± SD) measured after feeding a nitrogen-free diet, or a diet based on whey protein isolate to pigs ($n = 16$ for nitrogen-free; $n = 12$ for whey protein isolate). *$P < 0.05$. ALA, alanine; ARG, arginine; ASP, aspartic acid;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

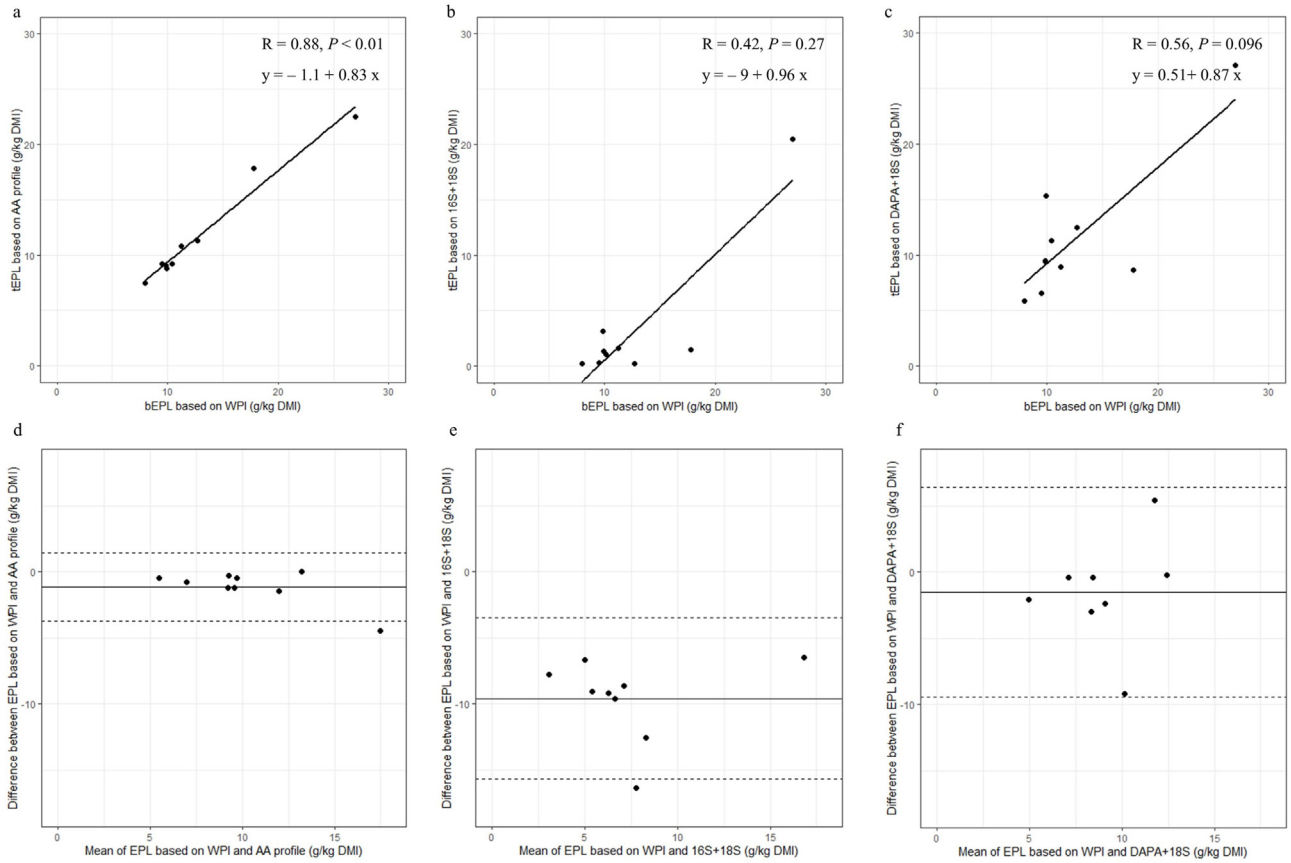

FIGURE 4. (A–C) Relations between basal and total endogenous protein losses in the ileum, analyzed after feeding a diet based on whey protein isolate (WPI method) to ileal cannulated pigs ($n = 9$). Ileal bEPL were quantified by the digesta protein content (WPI method), and ileal tEPL were quantified by the AA profile method, 16S+18S method, and DAPA+18S method. (D–F) Bland-Altman plots; the solid line represents the average difference between basal endogenous protein losses and total endogenous protein losses (g/kg DMI) and the dashed lines represent the 95% confidence interval limits. AA, amino acid; bEPL, basal endogenous protein loss; DAPA, diaminopimelic acid; DMI, dry matter intake; EPL, endogenous protein loss; tEPL, total endogenous protein loss.

