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A growing-finishing diet formulated to reduce the soybean meal does not compromise the growth performance, health, behaviour and gut health of Italian heavy pigs

Maria Vittoria Graziosi[a], Diana Luise[a], Roxana Elena Amarie[b], Federico Correa[a], Alberto Elmi[c], Sara Virdis[a], Clara Negrini[a], Francesco Palumbo[a], Giacomo Biagi[d], Maria Laura Bacci[d], Nadia Govoni[d], Andrea Serra[b] and Paolo Trevisi[a]

[a]Dipartimento di Scienze e Tecnologie Agro-alimentari, Università di Bologna, Bologna, Italia; [b]Dipartimento di Scienze Agrarie, Alimentari e Agro-ambientali, Università di Pisa, Pisa, Italia; [c]Dipartimento di Scienze Veterinarie, Università di Pisa, Pisa, Italia; [d]Dipartimento di Scienze Mediche Veterinarie, Università di Bologna, Ozzano dell'Emilia, Italia

ABSTRACT

Soy contributes to the environmental impact of Italian pork production. This study aimed to evaluate the effect of a reduced soybean meal (SBM) and crude protein (CP) finishing diet on growth performance, health, behaviour and gut health of Italian heavy pigs. 1920 pigs (35.6 kg body weight; (BW)) balanced by sex and BW were assigned to the control diet (CO), or the treated diet (TRT) formulated by reducing SBM by 31%, 67% and 69% (replaced by pea and sunflower meal) in 3 feeding phases, respectively, and 1.2% CP in the third phase. 251 pigs were individually weighed at d11, d94 and d181. Feed intake (FI), behavioural indices and air gases at pen-level were monitored monthly. Faecal samples (20/pigs/group) for microbiota, ammonia and volatile fatty acids (VFAs) and hair for stress biomarkers were collected. Diet did not affect final BW, faecal ammonia, cortisol and dehydroepiandrosterone. From d11–d94, the CO group had higher gain to feed (G:F) ($p = .007$), favourable faecal VFAs profile and a lower environmental ammonia ($p < .0001$). From d10–d181, the TRT diet increased the ADG ($p = .04$) and G:F ($p = .01$), reduced FI ($p < .0001$) and the lesions score index at d102 ($p = .03$) and promoted *Methanobrevibacter* (d94; padj. = 0.013) and *Clostridium sensu stricto* (d181; padj. = 0.001). Overall, the TRT diet combined with the stress of the transport and acclimatisation to the farm may limit the growth of pigs in the initial period, but it can increase their growth in the long term. Concluding, replacing 56% of SBM with sustainable alternatives seems promising for heavy pig.

HIGHLIGHTS

- Reducing dietary SBM and CP favoured the ADG and G:F of Italian heavy pigs under no stress condition.
- Reducing dietary SBM and CP did not stress the pigs and promoted positive behaviour and reduced tail lesion index.
- Reducing dietary SBM and CP slightly modulated specific faecal microbial taxa in growing-finishing pigs.

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Fattening pigs; sustainable feed; pea meal; sunflower meal; dietary fibre

Introduction

The efficiency of the agricultural phase is in synergy with societal demands. Indeed, a survey published by Eurobarometer in 2023 highlights that EU citizens prioritise animal welfare and environmental protection (European Commission 2023), confirming that the ethical quality and environmental impact of animal-derived products are almost equal to the organoleptic quality; Grunert et al. (2018) already reported this aspect in 2018.

According to the Life Cycle Assessment (LCA) carried out by Dourmad et al. (2014) on the environmental impacts of 15 pig farming systems in Europe, feed production had the most significant impact on climate change, eutrophication potential, and energy demand across farming systems, compared to animal housing

CONTACT Paolo Trevisi paolo.trevisi@unibo.it

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and manure storage and spreading. In fact, feed production accounts for 76% of the total nitrogen losses (Uwizeye et al. 2019). This could be particularly relevant in certain production systems including the Italian heavy pig's production system, which, according to Bava et al. (2017), has a higher impact (4.25 ± 1.03 kg CO_2 eq/kg) than the common EU production system based on pigs reared up to 100–120 kg of body weight (BW).

Furthermore, soybean production, as one of the main protein concentrates used in pig's formula, has been linked to deforestation and gas emissions due to its transport and processing (Chiozza et al. 2020). South America's tropical forests and savannas are under increasing pressure from agricultural expansion. Both cattle ranching and soy production contribute to forest loss (Fehlenberg et al. 2017). This issue has attracted the attention of the European Feed Manufacturers' Federation (FEFAC), which has been promoting responsible soy standards and programmes, with the objective of ensuring that there are adequate controls in place to guarantee that relevant soy expansion, forests, biodiversity and nature legislation is being complied with. In addition, the geographical separation between soybean cultivation and livestock production disrupts the nutrient cycle. Such discontinuity exacerbates the process of soil erosion and degradation, thereby threatening the biodiversity in the regions of origin (Taelman et al. 2015; Stødkilde et al. 2023). The main reasons for using soybean meal (SBM) in pig diets are related to its excellent balance of essential amino acids (AAs), low fibre concentration, continuous availability and cost-effectiveness compared to other feed ingredients (Wang et al. 2011). One of strategies proposed at the EU level is to differentiate the protein feed ingredients for the pig diets and to partially replace SBM with the inclusion of local protein sources, whose AAs profile can be corrected by the use of synthetic AAs. Among the local protein sources available in the EU, sunflower meal and pea as partial replacements for soy have been highlighted. Regarding pea, its cultivation is rising in the EU, however its availability is still uncontinuous and it is more expensive than SBM (Clément et al. 2018); furthermore, it is used with caution in pigs formulation due to the presence of antinutritional factors, such as tannins, protease inhibitors, lectins or phytate (Nikmaram et al. 2017) and its low content of sulphur AAs (Hanczakowska and Świątkiewicz 2014). In fact, the content of methionine in peas is 0.18% compared to 0.65% of SBM (Smith et al. 2013). Nevertheless, the use of pea as a local protein source in pig diets has been tested. According to the study by Smith et al. (2013), its inclusion above 20% reduced the growth performance, such as average daily gain (ADG), average daily feed intake (ADFI) and gain to feed (G:F) in finisher pigs.

