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Evaluating the effects of a low-protein diet with reduced amylose-amylopectin ratio in fattening pigs on performance, gut health and behaviour

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ABSTRACT

Reducing dietary crude protein (CP) and using synthetic amino acids (AAs) reduces environmental impact but may affect pigs' growth. This study aims to evaluate the effect of a low CP and a low amylose-to-amylopectin ratio (AM:AP) diet on performance, welfare, and faecal microbiota of growing-finishing pigs. 384 pigs (36.76 kg) were assigned to a control (CO) or a treated (TRT) diets blocked for sex and body weight. The TRT reduced the CP by 1.5%, 1.4%, and 0.7% across three feeding phases and the AM:AP ratio (TRT = 7 *vs* CO = 16.97). Pigs were weighed at day (d) 0, d36 and d160; faecal samples (16 pigs/group) were analysed for microbiota (v3–v4 16S rRNA gene sequencing), calprotectin, volatile fatty acids (VFAs) and ammonia concentrations at d11, d36 and d160. Behavioural indices and pen-level air gases were monitored monthly. No differences in performance were observed. Faecal microbiota alpha diversity was affected by diet-time interaction ($p < 0.0001$). At d36 and d160, the TRT had a higher abundance of Clostridium sensu stricto 1 ($p = 0.002$, $p = 0.009$, respectively) and *Terrisporobacter* ($p = 0.03$, $p = 0.02$, respectively). Total VFAs and butyric acid at d11 and iso-butyric and iso-valeric acids at d161 were higher in the males of the TRT group ($p < 0.05$). On d71, the TRT group had a higher environmental carbon dioxide ($p < 0.02$) concentration. TRT pigs exhibited greater activity ($p = 0.03$ at d50), with no differences in tail or ear lesions. In conclusion, a low CP and AM:AP diet can maintain fattening pigs performance and welfare while indirectly potentially reducing environmental impact.

HIGHLIGHTS

- A low CP and AM:AP diet can maintain pig growth and welfare while reducing environmental impact from farming.
- The study suggests healthier gut microbiota with a sustainable diet, benefiting both pigs and the environment.
- The synchrony between energy (from waxy maize) and AAs absorption may improve the efficiency of low CP diets.

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Soybean meal; tail and ear lesion; amylopectin; nutrient synchrony

Introduction

Population and economic growth are projected to lead to an escalation in annual meat consumption representing an annual increase in global meat consumption of 1.15%. This market expansion is expected to impact the demand for protein feed ingredients (Shannon et al. 2010; Wang et al. 2011; de Visser et al. 2014). According to a life cycle assessment analysis on heavy pig sector, the global warming potential of this production averaged 4.25 ± 1.03 kg carbon dioxide (CO_2) eq/kg of live weight (Bava et al. 2017). Feed was identified as the most impactful factor of CO_2 eq/kg production. In addition, the composition of feed influences the composition of manure in terms of ammonia (NH_3), phosphorus and nitrates, which, together with the pesticides and fertilisers used for cultivation, contribute to water, air and soil pollution. These findings underscore the importance of dietary strategies and the amount and type of protein used in livestock

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diets for the environmental impact of their production.

While generally reducing the amount of soya in pigs' diets is one strategy to curb the environmental impact of this industry, another equally valid strategy is the overall reduction of the protein content in the diet, through the use of synthetic amino acids (AAs). Thus, low crude protein (LCP) diets, balanced with synthetic AAs, are increasingly used as they increase protein conversion efficiency and reduce nitrogen excretion. Additionally, they could reduce feeding costs and promote intestinal health without compromising pig growth performance (Fan et al. 2017). The presence of free AAs or hydrolysed proteins may cause an increase in insulin release. Therefore, diets containing free AAs and small peptides appear to be linked to a reduced incidence of first-pass metabolism of AAs in the intestine and liver, compared to diets based on complex proteins. This suggests a more efficient utilisation of AAs (Eugenio et al. 2023).

However, the literature indicates that reducing dietary protein and supplementing with synthetic AAs does not result in a straightforward or linear reduction in nitrogen excretion in faeces (Canh et al. 1998). This could be related to the asynchrony that characterises the availability of AAs and energy following the meal. It is important to note that a balanced intake of AAs and high-energy substances is necessary for the synthesis of muscle proteins. Since starch is the main source of glucose in pig diets, incorporating more rapidly digestible starches with a lower ratio of amylose to amylopectin (AM:AP) can help to address the discrepancy between the availability of synthetic AAs and glucose (Zhou et al. 2022). The variety of maize known as waxy is a source of easily digestible starch. Its starch is composed almost entirely of amylopectin (Perez and Aumaitre 1979). According to the study by Zhou et al. (2021) a mix of maize starch and waxy maize starch (37% maize and 36% cassava; with a 4% AM:AP ratio) in LCP diets based on soybean meal enhanced whole-body protein deposition, growth performance of growing pigs, and reduced urinary nitrogen excretion.

In addition, the different starch source coupled with a reduction in crude protein (CP) can also affect the gut microbiota ecosystem, impacting various aspects, including pig's health and behaviours. Higher amylopectin content in the diet could reduce intestinal fermentation compared to amylose rich diets, resulting in lower short-chain fatty acid concentrations in the gut (Yang et al. 2021), which are essential for the energy needs of gut epithelial cells. Indeed, previous studies indicated that the use of waxy maize in LCP diets can significantly impact the caecal and faecal microbial ecosystem (Yang et al. 2021; Correa et al. 2024). However, data are limited to weaned piglets and a lack of knowledge is present for growing and fattening pigs. In fact, despite numerous studies on the use of LCP diets supplemented with synthetic AAs, it remains unclear to what extent protein and amino acids can be reduced without compromising performance, especially in heavy pigs (>160 kg) typical of Italian production systems. As indicated by the National Research Council (2012), the current recommendations for nutrient levels indicate a CP levels approximately 2–4% lower than those set out in the previous edition (NRC, 1998). However, most available evidence refers to pigs slaughtered at 110–120 kg (Wang et al. 2019), whereas the protein and AAs requirements of pigs slaughtered at higher body weights are still poorly defined (Tao et al. 2025). The growing–finishing phase is indeed a critical stage of swine production. It is distinct from the weaning phase, during which pigs consume nearly three quarters of the total feed required throughout their life cycle and excrete substantially larger amounts of manure, thus exerting a greater environmental impact. Another important knowledge gap concerns the combination of low-protein diets with starch sources rich in amylopectin, such as waxy maize, which has been investigated mainly in weaned or growing pigs (Zhou et al. 2021, 2022; Correa et al. 2024). Finally, the potential effects of a LCP and low AM:AP diet to behaviours, lesions, and health issues might be a key factor in improving pig welfare sustainability and productivity, particularly regarding the requirements for Parma Ham Protected Designation of Origin (PDO) production (Bottacini et al. 2018; Amatucci et al. 2023; Palumbo et al. 2023). The aim of this study was to evaluate the impact of a LCP diet (characterised by a reduction in soybean meal content, ultimately leading to its complete removal, and enriched with synthetic AAs and with a low AM:AP ratio on growth performance, health indicators, and the welfare of growing and finishing heavy pigs, as well as environmental parameters.

Material and methods

The procedures complied with the Italian law pertaining to experimental animals and were approved by the Ethic- Scientific Committee for Experiments on Animals of the University of Bologna (Protocol ID: 4525. Prot. n. 130114 15/05/2023).

Animals, housing and experimental design

The study was conducted on a commercial farm located in northern Italy. Upon arrival at the growing-fattening unit (day 0; d0), a total of 384 pigs were divided into two groups: the Control group (CO; $n = 192$ pigs; average body weight = 35.9 kg), which was fed a standard commercial diet, and the Treated group (TRT; $n = 192$ pigs; average body weight = 35.4 kg), which was fed a LCP diet with lower soybean meal, higher AAs and waxy maize replacing conventional maize. Each group of pigs was housed in 6 pens, with 64 pigs per pen, and each pen covering an area of 63 m^2 and featured fully slatted floors. The pens were equipped with enrichment materials, including a soft wooden log and a straw rack. Water was continuously available through six nipple drinkers per pen. The feeder front available for each pig was 0.59 m, and the farm was naturally ventilated. Feed, provided in pellet form, was offered *ad libitum* using an automatic distribution system that delivered feed to each pen *via* a valve.