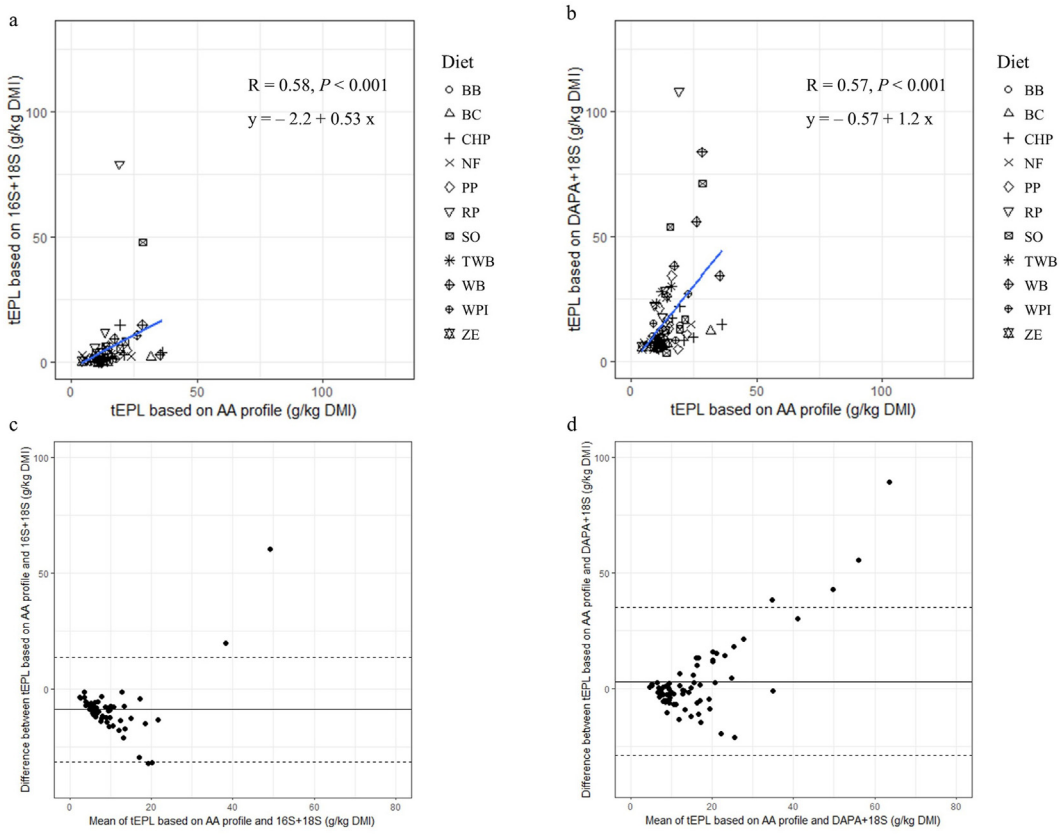

FIGURE 5. (A, B) Relations between total endogenous protein losses in the ileum, analyzed after feeding different protein sources to pigs. Ileal total endogenous protein losses were qualified by the AA profile method, compared with the 16S+18S method, and the DAPA+18S method. (C ,D) Bland-Altman plots; the solid line represents the average difference in total endogenous protein losses between the different methods (g/kg DMI) and the dashed lines represent the 95% confidence interval limits. AA, amino acid; BB, black beans ($n = 6$), BC, bovine collagen ($n = 5$); CHP, chickpeas ($n = 5$); DAPA, diaminopimelic acid; DMI, dry matter intake; NF, nitrogen free ($n = 11$); PP, pigeon peas ($n = 10$); RP, roasted peanuts ($n = 5$); SO, sorghum ($n = 10$); tEPL, total endogenous protein loss; TWB, toasted wheat bread ($n = 4$); WB, wheat bran ($n = 5$); WPI, whey protein isolate ($n = 12$); ZE, zein ($n = 6$).