Sunflower is the third largest oilseed produced in the world and in Europe, after soya and rapeseed, but it is the first European production in terms of the organic market. Its availability in the form of sunflower meal is still limited compared to SBM and depends on the time of harvest, but in recent years its production has been gaining interest and in fact only half of it is imported from Europe (Clément et al. 2018). From a nutritional point of view for swine, sunflower meal generally has lower levels of digestible protein and lysine and a higher fibre content than SBM (Lannuzel et al. 2022). However, among protein sources, except for SBM, sunflower meal, especially the fully decorticated one, has the highest CP digestibility. According to the study by Florou-Paneri et al. (2014), the average digestibility of AAs is 89% compared to 90.6% of soybean meal. It has a higher content of sulphur AAs, especially methionine, than other protein sources and is a good source of calcium, phosphorus and vitamin B. Furthermore, a recurring theme in the literature is the reduction of dietary crude protein (CP) to meet the AAs requirements with synthetic AAs. The findings consistently demonstrate that the incorporation of synthetic AAs offers significant benefits in mitigating the environmental footprint associated with soy production, processing, and transportation (Mosnier et al. 2011; Ogino et al. 2013; McAuliffe et al. 2016). Indeed, Ogino et al. (2013) showed that the use of synthetic AAs in a low-CP diet can reduce greenhouse gases emissions by 20% compared to a conventional pig diets containing 52% more SBM, and significantly reduce the impact during the manure management phase.

Therefore, the use of synthetic AAs in combination with low protein diets and based on the use of local protein sources could further promote the reduction of the environmental impacts of the pig production system. However, it is important to investigate whether this dietary strategy can also sustain the growth, welfare and well-being of pigs. Recent studies have shown that pigs fed with a low-CP diet can exhibit detrimental and harmful behaviours, such as tail biting (van der Meer et al. 2017; McAuley et al. 2022; Minussi et al. 2023). Tail and ear biting can cause stress and pain to the victims, as well as reduce their growth and overall health. This can lead to economic losses for farmers. Furthermore, the different

dietary composition may affect the gut microbiota and its metabolic activity, which can affect the health and performance of pigs (Trevisi et al. 2021).

Maintaining the health and welfare of pigs, together with improving the environmental sustainability of the production of PDO products, are key elements required by consumers. From this point of view, the production of Italian heavy pigs, characterised by the production of specific PDO products, must take this into account. However, the production of such high quality and PDO products implies the limitation of the use or percentage of use of raw materials in the feed formulas. However, in line with the European Union proposal to focus on the inclusion of the differentiation of different protein raw materials in the diet to comply with the reduction in the use of soybean meal, the combined use of pea and sunflower meal could represent a valid solution in the Italian context. To date, to the authors' knowledge, the effects of such a diet have not been studied in the Italian heavy pig supply chain. Therefore, the aim of the present study was to test the effect of a diet with reduced SBM content, replaced by local protein sources (pea and sunflower meal) and reduced CP in the last feeding phase on growth, health, gut health and aggressive behaviour of growing and fattening Italian heavy pigs.

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 (Trial ID 4525, Prot. n. 130114 – 5th May 2023).

Experimental design and sampling

The study was conducted on a commercial farm in the north of Italy. At the arrival at the growing-fattening unit (d0; average BW 35.6 kg), a total of 1,920 PIC × FOMEVA pigs were divided into two groups: (1) Control group (CO), receiving a commercial diet formulated including SBM as a main protein source and (2) Treated group (TRT), receiving a diet with reduced content of SBM (replaced with different doses of full de-hulling sunflower and pea meal in the three dietary phases) and decreased CP level in the last feeding phase. The control group consisted of 58% males and 42% females, while the treatment group consisted of 45% males and 55% females. From those 1,920 pigs, a sample of 251 pigs was selected for the assessments. The diets were formulated in agreement with the Parma ham PDO specification criteria. For both groups, pigs were fed with a three-phase feeding program. The diets were formulated to meet the nutritional requirements of the pigs at the different stages of growth (NRC 2012). The diet composition and calculated chemical composition are reported in Table 1.

The diets were analysed for their proximal and total AAs composition. In detail, the AAs composition was determined by the High-Performance Liquid Chromatography (HPLC) analysis. Dry matter was determined by drying samples at 103 °C. The Kjeldahl method (N*6.25) was used to assess crude proteins. Lipids were determined by petroleum ether extraction. The Wendee method was used to detect fibre. Ash content was determined by incineration at 550 °C and the polarimetric method was used to assess starch. Results of the analysed diets are reported in Table 2.

For each group, pigs were reared in 48 pens (20 pigs/pen) of 20.4 m^2. The pens were equipped with a soft wooden log and a steel chain as enrichment material. The pens had fully slatted floors. Water was continuously available and provided by a nipple drinker, the feeding space was 0.40 m/pig and the farm had a natural ventilation system. Liquid feed (water/meal ratio 3:1) was offered twice a day (at 8:00 am and 3:00 pm), using an automatic distributing system which provided the feed *via* a valve every two pens. The animals were not fed ad libitum but rather according to a nutritional curve normally used in the commercial farm. The feed consumption per pen was recorded on a monthly basis.