Diets and feeding

The diets were formulated in compliance with the Parma ham PDO specification criteria. Therefore, pigs were fed using a restricted list of raw material (Consortium for Parma Ham Protected Designation of Origin, 1992). Both groups followed a three-phase feeding program: phase I = d0–d38, phase II = d39–d70 and phase III = d71–d161, with diets designed to meet the pigs' nutritional requirements at different growth stages (National Research Council 2012). The diets were analysed for their composition and total AAs profile using High-Performance Liquid Chromatography (HPLC). Dry matter content was measured by drying samples at 103 °C, while crude protein content was assessed using the Kjeldahl method ($N \times 6.25$). Lipid content was determined *via* petroleum ether extraction, fibre content using the Weende method, and ash content through incineration at 550 °C. Starch content was analysed using the polarimetric method. The quantity of amylose and amylopectin was analysed as reported in the study by Correa et al. (2024). The composition and analytical results of the diets are presented in Table 1. The CO diet was characterised by a higher quantity of soybean meal (phase I = 9.8%; phase II = 7.4%; phase III = 5.2%), a higher CP content (phase I = 15.80%; phase II = 14.8%; phase III = 12.4%) and higher AM:AP ratio (phase I = 14.85%; phase II = 18.20%; phase III = 17.85%) compared to the TRT diet. The TRT diet was characterised by a lower soybean meal inclusion (only in phase I = 3%), a lower CP content (phase I = 14.3%; phase II = 13.4%; phase III = 11.7%) and a lower AM:AP ratio (phase I = 5.55%; phase II = 5.60%; phase III = 10.85%).

Growth performance and animal-based measures

On d0, pigs were individually identified with numbered ear tags and individually weighed on d0, d36 and d160 (end of the trial). The average daily gain (ADG) was calculated from these data. On monthly basis, from the selected piglets' pens, animal-based measures (ABMs) were provided following the Welfare Quality (2009) and Classyfarm (2019) protocols. These measures were divided into Behavioural Measures (BMs) and Lesions Measures (LMs). The BMs were assessed in the morning, between 10:00 am and 11:00 am, when the animals were most active. Observations were conducted directly in each pen, with growing-fattening pigs observed three times per pen, allowing for a 2-min break between each scan observation. Before the first observation, the assessor clapped their hands if necessary to ensure all animals were standing. After a 5-min acclimation period, observations began. The BMs recorded included abnormal behaviours, such as suckling behaviour defined as sucking any part of another pig's anatomy as though suckling milk (Torrey & Windowksi, 2006), and specific categories of behaviour as defined by Welfare Quality (2009). Social behaviour was further divided into negative interaction, including aggressive interactions, and positive interaction, which involved actions such as sniffing, nosing, licking, playing, or gently moving away without aggression. Exploratory behaviour was categorised as exploring the pen, which included sniffing, nosing, and licking the pen environment, or exploring enrichment materials, such as playing with straw or other enrichment items. Pigs not displaying social or exploratory behaviours were classified as resting if they were inactive and lying down, or as engaging in other activities, such as eating or drinking. The frequency of each BM was expressed as a percentage of the average across the five observations of animals exhibiting the behaviour relative to the total number of pigs in the pen, using the formula:

$$(n\ of\ pigs\ demonstrating\ the\ behaviour / total\ of\ pigs\ in\ the\ pen) * 100:$$

LMs were assessed in the afternoon. Each pen was observed for five minutes from 0.5 m, and the number of pigs displaying ear and/or tail lesions were recorded. Lesions were scored according to Welfare

Table 1. Composition and chemical composition of the experimental diets.

	Phase 1 d0–d38		Phase 2 d39–d70		Phase 3 d71–165	
Ingredients, %	CO	TRT	CO	TRT	CO	TRT
Corn	45.87	0.00	45.65	0.00	48.54	0.00
Waxy corn	0.00	48.23	0.00	46.23	0.00	51.99
Wheat midlings	19.25	19.25	19.25	17.76	9.31	11.95
Soybean meal	9.80	3.00	7.36	0.00	5.23	0.00
Pea protein	4.80	4.80	4.80	4.80	4.80	4.80
Sunflower meal	5.00	5.49	3.00	5.65	3.00	3.00
Barley	5.00	5.00	0.00	0.00	0.00	0.00
Wheat	5.07	8.87	15.12	20.61	24.20	23.28
Animal fat	1.55	0.80	1.52	1.00	1.89	1.43
Calcium carbonate 39.5%	1.03	1.00	0.95	0.88	0.91	0.89
Dicalcium phosphate	0.10	0.21	0.00	0.00	0.00	0.00
Sodium chloride	0.50	0.51	0.50	0.51	0.51	0.51
Choline 75%	0.08	0.08	0.08	0.08	0.00	0.00
Lysine 50%	0.75	1.06	0.69	1.00	0.66	0.89
Threonine 98%	0.20	0.29	0.18	0.26	0.17	0.24
Technological adjuvant	0.05	0.05	0.05	0.05	0.05	0.05
Phytase	0.11	0.11	0.10	0.10	0.10	0.10
Carbohydrase enzyme[a]	0.05	0.05	0.00	0.00	0.00	0.00
Mineral-vitaminic premix[b]	0.28	0.28	0.26	0.26	0.18	0.18
Benzoic acid	0.25	0.25	0.25	0.25	0.25	0.25
Tryptophan, Valine mix[c]	0.11	0.23	0.09	0.23	0.09	0.18
Histidine HCl 72%	0.00	0.03	0.00	0.02	0.00	0.01
Methionine 99%	0.05	0.05	0.05	0.05	0.05	0.05
Isoleucine	0.00	0.12	0.00	0.11	0.00	0.09
Leucine	0.00	0.07	0.00	0.04	0.00	0.00
Estimated chemical composition						
Dry matter, g/g%	87.59	87.22	87.47	87.20	87.58	87.25
Crude protein, g/g%	15.54	13.66	14.18	12.50	12.72	11.24
Crude lipids, g/g%	4.30	3.70	4.28	3.82	4.40	4.17
Crude fibre, g/g%	4.29	4.44	3.78	4.23	3.23	3.46
Ash, g/g%	4.59	4.33	4.18	3.83	3.68	3.47
Metabolised energy, MJ	2.280	2.283	2.330	2.330	2.425	2.425
Analysed composition, %						
Dry matter	87.7	88.1	88.4	88.2	87.9	87.6
Crude protein	15.8	14.3	14.8	13.4	12.4	11.7
Crude lipids	4.43	3.95	4.64	4.31	4.58	4.14
Crude fibre	4.25	3.72	4.25	3.64	3.14	2.80
Ash	4.46	4.33	4.18	4.11	3.63	3.41
Glycine	0.70	0.59	0.63	0.55	0.54	0.49
Glycine	0.73	0.60	0.67	0.56	0.59	0.52
Methionine	0.30	0.28	0.30	0.27	0.26	0.26
Cysteine	0.26	0.23	0.24	0.23	0.23	0.22
Lysine	1.10	1.05	0.94	0.96	0.87	0.89
Threonine	0.76	0.71	0.66	0.66	0.59	0.60
Tryptophan			0.19	0.19	0.17	0.17
Arginine	1.04	0.82	0.90	0.75	0.75	0.65
Isoleucine	0.62	0.57	0.53	0.50	0.46	0.48
Proline	1.00	0.90	0.90	0.90	0.80	0.80
Leucine	1.27	1.11	1.15	1.02	1.04	0.95
Valine	0.77	0.71	0.66	0.63	0.58	0.58
Histidine	0.40	0.35	0.36	0.33	0.31	0.29
Phenylalanine	0.74	0.59	0.66	0.55	0.58	0.51
Alanine	0.79	0.66	0.71	0.60	0.63	0.55
Aspartic acid	1.39	1.02	1.18	0.90	1.00	0.79
Glutamic acid	3.02	2.55	2.77	2.52	2.45	2.36
Tyrosine			0.44	0.36	0.39	0.33
Total starch	41.70	41.75	42.9	45.1	48.25	48.10
Amylose	5.40	1.80	6.6	2.4	7.30	4.70
Amilopectine	36.30	39.95	36.5	42.7	40.95	43.40
AM:AP	14.85	4.55	18.2	5.6	17.85	10.85

CO: normal protein diet; TRT: low protein and low amylose/amylopectin ratio diet.

Carbohydrase enzyme[a] = RONOZYME® MultiGrain DSM enzyme Guaranteed enzymatic activities (per gram of product): Endo-1,4-β-xylanase (EC 3.2.1.8): 2,700 U/g, Endo-1,3(4)-β-glucanase (EC 3.2.1.6): 700 U/g, Endo-1,4-β-glucanase (EC 3.2.1.4): 800 U/g.

Mineral-vitaminic premix[b] = Vitamins: Vitamin A 2,500,000 IU, Vitamin D3 700,000 IU, Vitamin D3 (25-hydroxy) HYD microencapsulated 300,000 IU, Vitamin E (all-rac-alpha-tocopheryl acetate) 25,000 IU, Vitamin K3 1,350 mg, Vitamin B1 800 mg, Vitamin B2 1,800 mg, Vitamin B6 (Pyridoxine hydrochloride) 1,100 mg, Vitamin B12 10.0 mg, Biotin 70.0 mg, Niacinamide 15,000 mg, Folic acid 600 mg; Minerals: Calcium D-pantothenate 4,445 mg, Iron (from iron(II) sulphate monohydrate) 6,750 mg, Manganese (from manganese(II) sulphate monohydrate) 10,000 mg, Manganese (from manganese chelate of hydroxy analogue of methionine) 10,000 mg, Zinc (from zinc sulphate monohydrate) 8,500 mg, Zinc (from zinc chelate of hydroxy analogue of methionine) 12,000 mg, Copper (from copper(II) sulphate pentahydrate) 1,500 mg, Copper (from copper chelate of hydroxy analogue of methionine) 1,500 mg, Iodine (from calcium iodate anhydrous) 400.0 mg, Selenium (from sodium selenite) 75.0 mg.