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

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

Ileal tEPL

Ileal EPL estimates based on the NF and WPI method were moderately to highly correlated, but because 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 with 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

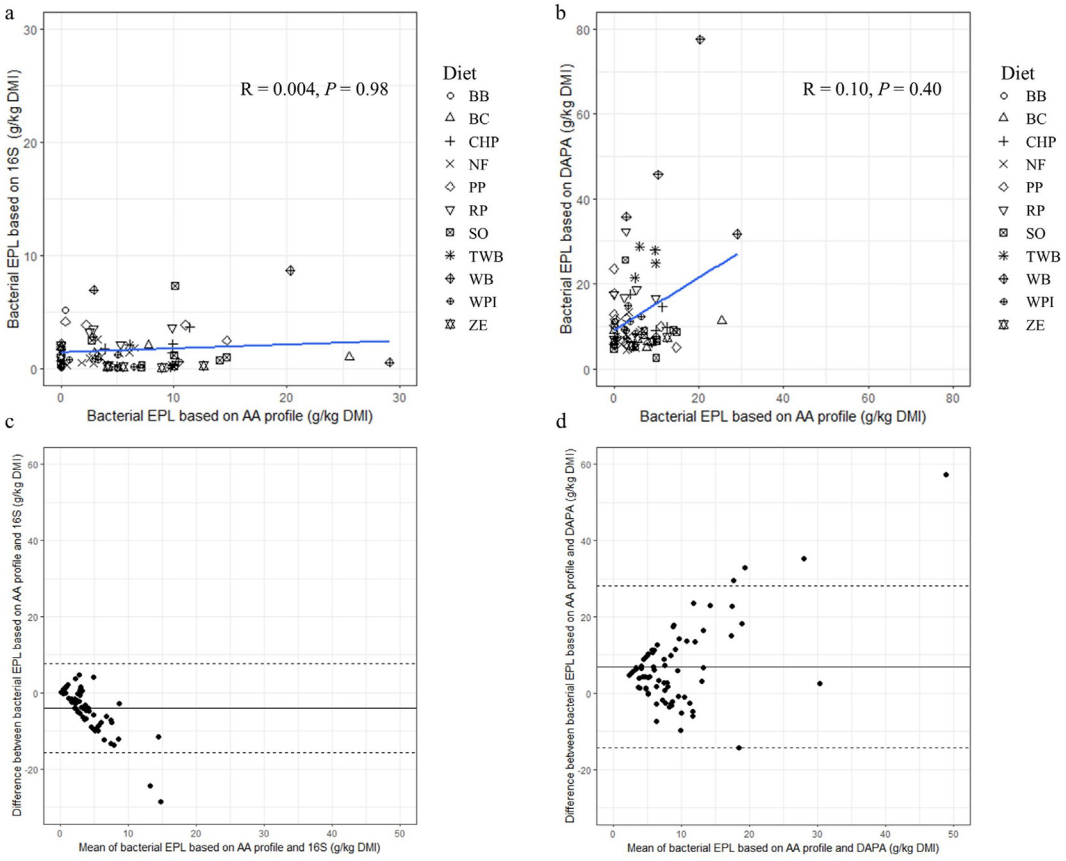

FIGURE 6. (A, B) Relations between bacterial endogenous protein losses in the ileum, analyzed after feeding different protein sources to pigs. Bacterial endogenous protein losses were quantified by the AA profile method, compared with the 16S method and DAPA method. (C, D) Bland-Altman plots; the solid 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. AA, amino acid; BB, black beans (n = 6); BC, bovine collagen (n = 5); CHP, chickpeas (n = 5); DAPA, diaminopimelic acid; DMI, dry matter intake; EPL, endogenous protein loss; 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).

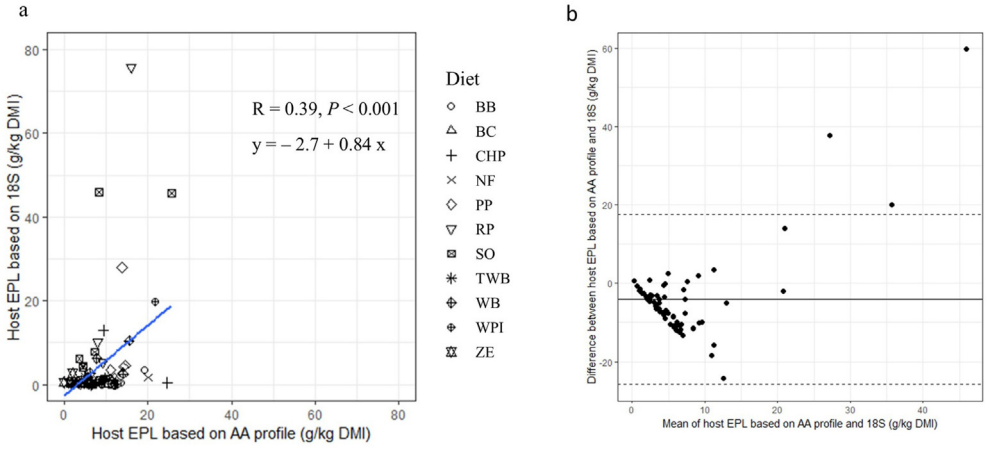

FIGURE 7. (A) Relations between host endogenous protein losses in the ileum, analyzed after feeding different protein sources to pigs. Host ileal endogenous protein losses were qualified by the AA profile method, and compared with the 18S method. (B) Bland-Altman plot; the solid line represents the average difference in host endogenous protein losses between the different methods (g/kg DMI) and the dashed lines represent the 95% confidence interval limits. AA, amino acid; BB, black beans (n = 6); BC, bovine collagen (n = 5); CHP, chickpeas (n = 5), DMI, dry matter intake; EPL, endogenous protein loss; 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).