Data and sample collection

At d11, from 6 pens/group, 251 pigs were selected based on BW and sex (CO = 126 pigs; TRT = 125 pigs) and individually identified with a numbered ear tag. The identified pigs were individually weighed during the growing phase at d11, d94 and during the finishing phase at d181. Among these animals, a subgroup of 48 animals (24 pigs/diet) balanced for pen and BW were selected to collect faecal samples at d11, d94 and d181 for microbiota, ammonia (NH_3) and volatile fatty acids (VFAs) analysis. Faecal samples were collected from the selected pigs by natural defaecation or in some cases by a slight stimulation with a sterile cotton swab. The samples were collected into a sterile tube, promptly frozen in liquid nitrogen and then stored at −80 C until analysis. The same pigs were shaved on the rump at d11, d94 and d181 and hair were collected at d94 and d181 for cortisol and

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

	Diet[a]					
	Phase 1 (d0–d28)		Phase 2 (d29–d94)		Phase 3 (d95–d184)	
Item	CO	TRT	CO	TRT	CO	TRT
Ingredients, %						
Barley	34.11	27.50	18.00	10.00	25.00	22.50
Wheat grain	14.28	20.00	0.00	19.17	0.00	13.63
Maize	24.00	26.47	43.11	22.67	49.60	47.43
Soybean meal 48	12.15	8.33	10.83	3.50	8.00	2.50
Pea	0.00	5.50	1.67	5.00	4.42	5.00
Full de-hulling sunflower meal	0.00	2.92	0.00	5.00	0.00	2.50
Wheat flour bran	0.00	0.00	5.00	5.03	4.85	0.00
Wheat bran	1.67	0.00	1.33	0.00	2.17	0.00
Triticale	3.75	0.00	10.00	0.00	0.00	0.00
White sorghum	0.00	0.00	0.00	20.50	0.00	0.00
Maize germ	0.00	0.00	0.00	2.50	0.00	0.00
Valine 96.5%	0.00	0.02	0.00	0.00	0.00	0.00
Mixed fat	3.00	2.83	1.83	2.00	1.50	1.67
Sugar beet pulp	3.30	2.50	5.50	2.50	2.50	2.50
Calcium carbonate	0.94	0.93	0.87	0.93	0.77	0.77
Sodium bicarbonate	0.00	0.00	0.55	0.50	0.58	0.47
Formic acid	0.83	0.83	0.00	0.00	0.00	0.00
Liquid lysine 50%	0.62	0.67	0.44	0.66	0.18	0.40
Sodium chloride	0.43	0.43	0.00	0.00	0.00	0.00
Mineral-vitaminic premix[b]	0.25	0.25	0.25	0.25	0.20	0.20
Enzymesc	0.00	0.00	0.05	0.05	0.05	0.05
Monocalcium Phosphate	0.25	0.33	0.30	0.08	0.10	0.22
Phytase	0.10	0.10	0.10	0.11	0.10	0.10
Threonine	0.14	0.16	0.10	0.13	0.01	0.06
2–hydroxy-4(methylthio) – HMB	0.12	0.13	0.07	0.07	0.00	0.00
Liquid choline 75%	0.05	0.05	0.00	0.00	0.00	0.00
L-Tryptophan	0.02	0.04	0.00	0.00	0.00	0.00
Calculated chemical composition						
Dry matter, %	86.73	86.72	87.21	87.38	87.15	87.01
Crude Protein, %	14.10	13.98	13.37	13.27	12.28	10.99
Crude Lipids, %	5.24	5.07	4.36	4.29	4.27	4.35
Crude Fibre, %	3.75	3.94	3.84	4.05	3.70	3.72
NDF, %	13.47	13.34	13.07	13.64	13.36	13.04
Ash, %	4.41	4.39	4.21	3.83	3.68	3.48
Starch, %	43.83	45.00	45.73	48.79	48.16	51.75
Net Energy, kcal/kg	2.41	2.45	2.37	2.26	2.35	2.46

Diet[a]: CO: diet formulated including soybean meal as main protein source; TRT: diet with reduced content of soybean meal and a decreased CP level in the last feeding phase.
Mineral-vitaminic premix[b:] Vitamin A 600.000 U.I./kg; Vitamin D3 600.000 U.I./kg; Vitamin E 22.000 mg/kg; Vitamin K3 1.600 mg/kg; Vitamin B1 1.000 mg/kg; Vitamin B2 2.000 mg/kg; Calcium D-Pantothenate 6.666 mg/kg; Vitamin B6 1.500 mg/kg; Vitamin B12 12 mg/kg; Niacin 12.000 mg/kg; Folic Acid 320 mg/kg; Biotin 40 mg/kg; Iron 30.000 mg/kg; Copper 4.800 mg/kg; Manganese 14.000 mg/kg; Zinc 30.000 mg/kg; Iodine 320 mg/kg; Selenium 120 mg/kg; Citric Acid 560 mg/kg; BHT (E 321) 2.249 mg/kg; Propylgallate (E 310) 185 mg/kg; Silicic Acid 1.400 mg/kg; Magnesium oxide 25.00%; Grape pomace 20.19%, Calcium carbonate 19.48%.
Enzymes[c]: Endo-1,4-β-xilanasi/EC 3.2.1.8/; Endo-1,3(4)-ß-glucanasi/EC 3.2.1.6/.
HMB: beta-hydroxy-beta-methylbutyrate; NDF: Neutral Detergent Fiber

dehydroepiandrosterone (DHEA) quantification. The throw-away of the hair collected at d11 ensured that the measurement of cortisol and DHEA as markers of chronic stress, can be attributed to a specific time interval of the trial (Koren et al. 2002).