Tryptophan, Valine mix[c] = Tryptophan 35.38% + Valine 15.44%.

Quality (2009). Tail lesions were scored as follows: 0 = no injuries; 1 = superficial bite marks without swelling; and 2 = open lesions, swelling, scarring, or partial tail loss. Ear lesions were scored as follows: 0 = up to 4 visible lesions; 1 = 5 to 10 visible lesions; and 2 = 11 to 15 visible lesions. Results for each pen were expressed as the prevalence of scores (0, 1, or 2). To quantify the frequency and severity of lesions, a Lesion Score Index (LSI) was calculated, ranging from 0 to 200. A score of 0 indicated no lesions, while a score of 200 indicated all animals had severe lesions. The LSI was calculated as follows:

$$\textit{Lesion score index } (\textit{LSI}) = [\%\ \textit{lesion type } 1 + (2 * \%\ \textit{lesion type } 2)].$$

Finally, BMs and LMs were always assessed by the same evaluator trained on how to apply the Welfare Quality (2009) and Classyfarm (2019) protocols.

Environmental parameters

The environmental parameters were assessed in the morning included light intensity and the proportions of gases in the air, such as CO_2 and NH_3. Measurements were conducted at the pigs' level (approximately 50 cm above the floor) in three locations within each pen: the corner nearest the middle of the room, the centre of the pen, and the corner closest to the outer wall. The average of these three measurements was calculated for each parameter. Light intensity was measured using a Mini Light Metre (UNI-T UT383, Dongguan City, China), while gas concentrations were recorded using a portable Dräger X-am 8000 multi-gas detector (Drägerwerk AG & Co, Lübeck, Germany). These measurements were carried out by trained personnel on each observation day, ensuring consistency and accuracy across the experimental pens.

Sample collection

A subgroup of 32 pigs (16 per group), balanced for sex (8 per sex in each group) and body weight, was chosen for faecal sample collection on d11, d36, and d161. Faecal samples were collected from the same individual into a sterile collection tube after rectal stimulation. The samples were immediately frozen in dry ice and then stored at −80 °C until processing for microbiota composition, volatile fatty acids (VFAs) and lactic acid profiles, NH_3 levels, and calprotectin concentrations.

Volatile fatty acids, lactic acid, ammonia and calprotectin concentrations

The quantification of VFAs and lactic acid concentrations in faecal samples was performed using HPLC following the procedure described by Correa et al. (2024). Briefly, 1 g of each faecal sample was diluted with 5 mL of 0.1 N sulphuric acid (H_2SO_4) and homogenised using an UltraTurrax (IKA®-Werke GmbH & Co. KG, Staufen, Germany). The mixture was centrifuged at 5,000 × g for 15 min at 4 °C to obtain the liquid phase, which was then microfiltered (SLMV033RS, 0.45-μm Millex-HV, Merck-Millipore, Billerica, MA). The filtered sample was directly injected into an HPLC system equipped with an Aminex 85 HPX-87 H ion exclusion column (300 mm × 7.8 mm; particle size 9 μm; Bio-Rad, Milan, Italy). Detection was performed at a wavelength of 220 nm. The analysis used isocratic elution with a flow rate of 0.6 mL/min and 0.008 N H_2SO_4 as the mobile phase. The injection loop volume was 20 μL. Individual VFAs (acetate, propionate, isobutyrate, butyrate, valerate, and isovalerate) and lactic acid were identified and quantified using an external calibration curve created from a standard solution containing the target organic acids (69775, 338826, 402907, B103500, 58360, 75054, 129542; Sigma–Aldrich, Milan, Italy) diluted in 0.1 N H_2SO_4.

The concentration of NH_3 in faecal samples was determined using an enzymatic colorimetric assay. Thawed faecal samples were diluted with deionised water at a weight/volume ratio of 1:10, vortexed, and centrifuged at 7,000 ×g for 10 min at 4 °C. The supernatant was analysed using the Urea/BUN-Color enzymatic assay kit (BioSystems S.A., Barcelona, Spain) according to the manufacturer's protocol. Results were expressed as μmol of NH_3 per gram of faeces.

Calprotectin concentration was analysed using the MBS033848 ELISA kit (Mybiosource, San Diego, CA, USA) according to the manufacturer's protocol. Prior to analysis, faecal samples were diluted 1:10 in phosphate-buffered saline (PBS) at pH 7.4. Each sample was analysed in duplicate. Absorbance was measured at 450 nm using a Multiskan FC Microplate Photometer (Thermo Fisher Scientific). Calprotectin concentration was calculated using a four-parameter logistic curve and expressed in ng/mL.

Faecal microbial profile analysis

Total bacterial DNA was extracted following the manufacturer's instructions of the SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA). The extracted DNA was then processed as described by Luise et al. (2023).

In brief, the DNA samples were quality-checked, diluted, and amplified for the V3–V4 regions of the 16S ribosomial RNA gene using Pro341F and Pro805R primers, which were modified with the Nextera XT universal tail (Takahashi et al. 2014). Amplification was performed with Platinum™ Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific). Library preparation and sequencing were conducted using the MiSeq® Reagent Kit V3 with the 300PE strategy on the Illumina® MiSeq platform.

Microbial data analysis was carried out using the DADA2 pipeline (Callahan et al. 2016), and taxonomy was assigned using the Silva Database (release 138·1) as a reference (Quast et al. 2013).

Statistical analysis

Statistical and bioinformatic analyses were performed using RStudio (RStudio, PBC, Boston, MA, USA) using the 'car' (Fox and Weisberg 2019), 'lme4' (Bates et al. 2015), 'emmeans' (Lenth 2016) and 'jmv' (Selker et al. 2020) packages. Before model fitting, the assumption of normality for the dependent variable was evaluated visually with a quantile–quantile plot (qqnorm) and statistically with the Shapiro–Wilk test. Data on body weight, ADG, faecal VFAs, calprotectin and NH_3 were fitted using a linear mixed model and analysed using an ANOVA model in which the diet and sex were included as fixed factors, and the pen was included as a random factor. For ABMs and environmental parameters, the pen was used as the experimental unit, and data were fitted using a linear model and ANOVA analysis, including diet as a fixed factor. The distribution of residuals was examined visually using a quantile-quantile (Q–Q) plot and an overlaid reference line (qqnorm and qqline), to evaluate the assumption of normality. In addition, residual versus fitted value plots, obtained *via* the 'plot' function, were inspected to verify the assumption of homoscedasticity and to detect any potential model misspecification or influential data points.

For the microbial data the analysis was carried out using the 'phyloseq' (McMurdie and Holmes 2013), 'Vegan' (Dixon 2003) and 'microbiome' (Lahti et al. 2017) and 'microbiomeMarker' (Cao et al. 2022) packages on R environment. Both α-diversity (measured by Shannon, Chao1, and InvSimpson indices) and β-diversity (assessed using the Bray-Curtis distance matrix) were calculated. The α-diversity indices were analysed using a linear mixed model and ANOVA model, with sex, diet, time, and the diet × time interaction as fixed factors, and pig replicate by time as a random factor. The impact of sex, diet, time, and the diet × time interaction on β-diversity was evaluated using a PERMANOVA model (Adonis test) with 9,999 permutations. The effect of diet at each timepoint was further analysed using the Adonis test and visualised through a Principal Coordinates Analysis (PCoA) plot. Prior to the adonis analysis, permutational analysis of multivariate dispersion (PERMDISP) was performed to assess potential differences in the dispersion of multivariate data among groups. To identify taxa differentially expressed between dietary groups at each timepoint, a linear discriminant analysis (LDA) effect size algorithm at the genus level was applied, with taxa considered significantly different if the LDA score was >3 and the adjusted *P*-value (*P*.adj) was <0.05. Differences with *P*-values (P) ≤ 0.05 were considered significant and $0.05 < P < 0.10$ were considered as a tendency. When an effect was considered significant, a Tukey–Kramer test was used to differentiate least squares means.

Results

Growth performance

The effect of the diet on growth performance is shown in Table 2. Neither diet or sex had an impact

Table 2. Effect of a low protein and low amylose/amylopectin ratio diet on body weight and average daily gain of growing and finishing pigs.