16S+18S method and DAPA+18S methods were both moderately correlated with the AA profile method. Because 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 section "Methodology").

The fraction of bacterial protein in EPL was estimated to range between ~37% and 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. On the basis of the AA profile method, the bacterial protein fraction in ileal tEPL were highest (>50% of tEPL) in pigs fed with BC, WB, ZE, and SO. These are all well fermentable protein sources, which may enhance the bacterial growth in the ileum [80–82].

Methodology

This study introduces new methods to quantify tEPL. On the basis of the current results, the AA profile method is most promising. Nevertheless, the 16S+18S- and DAPA+18S methods also have potential, after refinement of the methods, and may be less laborious in the future. Current assumptions, limitations, and suggested adjustments are discussed below.

Endogenous losses calculated by AA profile

Bacterial and host EPL were estimated using the AA profiles of the diet, endogenous protein, bacterial protein, and ileal digesta. For this method, it was assumed that all dietary AAs had identical digestibility coefficients. In reality, the digestibility differs among the different AAs, depending on, among other things, the position in the chain and the presence of S- or H linkages [83,84]. This results in a different AA profile of the undigested feed compared with that of the ingested feed, affecting the estimates of EPL (Figure 8). By excluding the AAs with an AID greater or lower than the average AID for all AAs combined plus or minus the SD, we attempted to minimize the impact of digestibility differences. Furthermore, for this calculation, AA profiles of the host and bacterial EPL were based on literature. Although AA profiles of EPL analyzed in various studies were