Mortality and exclusion for sanitary reasons were recorded daily throughout the study. Furthermore, on monthly basis, from the pens of ear tagged pigs, behavioural observations and lesion assessments were done using the Welfare Quality® 2009 protocol. Environmental parameters were collected from the same pens using a XAM8000 Multigas Detector (Dräger, Lübeck, Germany). At the end of the fattening phase, all pigs on the farm were slaughtered in a commercial abattoir.

Volatile fatty acids and ammonia in faeces

The quantification of VFAs (acetate, propionate, isobutyrate, butyrate, valerate, isovalerate) and lactic acid in faecal samples was performed using HPLC according to the procedure described by Trevisi et al. (2023). Briefly, 1 g of each faecal sample was diluted with 5 mL of 0.1 N H SO and homogenised using an UltraTurrax (IKA®-Werke GmbH & Co. KG, Staufen, Germany). The mixture was centrifuged (5000 × g for 15 min at 4 °C) to obtain the liquid phase which was microfiltered (SLMV033RS, 0.45-µm Millex-HV, Merck-Millipore, Billerica, MA). The sample was injected directly into HPLC using an Aminex 85 HPX-87 H ion exclusion column (300 mm × 7.8 mm; particle size 9 µm; Bio-Rad, Milan, Italy); the detection

Table 2. Chemical and amino acid composition of the experimental diets.

	Diet[a]					
	Phase 1 (d0–d28)		Phase 2 (d29–d94)		Phase 3 (d95–d184)	
Item	CO	TRT	CO	TRT	CO	TRT
Analysed chemical composition (%)						
Dry matter	89.40	88.50	88.50	88.55	89.45	89.30
Crude Protein	13.90	13.69	13.12	13.20	12.54	11.30
Crude Lipids	5.28	5.07	4.77	4.51	4.20	4.34
Crude Fibre	3.63	3.94	3.68	4.19	3.78	3.74
Ash	4.25	4.28	4.20	3.84	3.78	3.48
Starch	44.00	44.81	46.14	48.13	50.05	52.89
Analysed amino acid composition (%)						
Cysteic acid	1.03	1.19	1.23	1.15	1.05	1.08
Aspartic acid	1.12	1.39	1.16	1.32	1.01	1.21
Glutamic acid	2.61	2.80	2.40	2.60	2.20	1.90
Serine	0.44	0.52	0.53	0.42	0.45	0.32
Glycine	0.63	0.55	0.61	0.61	0.58	0.55
Histidine	0.41	0.45	0.41	0.22	0.39	0.12
Arginine	0.33	0.36	0.32	0.30	0.34	0.21
Threonine	0.51	0.48	0.56	0.46	0.48	0.31
Alanine	0.70	0.82	0.72	0.62	0.63	0.56
Proline	0.52	0.65	0.55	0.71	0.48	0.66
Tyrosine	0.21	0.15	0.15	0.14	0.16	0.10
Valine	0.53	0.51	0.51	0.46	0.44	0.39
Methionine	0.56	0.50	0.41	0.49	0.42	0.44
Cystine	0.31	0.22	0.32	0.16	0.29	0.13
Isoleucine	0.45	0.44	0.42	0.44	0.41	0.38
Leucine	0.68	0.55	0.58	0.64	0.52	0.59
Phenylalanine	0.61	0.61	0.56	0.49	0.58	0.45
Lysine	0.92	0.91	0.85	0.85	0.71	0.68
Tryptophan	0.28	0.28	0.24	0.22	0.21	0.19

Diet[a]: CO: diet formulated including soybean meal as main protein source; TRT: diet with a reduced content of soy and a decreased CP level in the last feeding phase.

wavelength was 220 nm. Analyses were performed using isocratic elution (flow rate 0.6 mL/min) with 0.008 N H SO solution as mobile phase and theinjection loop was 20 μL. Individual VFAs and lactic acid were identified and quantified by means of an external calibration curve using a standard solution with 5 points at different concentration of a mixture containing the detected organic acids (69775, 338826, 402907, B103500, 58360, 75054, 129542, Sigma-Aldrich, Milano Italy), diluted in 0.1 N H_2SO_4. For NH_3 determination, faecal samples were thawed and 1 g of faeces was diluted with deionised water to a weight/volume ratio of 1:10.After vortexing, the samples were centrifuged at 7000 rpm for 10 min at 4 °C. Faecal NH was determined by an enzymatic colorimetric assay on the supernatant according to the manufacturer's protocol (Urea/BUN-Color; BioSystems S.A., Barcelona, Spain) and data were expressed as μmol/g faeces.

Microbial profile from faeces

Bacterial DNA extraction was performed on a total of 120 faecal samples using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, Ca, USA) according to the manufacturer's instructions. The DNA concentration and purity (absorbance ratios 260/280 and 260/230, respectively) of the isolated DNA were checked using NanoDrop spectrophotometry (Fisher Scientific, 13 Schwerte, Germany). The V3-V4 region of the 16S rRNA gene (∼460 bp) was amplified; amplicons were generated using the universal primers Pro341F: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGACCCTACGGGNBGCASCAG-3′ and Pro805R:5′GTCTCGTGGCTCGGAGATG TGTATAAGACAGGACTACNVGGTATCTAATCC-3′ (Takahashi et al. 2014) using Platinum™ Taq DNA Polymerase High Fidelity (Termo Fisher Scientific, Italy) was sequenced on the Illumina MisSeq 300 × 2 bp platform. Library construction and 16S Rrna gene sequencing were performed using MiSeq® Reagent Kit V3-V4 on the MiSeq-Illumina® platform. Microbiota analysis was performed 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). Alpha diversity indices (Chao1, Shannon, and Simpson diversity) and Bray-curtis distance matrix were then calculated.