	Diet[1]			Sex			*P*-value	
Item	CO	TRT	SEM	F	M	SEM	Diet	Sex
Body weight, Kg								
d 0[2]	35.9	35.4	0.38	35.6	35.7	0.33	0.48	0.32
d 36[3]	70.2	72.7	2.14	71.0	72.7	1.60	0.39	0.38
d 161[4]	190.0	192.0	1.38	190.0	192.0	1.34	0.23	0.34
Average daily gain, g/day								
d 0–d 36[3]	923	1029	60.70	960	992	46.70	0.19	0.38
d 36–d 161[3]	954	973	32.00	974	953	25.40	0.65	0.35
d 0–d 161[4]	951	983	25.20	969	965	20.00	0.31	0.83

CO: normal protein diet; TRT: low protein and low amylose/amylopectin ratio diet;
[1]Diet
[2]Values are means ± SEM of 192 pigs per group.
[3]Values are means ± SEM of 81 pigs of CO group and 77 pigs of TRT group.
[4]Values are means ± SEM of 159 pigs of CO group and 157 pigs of TRT group.

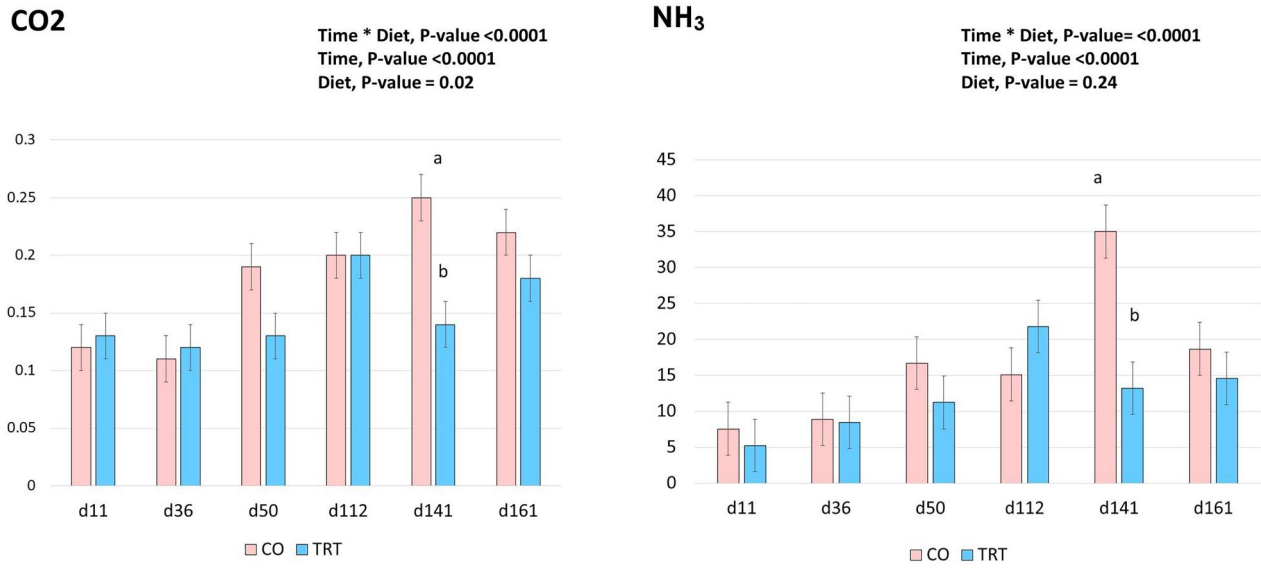

Figure 1. Effect of a low protein and low amylose/amylopectin ratio diet on carbon dioxide ammonia concentration in pens of growing and finishing pigs. Statistics and relative means of the environmental parameters recorded in the pens where the ear-tagged animals were housed as a function of diet and time. The superscript letters next to the result represent the statistically significant difference ($P < 0.05$) between the CO and TRT groups at the same timepoint. Diet: CO = normal protein diet; TRT = low protein and low amylose/amylopectin ratio diet. a,b: Means with different superscripts within a row differ significantly ($P < 0.05$).

on the BW and ADG of the growing-fattening pigs at any time point.

Behavioural and lesion animal-based measures

Table 3 shows the effect of diet on the BMs and LMs observed monthly during the study. The dietary treatment did not affect the frequency and severity of ear and tail lesions at any of the timepoints.

Regarding the BMs, at d11, a higher proportion of pigs in the TRT group exhibited negative interaction compared to the CO group ($p = 0.01$). At d50, the TRT group showed a higher proportion of pigs exploring the pen ($p = 0.03$). At d112, the TRT group tended to have a higher proportion of pigs engaging with enrichment materials ($p = 0.06$), while the CO group showed a tendency for more positive interactions ($p = 0.07$). No other significant differences in BMs were observed between the two experimental groups at the other time points.

Environmental parameters

Figure 1 shows the effect of diet, time point, and their interaction on the environmental parameters recorded monthly in all the selected pens. The data shows a significant effect of the interaction between timepoints and diet ($P < 0.001$) and timepoints ($P < 0.001$) for CO_2, NH_3. The diet significantly influences the concentration of CO_2 ($P = 0.02$). In detail, the CO_2 and NH_3 were higher in the CO group compared with the TRT group at d141 ($P < 0.05$).

Faecal volatile fatty acids

Table 4 presents the concentrations of VFAs and lactic acid in faeces sampled on d11, d36, and d161 from 32 pigs (16 per group, balanced for sex and BW).

Total VFAs were affected by the interaction between diet and sex ($p < 0.01$) and by sex ($p = 0.01$) at d11; the males of the TRT group had a higher concentration compared to females of the TRT and to both male and female of the CO group ($p < 0.05$). Total VFAs were influenced by diet at d36 ($p = 0.01$) and tended to be affected by the interaction between diet and sex at d161 ($p = 0.09$); the males of the TRT group had a higher concentration compared to females of the TRT and to both males and females of the CO group ($p < 0.05$).

Acetic acid was influenced by the interaction between diet and sex at d11 ($p < 0.05$), but no significant differences were observed between the groups.

Butyric acid was affected by the interaction between diet and sex at d11 ($p < 0.01$), and the males of the TRT group had a higher concentration compared to the females of the TRT and to both male and female of the CO group ($p < 0.05$).

Table 3. Effect of a low protein and low amylose/amylopectin ratio diet on behaviours and injuries' prevalence of growing and finishing pigs.

Item	Diet[1]		SEM	*P*-value
	CO	TRT		
d 11[2]				
Behaviour, %				
Rest	81.5	77.5	1.93	0.22
Suckling	0.31	0.42	0.30	0.81
Positive interaction	3.23	3.35	0.20	0.70
Negative interaction	0.94	1.36	0.07	0.01
Enrichment investigation	0.21	0.63	0.37	0.47
Pen exploration	8.02	9.10	1.34	0.60
Other activities	5.83	7.63	0.70	0.14
Lesions				
LSI ears	69.8	79.4	16.1	0.69
LSI tail	97.9	79.9	18.2	0.52
d 36[3]				
Lesions				
LSI ears	71.5	60.6	10.8	0.52
LSI tail	41.2	44.4	14.7	0.89
d 50[4]				
Behaviour, %				
Rest	60.1	54.1	2.45	0.16
Suckling	0.11	0.11	0.11	0.98
Positive interaction	4.43	4.37	0.68	0.95
Negative interaction	1.99	1.81	0.14	0.42
Enrichment investigation	2.85	2.77	0.85	0.95
Pen exploration	18.1	23.8	1.21	0.03
Other activities	12.2	13.0	1.11	0.66
Lesions				
LSI ears	61.0	70.7	6.58	0.35
LSI tail	45.1	57.4	11.7	0.50
d 112[5]				
Behaviour, %				
Rest	69.0	69.9	5.47	0.91
Suckling	0.12	0.00	0.08	0.37
Positive interaction	1.95	0.46	0.42	0.07
Negative interaction	1.61	1.39	0.31	0.65
Enrichment investigation	3.58	5.00	0.39	0.06
Pen exploration	15.6	13.9	4.16	0.79
Other activities	8.22	9.42	1.09	0.48
Lesions				
LSI ears	1.17	2.89	1.16	0.35
LSI tail	21.2	23.9	6.60	0.79
d 141[6]				
Behaviour, %				
Rest	82.3	85.8	5.67	0.68
Suckling	0.23	0.00	0.17	0.37
Positive interaction	1.42	1.23	0.64	0.84
Negative interaction	0.48	0.13	0.19	0.27
Enrichment investigation	0.59	0.00	0.30	0.23
Pen exploration	8.67	7.58	3.20	0.82
Other activities	6.32	5.26	1.56	0.65
Lesions				
LSI ears	4.81	1.89	1.17	0.15
LSI tail	19.0	24.8	7.62	0.62
d 161[7]				
Lesions				
LSI ears	18.9	12.9	4.87	0.43
LSI tail	7.39	10.42	2.17	0.38

Diet[1]: CO = normal protein diet; TRT = low protein and low amylose/amylopectin ratio diet.
[2]Values are means ± SEM of 192 pigs per group.
[3]Values are means ± SEM of 183 pigs of CO group and 191 pigs of TRT group.
[4]Values are means ± SEM of 181 pigs of CO group and 188 pigs of TRT group.
[5]Values are means ± SEM of 173 pigs of CO group and 172 pigs of TRT group.
[6]Values are means ± SEM of 167 pigs of CO group and 163 pigs of TRT group.
[7]Values are means ± SEM of 159 pigs of CO group and 157 pigs of TRT group.