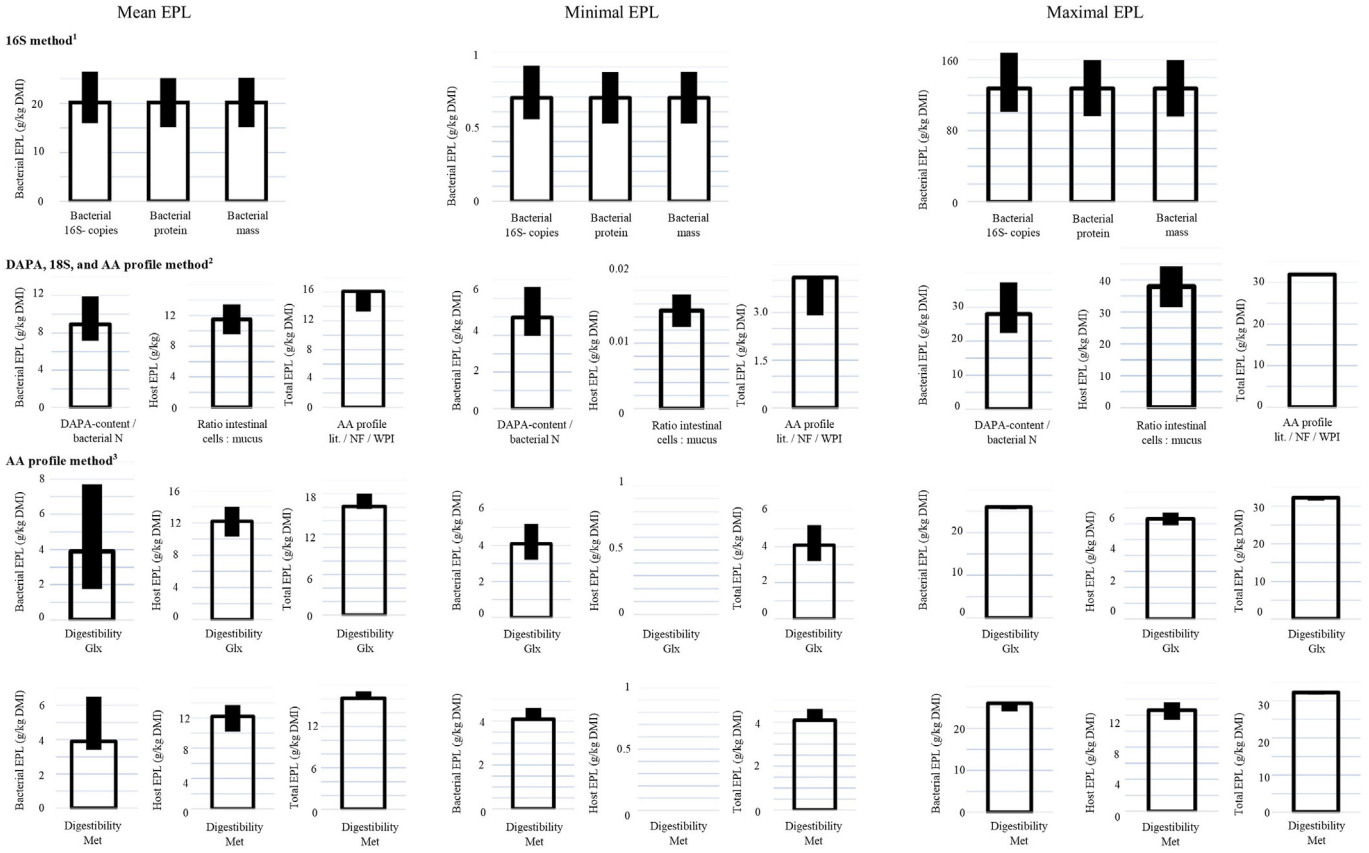

FIGURE 8. Impact of assumptions made in the calculations for bacterial, host, and total endogenous protein losses. For each method, the mean, minimal, and maximal endogenous protein losses estimations were used and the impact of a 25% change in each assumed value in the equations on the endogenous protein losses estimates was evaluated. The white bars represent the estimated endogenous protein losses calculated using the default assumption as described in section "Methods to quantify EPL," 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 1×10^{-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; error bars represent total endogenous protein losses calculated with the average digesta amino acid profile in pigs fed NF and WPI. [3]Assumptions AA profile method: digestibility of all amino acids is equal. Impact of digestibility was checked for 2 selected amino acids, glutamic acid + glutamine with a high concentration in endogenous protein losses, and methionine with a low concentration in endogenous protein losses. For both amino acids, endogenous protein losses were calculated when the amino acids had a 25% lower or 25% higher digestibility compared to the other amino acids. AA, amino acid; DAPA, diaminopimelic acid; EPL, endogenous protein loss; Glx, glutamic acid + glutamine; lit., literature; Met, methionine; N, nitrogen.

comparable [3,7], [this study], even small differences in AA profiles, for example caused by the different protein sources fed, can lead to different results.

Ileal bacterial tEPL analyzed by 16S and DAPA

In this investigation, we used ddPCR to quantify the ileal bacterial mass by the number of 16S RNA gene copies. While implementing this technique, we acknowledged that variations in cell lysis efficiency and technical nuances in DNA extraction could influence the total biomass estimations [85]. Given the anticipated high bacterial density in our ileal samples, the impact of contamination, commonly a concern in low biomass samples, was deemed to be 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 that 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/g 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 [50,88]. 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]. Second, we assumed that the bacterial protein content was 12.5 g/100 g 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]. 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].

Ileal host tEPL analyzed by 18S

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

Implications for true digestibility

Correcting the AID by bEPL analyzed in pigs fed with the WPI diet resulted in an 2%–7% unit increased SID compared with the NF diet (Table 2, [11]). Following the lower amount of EPL estimates based on the NF method compared with WPI, using the NF diet for estimating bEPL led to lower SID estimates. As anticipated, the TID of CHP, TWB, and WB was greater than the SID based on the WPI method, but for the other protein sources, no significant differences were observed. The differences between SID and TID may be important for protein evaluation and

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 (%)	SID (%)	TID (%)	*P* value[3]
			NF method	WPI method	AA profile method	
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

Abbreviations: AA, amino acid; NF, nitrogen-free; WPI, whey protein isolate.
[1] Data are presented as calculated means of untransformed data (SD).
[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 ($P < 0.05$).

diet formulation, emphasizing the relevance to more accurately quantify tEPL.

Conclusions

In light of our current findings in pigs, the AA profile method emerges as the most promising approach for quantifying 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 bEPL, the WPI method is preferred over the NF method, because the NF method may result in an underestimation of bEPL.

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Author contributions

The authors' responsibilities were as follows – 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 the article; LN, SV, WJJG: had primary responsibility for the final content; and all authors: read and approved the final manuscript.

Conflict of interest

The authors report no conflict of interest.

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Data availability

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2024.10.029.

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