Hair cortisol and dehydroepiandrosterone analysis

The collected hair samples were subjected to the washing and extraction process prior to the cortisol and DHEA analysis. The sample washing protocol consisted of an initial washing of hairs in a beaker with distilled water (10 mL per 50 mg) using tweezers to

remove impurities. This washing was repeated twice if the samples were very dirty. The samples were then dried on absorbent paper for 12 h and placed in a 50 mL Falcon tube with a fume hood and isopropanol (5 mL per 250 mg sample) for 3 min. The supernatant was then removed under a fume hood and discarded before repeating the wash with isopropanol. Finally, the falcon with the sample was fully air dried and pulverised using a pestle. From the pulver an aliquot of 120 mg was obtained and methanol was added (2 mL for each 60 mg of the sample). The sample was incubated overnight at RT with continuous gentle agitation and then centrifuged for at least 30 min. The supernatant was then collected under a fume hood and dried before being subjected to the radioimmunoassay (RIA). The dry extracts were reconstituted in assay buffer (phosphate buffered saline, 0.1% BSA, pH 7.4) for measurement of cortisol (6 mg hair equivalent) and DHEA (4 mg hair equivalent) by radioimmunoassay as previously described (Bacci et al. 2014; Elmi et al. 2020).

Animal based measurement

A three-point scoring system from 0 to 2 was used to score ear and tail lesions, where score 0 represented intact ears/tails and no injuries, score 2 represented ears or tails with erosive and/or necrotic lesions and score 1 as intermediate value with superficial bite along the tails and ears but with no evidence of swelling. Data are expressed according to the Lesion Score Index (LSI) proposed by Vitali et al. (2021). Behavioural observations included suckling', social, exploratory, inactive or resting behaviour and other behaviours as defined by the Welfare Quality (2009) and Palumbo et al. (2023).

Environmental parameters

Carbon dioxide, NH_3 and hydrogen sulphide were collected. Light intensity and gas percentage were recorded at pig height (approximately 50 cm) at three different points: the right corner, centre and left corner of the pen. The average of the three measurements was then calculated. Gas percentage was recorded using the Dräger X-am® 8000 multi-gas detector (Drägerwerk AG & Co, Lübeck, Germany).

Statistical analysis

Data of BW, ADG, faecal NH_3 and VFAs, cortisol and DHEA were fitted using a linear mixed model in which the diet and sex were included as fixed factors and the pen was included as a random factor. For FI and gain to feed ratio (G:F), behavioural and environmental parameters the pen was used as the experimental unit and data were fitted using a linear model including diet as a fixed factor. Data on alpha diversity indices (Chao1, Shannon, and Simpson diversity) of microbiota were fitted using a linear mixed model in which the diet, sex, time and interaction between diet and time were included as fixed factors and the pig was included as a random factor. For the beta diversity, a dissimilarity matrix using Bray Curtis distances of centred log-ratio transformed data was constructed and results were plotted using a Principal Coordinates Analysis plot. Differences were tested using a PERMANOVA model (Adonis test) with 9999 permutations, including diet, time and their interaction as factors. Linear discriminant analysis (LDA) effect size algorithm at genus levels was applied to identify taxa differentially expressed (LDA score >3 and P. adj <0.05) between the dietary treatments.

All data were analysed within the R software (Core Team, 2021) using the packages 'car' (version 3.0.12), 'lm4' (version 1.1.27.1) and 'lsmeans' (version 2.30), 'phyloseq' (McMurdie and Holmes 2013) v1.38, 'vegan' v2.6 (Dixon 2003) and 'microbiomeutilities' v1.0 (https://github.com/microsud/microbiomeutilities).

Results

Growth and health

At the housing, the CO group consisted of 920 pigs with an average BW of 36.2 kg ± 6.53, while the TRT group was composed by 1000 pigs with an average BW of 35.0 kg ± 3.85.

The effect of the diet on the growth performance is shown in Table 3. The diet had no effect on the BW of the pigs at any time point, while it was influenced by the sex which led to higher BW in castrated males than in females at d11 ($p = .02$), d94 ($p = .0002$) and d181 ($p = .002$). The diet did not affect the ADG of the period d11–d94, while it influenced the ADG of the periods d94–d181 ($p = .01$) and d11–d181 ($p = .04$); the TRT group had higher ADG than the CO group in both periods. The sex influenced the ADG from d11 to d94 ($p < .0001$) and from d11 to d181; in both periods, castrated males had a higher ADG than females.

Data on FI, G:F and the number of animals excluded from the trial due to mortality or health reasons are shown in Table 4. Pigs in the TRT group had a higher FI than pigs in the CO group from d11 to d94 ($p = .004$), while from d94 to d181 ($p < .0001$) and

Table 3. Effect of partial replacement of soybean meal with peas and sunflower meal on body weight and average daily gain of growing and finishing Italian heavy pigs on body weight and average daily gain of growing and finishing Italian heavy pigs.