Iso-butyric acid was affected by the interaction between diet and sex at d161 ($p < 0.001$), and the males of the TRT group had a higher concentration compared to females of the TRT and to both males and females of the CO group ($p < 0.05$).

Table 4. Effect of a low protein and low amylose/amylopectin ratio diet on concentration of volatile fatty acids and calprotectin in faeces of growing and finishing pigs.

	Sex × Diet[1]					*P*-value		
	F		M					
Fatty acid, micromoles/g	CO	TRT	CO	TRT	SEM	Diet	Sex	Sex × Diet[1]
N samples	8	8	8	8				
Total fatty acids								
d 81	414[a]	474[a]	401[a]	520[b]	35.7	0.18	**0.01**	**<0.01**
d 106	541	478	451	397	42.6	0.26	**0.01**	0.83
d 231	490[x]	495[x]	357[x]	503[y]	63.6	0.10	0.94	**0.09**
Lactic acid								
d 81	13.7	14.1	10.8	10.7	2.58	0.38	0.91	0.91
d 106	11.91	9.9	16.57	8.66	3.08	0.23	0.57	0.24
d 231	16	17.2	14.3	15.0	3.29	0.79	0.80	0.75
Acetic acid								
d 81	209	238	241	211	17.3	0.18	0.12	**<0.05**
d 106	282	246	241	225	32.1	0.35	0.13	0.54
d 231	261	252	166	229	36.6	0.04	0.81	0.16
Propionic acid								
d 81	68.9	91.5	83.5	86.3	8.4	0.03	0.15	0.18
d 106	84.3	68.5	72.2	67.0	9.68	0.35	0.07	0.40
d 231	67.7	70.5	46.9	72.7	13.2	0.22	0.82	0.18
Butyric acid								
d 81	96.9[a]	99.6[a]	61.41[a]	148.9[b]	15.8	0.89	**<0.01**	**<0.01**
d 106	129.3	113.1	120.2	79.8	15.6	0.64	0.39	0.38
d 231	116	116	106	123	18.2	0.66	0.99	0.45
Iso-butyric								
d 81	12.3	12.3	9.6	14.4	2.35	0.99	0.47	0.24
d 106	12.20	7.39	11.23	7.59	1.87	0.67	0.04	0.72
d 231	12.6[a]	17.8[a]	11.3[a]	35.1[b]	2.89	0.72	0.15	**<0.001**
Valeric acid								
d 81	3.07	5.03	4.78	4.83	1.02	0.12	0.17	0.28
d 106	3.64	2.81	2.83	1.72	0.79	0.41	0.32	0.80
d 231	3.15	3.52	1.83	3.63	0.83	0.21	0.65	0.21
Iso-valeric acid								
d 81	10.2	15	12.8	17.4	4.26	0.37	0.53	0.96
d 106	13.7	11.6	13.2	8.1	2.09	0.87	0.41	0.40
d 231	15.40[a]	16.90[a]	7.41[a]	24.29[b]	8.92	0.28	0.83	0.13

Sex * Diet[1]: interaction between sex and diet; Sex: F = female; M = male; Diet: CO = normal protein diet; TRT = low protein and low amylose/amylopectin ratio diet.
a,b: Means with different superscripts within a row differ significantly ($p < 0.05$).
x,y: Means with different superscripts within a row tend to differ ($0.05 < p < 0.10$).

No other differences were detected for the other VFAs and for lactic acid at any timepoints.

Faecal ammonia concentration

Table 5 shows the concentrations of NH_3 in faeces sampled on d11, d36, and d161. NH_3 concentration was not influenced by either diet, sex or their interaction at any timepoints.

Faecal calprotectin

Table 5 shows the concentrations of calprotectin in faeces sampled on d11, d36, and d161. Calprotectin levels were significantly affected by the interaction between sex and diet at d161 ($p = 0.04$), although no significant differences were observed between the groups. No differences on calprotectin concentration were detected at d11 and d36.

Faecal microbiota profile

As outlined in the experimental protocol, faecal samples were collected from 32 pigs (16 per group, balanced for sex and body weight) at days 11, 36, and 161. DNA extraction and sequencing yielded a total of 96 valid samples, consistent with the study design. Overall, the sequencing procedure produced a total of 3,507,742; after quality control, an average of 36,539 sequences per sample were retained, which, after bioinformatic analysis, produced a total of 3,849 amplicon sequence variants (ASVs). The rarefaction curves showed that the number of different species, observed as a function of the number of sequences, ranched a plateau trend, indicating that the sequencing procedure was able to capture all the variability present in the samples (Supplementary Figure 1).

Among the 6,541 recovered ASVs, 17 phyla, 81 families, and 211 genera were identified. The most abundant phyla were Firmicutes (89.10 ± 5.10%),

Table 5. Effect of a low protein and low amylose/amylopectin ratio diet on concentration of ammonia and calprotectin in faeces of growing and finishing pigs.

	Sex × Diet[1]							
	F		M			*P*-value		
Items	CO	TRT	CO	TRT	SEM	Diet	Sex	Sex × Diet[1]
N samples	8	8	8	8				
NH_3, μl/g								
d 11[2]	18.4	28.8	24.9	25.1	5.31	0.33	0.1	0.27
d 36[2]	25.3	24.8	23.8	22.5	4.61	0.79	0.9	0.92
d 161[2]	26.5	24.4	18.7	25.3	4.44	0.17	0.69	0.27
Calprotectin, ng/ml								
d 11[2]	1044	1047	1092	1125	45.6	0.96	0.36	0.70
d 36[2]	1043	1079	1074	1070	39.8	0.47	0.41	0.33
d 161[2]	991	1058	990	1059	37.1	0.16	0.14	0.04

Sex * Diet[1]: interaction between sex and diet; Sex: F = female; M = male; Diet: CO = normal protein diet; TRT = low protein and low amylose/amylopectin ratio diet.

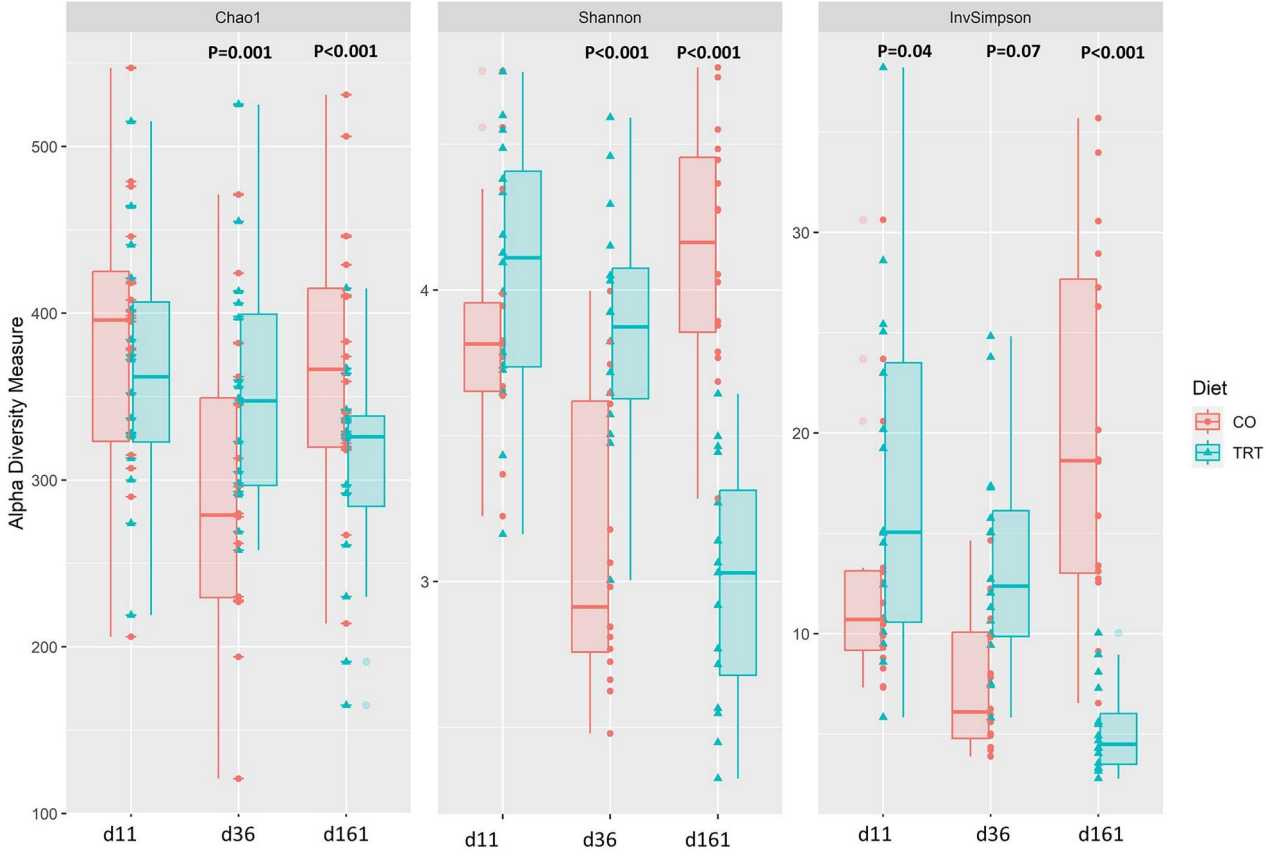

Figure 2. Effect of a low protein and low amylose/amylopectin ratio diet on the Chao1, Shannon and InvSimpson index values of pigs' faecal samples collected at days 11, 36, and 161. Diet: CO = normal protein diet; TRT = low protein and low amylose/amylopectin ratio diet. The presented *P*-value depicts the overall dietary treatment effect. Each group included 16 samples, with 8 samples per sex within each group.

Bacteroidota (7.26 ± 3.35%), and Euryarchaeota (1.64 ± 1.46%). The most abundant families were Streptococcaceae (23.75 ± 16.61%), Clostridiaceae (13.63 ± 7.68%), Lactobacillaceae (14.71 ± 15.05%), and Lachnospiraceae (8.02 ± 4.52%). The most represented genera were *Streptococcus* (25.26 ± 17.26%), *Clostridium sensu stricto* 1 (14.45 ± 7.99%), Lactobacillus (12.08 ± 13.12%) and *Turicibacter* (6.91 ± 11.21%).

The results for the α diversity indices at d11, d36 and d161 for the CO and TRT groups are shown in Figure 2. The interaction between the diet and time significantly affected all three alpha indices parameters ($p < 0.0001$) as well as the time (Chao1 and Shannon, $p < 0.001$; InvSimpson, $p = 0.02$) and the sex (Chao1, $p = 0.010$; Shannon, $p = 0.02$; InvSimpson, $p = 0.04$). Considering the effect of diet within each timepoint, it

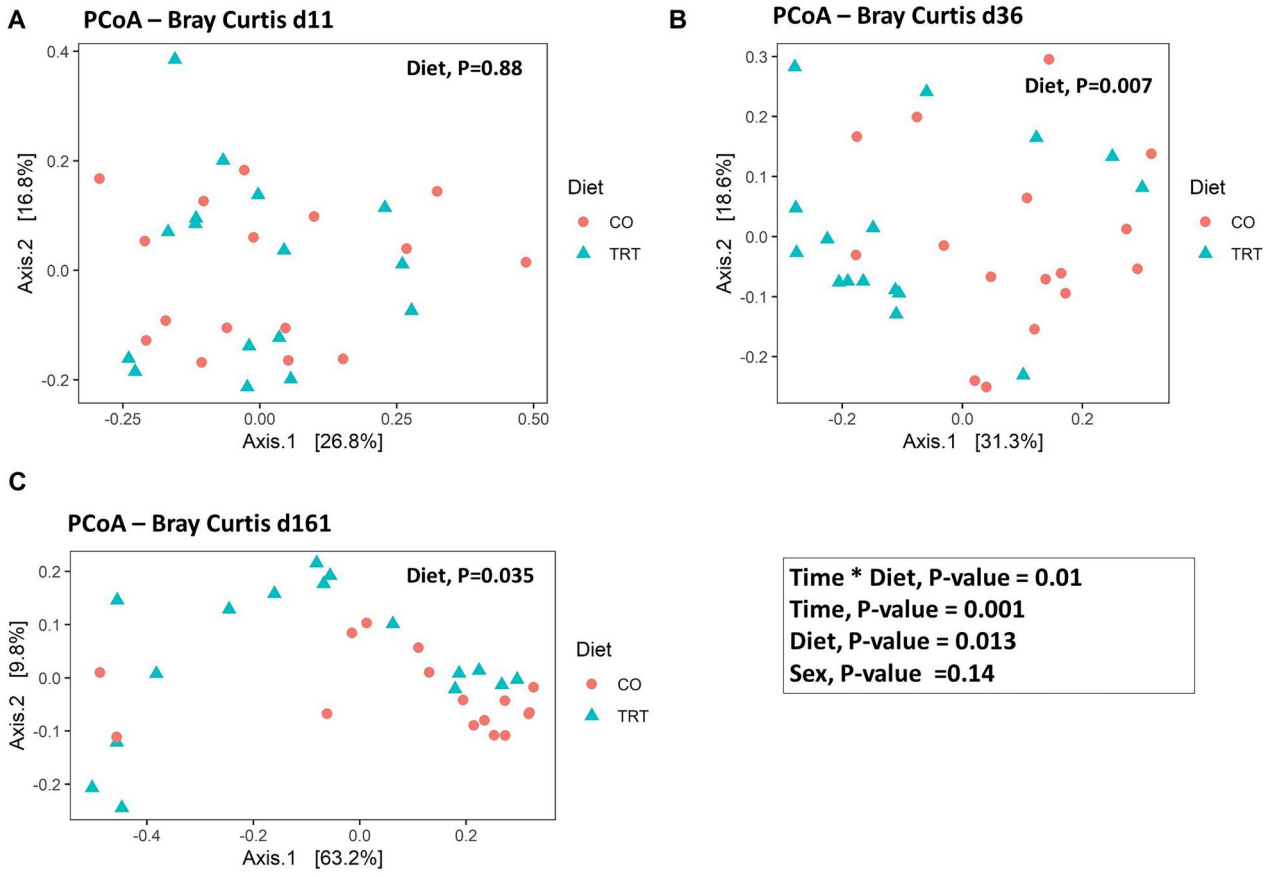

Figure 3. PcoA Plot on bray curtis distance matrix on faecal samples of pigs fed the different diets and collected at days 11, 36 and 161. Diet: CO = normal protein diet; TRT = low protein and low amylose/amylopectin ratio diet. Each group included 16 samples, with 8 samples per sex within each group.

results that at d11, the diet did not affect the Chao1 and Shannon indices, while the InvSimpson index was higher in the TRT group than CO ($p = 0.04$); at d36, the Chao1 ($p = 0.001$) and the Shannon ($p < 0.001$) indices were higher in the TRT group and the InvSimpson tended to be higher in the TRT group ($p = 0.07$). At d161, all the indices were lower in the TRT group ($p < 0.001$).

For β diversity, three PCoA plots were generated using a Bray-Curtis distance matrix for the data at d11, d36 and d161 (Figure 3). The interaction between diet and time ($p = 0.01$; $R^2 = 0.23$), the diet ($p = 0.013$, $R^2 = 0.023$), and time ($p = 0.001$; $R^2 = 0.052$) affected the β diversity, while no effect was observed for the sex. Considering the three timepoints separately, the diet did not affect the β diversity at d11, while it influenced it at d36 ($p = 0.007$, $R^2 = 0.091$) and at d161 ($p = 0.035$; $R^2 = 0.10$. The plots show that the samples from the different diets did not form separate clusters at d11, indicating that the overall microbial composition of the experimental diets was similar, while clusters due to the diet were valuable at d136 and d161.

To identify specific bacterial markers that were differentially expressed between treatments, the LDA effect size analysis was conducted. At d11, no specific bacterial markers associated with the diets were identified. Results for d36 and d161 are reported in Figure 4. At d36, the TRT group was characterised by a higher abundance of 9 specific microbial taxa including: *Clostridium sensu stricto 1* (LDA score = 4.81, *P* adj. = 0.002), *Terrisporobacter* (LDA score = 4.46, *P* adj. = 0.003), *Turicibacter* (LDA score = 4.44, *P* adj. = 0.004), *UCG-005* which belong to the Oscillospiraceae family (LDA score- = 4.26, *P* adj. < 0.0001) and *Romboutsia* (LDA score- = 3.69, *P* adj. = 0.01); the CO group was characterised by a higher abundance of 8 specific microbial taxa including *Lactobacillus* (LDA score = 4.97, *P* adj. = 0.02), *HT002* which belong to the *Lactobacillus* (LDA score- = 4.34; *P* adj. = 0.02), *Methanosphaera* (LDA score = 3.64; *P* adj. = 0.02), *Subdoligranulum* (LDA score = 3.61; *P* adj. = 0.03) and *Ruminococcus gauvreauii group* (LDA score = 3.44; *P* adj. = 0.01).