	Diet[a]			Sex			*p*-value	
Item	CO	TRT	SEM	F	M	SEM	Diet	Sex
Body weight, Kg								
d11	46.8	45.6	1.3	45.2	47.2	1.00	0.48	0.02
d94	124	121	2.10	119	126	1.70	0.33	0.0002
d181	177	180	1.55	175	182	1.50	0.26	0.0020
Average daily gain, g/day								
d11–d94	926	908	11.00	885	948	21.00	0.28	<0.0001
d94–d181	615	677	16.50	643	649	15.30	0.01	0.76
d11–d181	765	788	8.00	761	792	8.10	0.04	0.01

Diet[a]: CO: diet formulated including soybean meal as main protein source; TRT: diet with a reduced content of soy and a decreased CP level in the last feeding phase.

Table 4. Effect of partial replacement of soybean meal with peas and sunflower meal on body weight and average daily gain of growing and finishing Italian heavy pigs on feed consumption, gain to feed, mortality and exclusion of growing and finishing Italian heavy pigs.

	Diet[a]			*p*-value
Item	CO	TRT	SEM	Diet
Feed Intake (g/day)				
d11–d94	2285	2328	5.95	0.004
d94–d181	2783	2566	12.2	<0.0001
d11–d181	2619	2525	7.89	<0.0001
Gain to feed				
d11–d94	0.41	0.39	0.003	0.007
d94–d181	0.22	0.26	0.005	0.001
d11–d181	0.30	0.31	0.004	0.01
Mortality and exclusions				
d11–d94	7.94	7.26	4.73	0.92
d11–d181	7.94	8.85	4.78	0.89

Diet[a]: CO: diet formulated including soybean meal as main protein source; TRT: diet with a reduced content of soy and a decreased CP level in the last feeding phase.

from d11 to d181 ($p < .0001$) the TRT group showed a lower FI than the CO group. With regard to the G:F, the diet had a significant effect in all three periods and the TRT group had a lower G:F than the CO group at d11–d94 ($p = .007$) and a higher G:F at d94–d181 ($p = .001$) and d11–d181 ($p = .01$). The diets did not influence the mortality and rate of exclusion of pigs from the study.

Volatile fatty acids and ammonia in faeces

Table 5 shows the effect of diet and sex on the concentration of NH_3 and VFAs in the faecal samples collected at d11 and d181. The diet did not affect faecal NH_3 concentration. The sex affected the faecal concentration of NH3 at d11. Castrated males showed higher faecal concentration of NH3 than female animals ($p = .01$). At d181, no difference due to the sex was observed on faecal NH3.

The interaction between diet and sex influenced the faecal concentration of acetic acid at d11 ($p = .009$), and, within the TRT group, male pigs had higher faecal concentration of acetic acid than females ($p = .04$). At the same timepoint, higher faecal concentrations of propionic ($p = .05$) and butyric acid ($p = .0005$) was observed in the CO group. At d181, faecal propionic acid tended to be higher in the TRT group ($p = .09$). Butyric acid was influenced by the interaction between diet and sex ($p = .02$) and its concentration was lower in female pigs in the CO group than in females in the TRT group ($p = .05$), males in the CO group ($p = .01$) and males in the TRT group ($p = .03$). Sex and diet did not affect the faecal concentration of the other VFAs.

Faecal microbial profile

Bacterial DNA from faecal samples was successfully extracted and amplified from a total of 120 samples. After quality control, the total of sequences obtained was 3,924,264, with an average of 32,702 per sample and a total of 2880 amplicon sequence variants were generated. Rarefaction curves are shown in Supplementary Figure S1, which shows the number of different species observed as a function of the number of sequences; the tendency of the curves to plateau indicates that the sequencing procedure was able to capture all the variability present in the samples.

Among the 2,880 amplicon sequence variants recovered, 16 phyla, 68 families and 160 genera were identified. The most abundant phyla (mean and SD) were Firmicutes 87.1 ± 7.02%, Bacteroidota 9.38 ± 5.82% and Spirochaetota 1.66 ± 1.76%. The most abundant families were Clostridiaceae 36.41 ± 16.51%, Lactobacillaceae 19.2 ± 12.8%, Peptostreptococcaceae 7.33 ± 4.22% and Erysipelotrichaceae 3.99 ± 4.88%. The most represented genera were *Clostridium_sensu_stricto_1* 37.8 ± 17.1%, *Lactobacillus* 20. 5 ± 13. 5%, *Terrisporobacter* 5.93 ± 3.48% and *Turicibacter* 3.86 ± 5.20%.

Figure 1A shows the values for Chao1, Shannon and InvSimpson diversity indices for each diet at d11,

Table 5. Effect of partial replacement of soybean meal with peas and sunflower meal on body weight and average daily gain of growing and finishing Italian heavy pigs and sex on concentration of ammonia and volatile fatty acids in faeces of growing and finishing Italian heavy pigs.

	Diet[a]			Sex			*p*-value	
Item	CO	TRT	SEM	F	M	SEM	Diet	Sex
NH3, (umol/g)								
d11	32.50	35.90	5.30	27.40	41.00	4.60	0.66	0.01
d181	31.00	36.00	3.90	34.00	32.80	13.70	0.85	0.52
VFAs, (mmol/g)								
d11								
Lactic	0.04	0.04	0.04	0.04	0.03	0.05	0.95	1.00
Acetic[b]	0.26	0.28	0.01	0.26	0.28	0.01	0.54	0.34
Propionic	0.12	0.10	0.00	0.11	0.11	0.01	0.05	0.66
Butyric	0.07	0.04	0.01	0.06	0.05	0.01	< 0.01	0.19
Isobutyric	0.01	0.04	0.03	0.02	0.02	0.04	0.70	0.99
Isovaleric	0.03	0.04	0.04	0.03	0.03	0.04	0.92	0.97
d181								
Lactic	0.02	0.03	0.04	0.03	0.02	0.04	0.97	0.81
Acetic	0.26	0.25	0.01	0.24	0.25	0.01	0.17	0.56
Propionic	0.09	0.10	0.01	0.09	0.09	0.01	0.09	0.33
Butyric[c]	0.04	0.05	0.01	0.04	0.06	0.01	0.01	< 0.01
Isobutyric	0.01	0.01	0.02	0.01	0.01	0.02	1.00	1.00
Isovaleric	0.02	0.02	0.03	0.02	0.01	0.03	0.96	0.91