At d161, the TRT group was characterised by a higher abundance 3 specific microbial taxa including, *Clostridium sensu stricto 1* (LDA score = 4.91, *P*

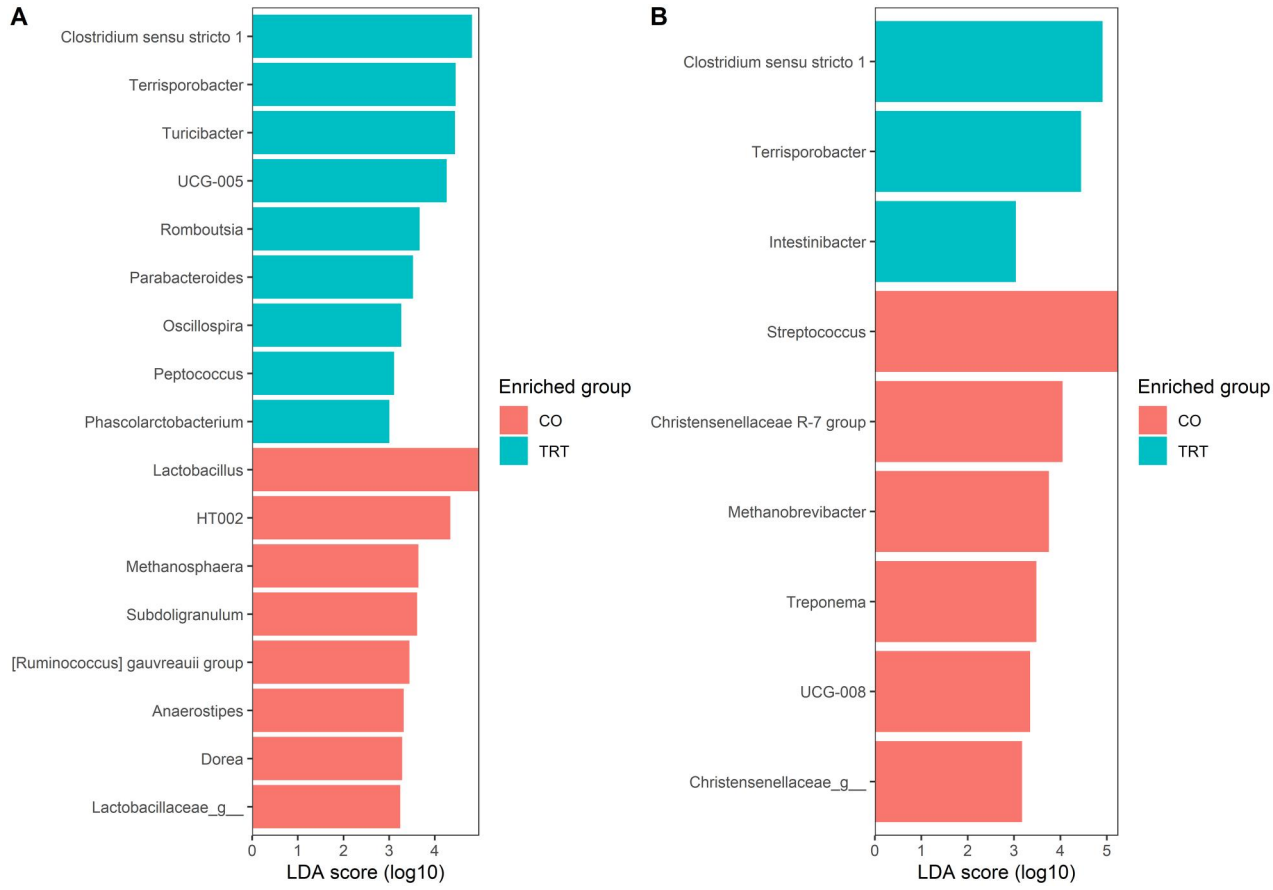

Figure 4. Barplot of linear discriminant analysis (LDA) effect size (LEfSe) at genus level for the faecal microbiota of pigs fed the different diets and collected at days 36 (A) and 161 (B). Horizontal bars represent the effect size for each taxon. The length of the bar represents the LDA score. LDA threshold score for discriminative features was set to 3.0. Diet: CO = normal protein diet; TRT = low protein and low amylose/amylopectin ratio diet. Each group included 16 samples, with 8 samples per sex within each group.

adj. = 0.009), *Terrisporobacter* (LDA score = 4.45, *P* adj. = 0.02) and *Intestinibacter* (LDA score = 3.04, *P* adj. = 0.04); the CO group was characterised by a higher abundance of 6 specific microbial taxa, including *Streptococcus* (LDA score = 5.24, *P* adj. = 0.014), *Christensenellaceae R-7 group* (LDA score = 4.04; *P* adj. = 0.04), *Methanobrevibacter* (LDA score = 3.74; *P* adj. = 0.04), *Treponema* (LDA score = 3.48; *P* adj. = 0.03) and *UCG-008* which belong to the Butyricicoccaceae family (LDA score = 3.34; *P* adj. = 0.02).

Discussion

The study demonstrates that the administration of an LCP diet supplemented with synthetic AAs, alongside utilising an alternative maize variety capable of reducing the AM:AP ratio during the fattening and finishing phases, did not affect the growth performance of pigs until they reach their heavy BW suitable for PDO Italian production.

The present results are consistent with previous studies in which the dietary CP was reduced in growing and finishing pigs till 10.8–12% (Cho and Kong 2025; Monteiro et al. 2017; Gallo et al. 2014; Wang et al. 2022). Knowing that these studies have been conducted on pigs weighing between 30 and 130 kg, it was important to evaluate the effect of a LCP in animals raised up to 190 kg. This target BW is of particular relevance to the Italian PDO production system, and has rarely been investigated in previous studies focusing on low CP diet. Comparing our results at the initial phase of rearing with the results by Wang et al. (2019) in which performance was maintained with a CP level of 14–15%, we can say that our TRT group achieved better results even though a lower CP level (13–12% CP). The maintenance of performance in the face of diminished CP levels indicates that the synchrony between glucose release from waxy maize and AAs absorption may have augmented protein utilisation efficiency. This mechanism, already hypothesised in experimental studies (Zhou et al. 2021), gains

practical confirmation in our trial, which was conducted on a large population of heavy pigs under commercial conditions. In fact, a salient aspect that merits consideration is that, in contrast to numerous preceding studies conducted on a limited number of animals in experimental facilities, our experiment encompassed a substantial population raised under commercial conditions. In fact, previous studies in heavy pigs but under experimentally controlled conditions have demonstrated that the level of CP can be further reduced to 10% without any negative effects on the animals' growth performance (CP 11.9–10.3%; Schiavon et al. (2015); 9.8% and 11.7–10.9%, Galassi et al. (2010) and Gallo et al. (2014). Furthermore, it should be noted that these studies were conducted about ten years ago and that genetics selection has led to leaner and less precocious pigs, possibly modifying protein requirements, then, updating data on the effect of LCP diets on heavy pigs is important. Our finding lends solid practical validity to the evidence that CP can be reduced till 12% in heavy pigs' diets, allowing to avoid also the use of soybean meal in the diets from d39 till d165. In fact, adding to the sustainability of the approach tested in this trial is the significant reduction in soybean meal that characterises the TRT group's diet. Indeed, in the three phases, following the reduction of CP, soybean meal was also reduced by 63%, 100% and 100%, respectively. It is important to note that the significant reliance on soybean imports from South America represents a key factor contributing to the suboptimal sustainability of the Italian and European pig supply chain (Wilke et al. 2023). Consequently, a substantial reduction in soybean utilisation would prove to be a substantial advantage.

As previously mentioned briefly, the positive effect obtained in the present study can also be attributed to the particular maize cultivar that has been used. In fact, synthetic AAs exists in the form of AAs monomers and can either be absorbed by intestinal cells or oxidised upon reaching the gut. In contrast, the starch in the ration releases glucose through hydrolysis during intestinal digestion. As a result, synthetic AAs from the diet can be released much more rapidly than glucose derived from starch and LP diets supplemented with large amounts of synthetic AAs can cause asynchronous kinetics of glucose and AAs and in turn reduce the efficiency of AAs utilisation, as more AAs may be consumed in first-pass metabolism. Additionally, the efficiency of AAs used for protein synthesis may decrease due to the lack of energy in the short period after the meal (Nolles et al. 2009). Therefore, optimising dietary glucose release to promote synchronous glucose and AAs supply may be a viable approach to improve AAs utilisation efficiency in LCP diets. To better highlight this effect, the diet could have involved more pronounced protein restriction. For future studies, it is therefore recommended that diets with varying protein levels are tested across multiple experimental groups. Within groups with the same protein level, the effects of waxy maize and conventional maize should be compared. However, such an experimental design was not feasible in the present study, which was conducted in a commercial facility involving a large number of animals.

In the present study, the faecal NH_3 concentration was also investigated, but it was not affected by the diet. Faecal NH_3 is produced in the large intestine because of fermentation of indigestible proteins by the microbial population; therefore, higher levels of NH_3 in faeces may indicate sub-optimal digestion or utilisation of dietary protein by the animals. The same result observed in this study was made in the study by Graziosi et al. (2024), where, during the fattening phase, the CP content of the diet was reduced by 1.3%. In the past, several studies have shown that feeding reduced-protein diets can improve N utilisation efficiency, resulting in higher N retention (Monteiro et al. 2017; Galassi et al. 2010) or lower excretion for the same amount of N ingested (Gallo et al. 2014). Osada et al. (2011) reported 28.7% less excreted N in urine and faeces of pigs fed a LP diet than those fed the standard diet. Considering that GHG production is dependent on the conversion of carbon and excreted N into CO_2, methane, and dinitrous oxide, reducing the protein content of the diet and consequently reducing N excretion by pigs has the potential to reduce GHG emissions (Atakora et al. 2003). Indeed, the study by Lee et al. (2017) showed lower NH_3 from pigs fed reduced protein diets (10% reduction in CP) compared to those receiving the control diet. Clearly, to obtain more realistic and usable data on the sustainability of this approach, the nitrogen content of urine must also be evaluated. This remains a critical issue in this experiment, despite the fact that concentrations of certain environmental gases were recorded where urine and faeces were present.

Our findings suggest that the TRT diet allowed to reduce the concentration of NH_3 and CO_2 at pen level in the final stages of the study, at d141 and d161. The discrepancy in results, particularly for NH_3 between faeces and air, could be due to the fact that the NH_3 in the air comes from microbial fermentations

originating from manure, where faeces and urine are mixed. Nevertheless, a reduction in the concentration of these gases, even if only at the pen level, has the potential to engender favourable outcomes for animal welfare.

Pigs health and welfare parameters were monitored in the present study since certain variations in nutrients (mainly nutrients deficiencies) and substances in the diet can directly affect the secretion of hormones or neurotransmitters, leading to abnormal behaviour and hyperactivity (Brunberg et al. 2016). Behavioural measurements showed that in the TRT group the pigs had a higher number of negative interactions at d11, a higher frequency of box exploration at d50, and at d112, they interacted more with environmental enrichments. The observations indicate that pigs in the TRT group were more active and restless than those in the CO group. In agreement with the observation of Almeida et al. (1994; performed in rats) it could be hypothesised that the reduction in CP could exacerbate aggressive behaviours or hyperactivity of the pigs. Furthermore, the presence of a cereal in the diet that is digested more quickly may have increased the animals' feeling of hunger, thereby further exacerbating the hyperactivity in the pigs (Menoyo et al. 2011). The present findings indicate that hyperactivity did not exacerbate negative behaviours, such as tail and ear biting, in piglets. This lack of difference in aggressive behaviour could still be attributed to the fact that both diets were balanced in nutrients such as AAs. In fact, the literature suggests that if the reduction in protein intake is not properly balanced with the correct supplementation of synthetic AAs, nutrient deficiencies can lead to aggressive behaviour in pigs, which can increase lesions (van der Meer et al. 2017).

As mentioned above, supplementing an LCP diet with a more digestible starch source may alter both the intestinal microbiota and fermentation processes. According to what was observed in the faecal microbiota analysis carried out at three time points, the inclusion of TRT diet led to a modification on the structure of the microbiota especially at days 36 and 161 and to an increase in alpha diversity indices at day 36. However, at day 161, the TRT diet had a reduction in α diversity indices compared to the CO diet. This divergent effect on alpha diversity deserves further considerations. At the intermediate stage (d36), the inclusion of highly digestible starch, alongside a reduction in dietary protein content, may have decreased undesirable protein fermentation and encouraged the development of a more diverse microbial community. This has been reported previously in a weaning trial involving a low-CP and low-AM:AP ratio diet (Correa et al. 2024). Similarly, Chen et al. (2018) and Liu et al. (2021) observed higher microbial diversity in pigs fed moderately reduced-protein diets, highlighting the role of a gut environment with fewer undigested protein substrates in supporting a more complex community. In contrast, at the finishing stage, the decrease in diversity may reflect the progressive selection of taxa that are better adapted to the dietary and physiological conditions. The reduced availability of complex substrates due to the diet's higher digestibility may have favoured the proliferation of genera such as *Clostridium sensu stricto* 1 and *Terrisporobacter*. In fact, these genera were found to be more abundant in the TRT group at both d36 and d161. Previous studies have reported that these genera are typically promoted by LCP diets in different production phases (Chen et al. 2018; Liu et al. 2021, 2023). Their presence may have contributed to lower bacterial distribution evenness, leading to a decline in α-diversity indices at d161. Furthermore, it should be noted that the literature describes a general tendency for diversity to decrease with increasing pig age, in relation to progressive gut microbiota stabilisation (Liao et al. 2024). Therefore, a long-term reduction in α-diversity should not necessarily be interpreted as a negative outcome, but rather as an indication that the microbial community is becoming more specialised and adapted to a low-protein, high-carbohydrate diet.

Some studies suggested that *Clostridium sensu stricto 1* and *Terrisporobacter* are associated with intestinal inflammation and diarrhoea (Hu et al. 2021). However, it should be noted that other studies have shown these genera to be commonly and abundantly present in the large intestine of pigs (in this study, genus *Clostridium sensu stricto* 1 accounted for 14% of the relative abundance) (Liao et al. 2024; Wang et al. 2018). Furthermore, according to some studies, the genus *Clostridium sensu stricto* 1 was also more abundant in faeces and in the caecum of pigs characterised by better feed efficiency (McCormack et al. 2017; Quan et al. 2018). This trend was also noted for some *Clostridium* species (boltea, clostridiumforme, saccharolyticum, cellulosis, clariflavum) in the ileum, caecum or faeces (McCormack et al. 2019; Quan et al. 2018; Yang et al. 2017). This result could be due to the degradation capacity of polysaccharides of this genus. Furthermore, in the study by Liu et al. (2023), the group fed the normal protein content diet had a higher abundance of the genera *Lactobacillus* and *Streptococcus*, which were also the characterising genera of the control group in the present study at both the timepoints.

In general, the effects of diet on the microbiota seem to be more attributable to a reduction in the CP content of the diet; however, this finding could be skewed by the fact that there are no studies that have investigated the effect of the AM:AP ratio on the faecal microbiota of growing and finishing pigs; in fact, there are a limited number of studies that have evaluated the effect of such variation in ration on the gut microbiota but in the weaning stages (Correa et al. 2024; Wang et al. 2022).

Starch serves as the substrate for microbial fermentation and can directly regulate the bacterial community and the production of VFAs (Haenen et al. 2013). It is important to investigate the impact of various starch types in protein-reduced diets on gut microbiota and VFAs synthesis (Zhou et al. 2022).

Similarly, to the microbial profile, the effect of the diet was also not consistent on the VFAs over time. In addition, the main effects of the diet on VFAs depends on time and sex of the pigs. Total VFAs and butyric acid at d11 (when no effect on the microbiota was noted) and isobutyric and isovaleric acid at day 161 were higher only in male pigs fed the TRT diet. These results indicate that the taxonomic characterisation of the microbiota, based on the DNA, does not always reflect its fermentative activity and, secondly, it shows a different response to diet according to sex. The increase in VFAs in the faeces of TRT males could be due to the fact that males generally have a more voracious feeding behaviour than females (Andretta et al. 2016), which could lead to a more rapid intestinal transit, increasing the potential nutrient substrate for the microbiota in the large intestine, which could be reflected by an increase in the concentration of AGVs in the faeces (Lewis and Heaton 1997), as they are not absorbed by the host. This increase in male pigs' voracity may have been exacerbated by the reduced CP of the TRT diet compared to the CO diet (Minussi et al. 2023), explaining the higher VFAs in the faeces of male in the TRT diet.

Conclusions

This study shows that a low AM:AP and CP diet supplemented with synthetic AAs can sustain growth performance and welfare of heavy pigs while reducing soybean meal use and potentially indirectly lowering environmental emissions.

No adverse effects were observed on behaviour or health, supporting the suitability of this approach for swine production. For future research, it will be important to better highlight the specific benefits of a low AM:AP dietary ratio by isolating its contribution within different dietary protein level and synthetic AAs inclusion, in order to fully exploit its potential in LCP feeding strategies. Subsequent studies should also investigate whether this type of strategy has an effect on carcase characteristics and meat quality.

Ethics approval

The procedures complied with the Italian law pertaining to experimental animals and were approved by the Ethic- Scientific Committee for Experiments on Animals of the University of Bologna (Protocol ID: 4525. Prot. n. 130114 15/05/2023)

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Author contributions

PT and DL: Conceptualisation; MVG, DL, FC, FC, AS and PT: Data curation and Investigation; MVG, DL, FC: Formal analysis; MVG and DL: Writing – original draft; MVG, DL, FC, FC, AS and PT: Writing – review & editing; PT and DL: Supervision; PT: Resources and Funding acquisition. All authors read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All dataset used in this paper are available from the authors upon reasonable request. Raw sequence data are freely available at NCBI Sequence Read Archive under the accession number PRJNA1271734.

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