Diet[a]: CO: diet formulated including soybean meal as main protein source; TRT: diet with a reduced content of soy and a decreased CP level in the last feeding phase.
Acetic[b]: for acetic acid at d11 the interaction between diet and sex was significant ($p = .009$). TRT F *vs* TRT M, $p = .04$; CO M *vs* TRT M, $p = .01$.
Butyric[c]: for butyric acid at d181 the interaction between diet and sex was significant ($p = .02$). CO F *vs* TRT F, $p = .05$; CO F *vs* CO M, $p = .01$; CO F *vs* TRT M, $p = .03$.

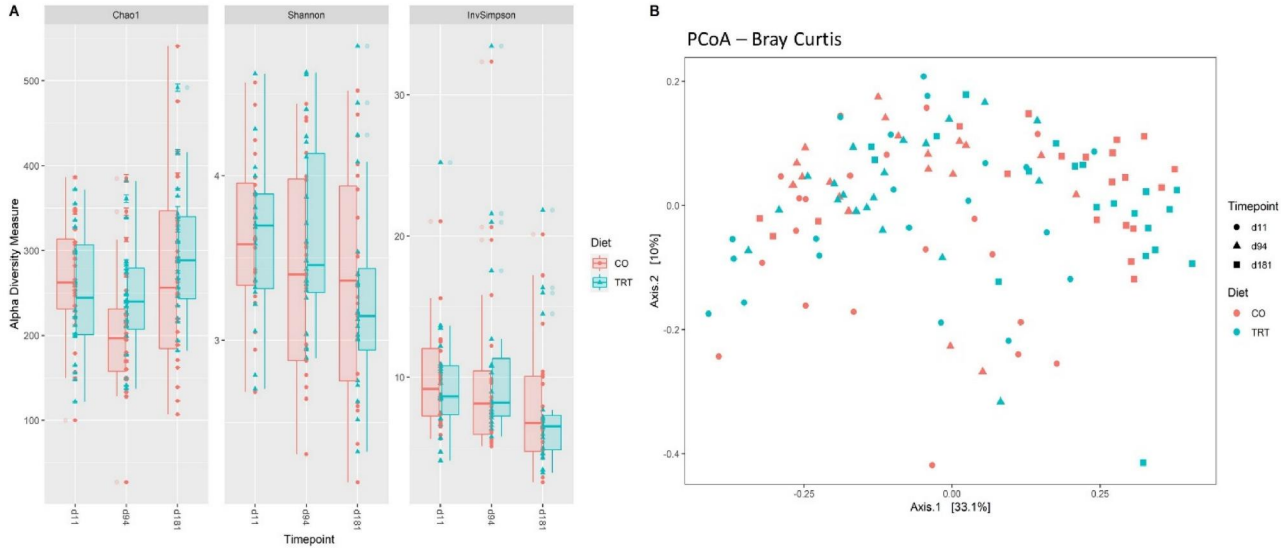

Figure 1. Effect of partial replacement of soybean meal with peas and sunflower meal and time on the alpha (A) and beta (B) diversity indices of faecal samples of growing-finishing heavy pigs. A: Chao1. Shannon and inverse Simpson (InvSimpson) indices of pigs faecal samples collected at days 11, 94 and 181. B: Principal Coordinates analysis plot (PCoA) were generated using a Bray Curtis distance matrix based on centred log-ratio transform transformed data. Diet: CO: diet formulated including soybean meal as main protein source; TRT: diet with a reduced content of soy and a decreased CP level in the last feeding phase.

d94 and d181. The interaction between diet and time, diet and sex were never significant for any of the alpha indexes. The time influenced the Shannon index ($p = .03$; d11 vs d181,3.61 vs 3.29; $p = .04$) and tended to influence the InvSimpson index ($p = .06$; d94 vs d181, 10.8 vs 8.0; $p = .06$). The Adonis test analysis was not affected by diet and time while time had a significant effect ($R^2 = 0.15$; $p = .001$) on the Bray Curtis matrix. Figure 1B shows the PcoA plot in which any potential cluster due to diet cannot be observed while it can be observed the clusterization of samples due to time.

To identify specific bacterial markers that were differentially expressed between diets, the LDA effect size analysis was conducted at each timepoint and results are reported in Figure 2. At d11, the CO diet was characterised by a higher abundance of

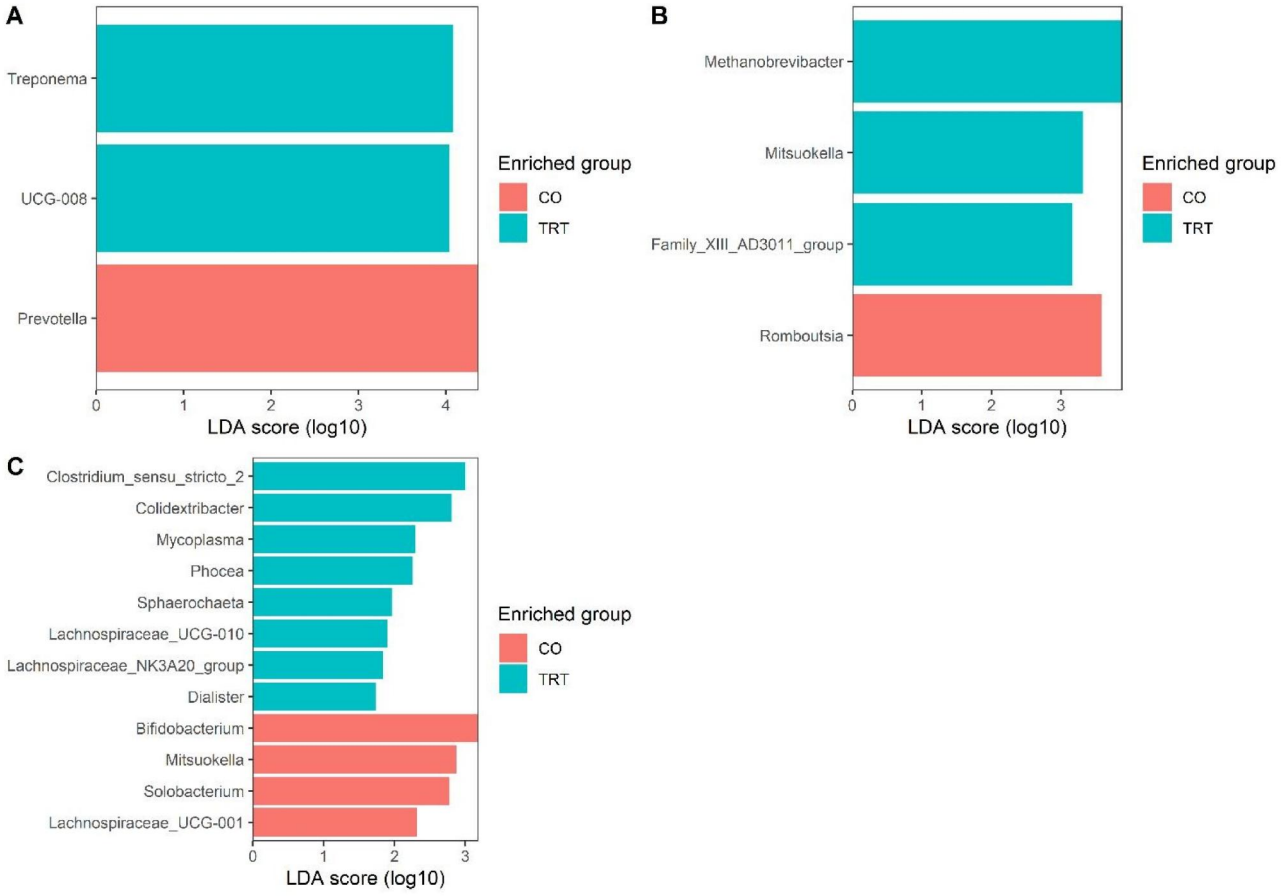

Figure 2. Effect of partial replacement of soybean meal with peas and sunflower meal on microbial biomarkers at genus level in pigs' faecal samples collected at days 11 (A) 94 (B) and 181 (C). LEfse = linear discriminant analysis effect size analysis; LDA score: Linear discriminant analysis score. Diet: CO: diet formulated including soybean meal as main protein source; TRT: diet with a reduced content of soy and a decreased CP level in the last feeding phase.

Prevotella (Linear Discriminant Analysis (LDA) score- = 4.36, *p*adj. = 0.005) and the TRT diet was characterised by higher abundance of *Treponema* (LDA score = 4.08, *p*adj = 0.028) and UCG-008 belonging to *Butyricicoccus* (LDA score = 4.03, *p*adj = 0.002). At d94, the CO diet was characterised by higher abundance of *Romboutsia* (LDA score = 3.58, *p*adj = 0.04) and the TRT diet was characterised by higher abundance of *Methanobrevibacter* (LDA score = 3.87, *p*adj = 0.013), *Mitsuokella* (LDA score = 3.31, *p*adj < 0.001) and Family_XIII_AD3011_group (LDA score = 3.16, *p*adj = 0.04). At d181, only one genus reached an LDA score higher than 3; the *Bifidobacterium* (LDA score = 3.17, *p*adj = 0.04) genus which was higher in the CO diet. Other genera had a significant *p*-value but LDA score ≤ 3; the CO diet was characterised by higher abundance of *Mitsuokella* (LDA score = 2.87, *p*adj. = 0.03), *Solobacterium* (LDA score = 2.77, *p*adj = 0.01) and Lachnospiraceae_UCG-001 (LDA score = 2.31, *p*adj = 0.05) and the TRT diet was characterised by higher abundance of Clostridium_sensu_stricto_2 (LDA score = 3.00, *p*adj = 0.001), Colidextribacter (LDA score = 2.80, *p*adj = 0.02), *Mycoplasma* (LDA score = 2.29, *p*adj = 0.013), *Phocea* (LDA score = 2.25, *p*adj = 0.006), *Sphaerochaeta* (LDA score = 1.96, *p*adj = 0.03), Lachnospiraceae_UCG-010 (LDA score = 1.89, *p*adj = 0.04), Lachnospiraceae_NK3A20_group (LDA score = 1.83, *p*adj = 0.03) and *Dialister* (LDA score = 1.73, *p*adj = 0.04).

Cortisol and dehydroepiandrosterone concentration on hair

Table 6 shows the effect of diet and sex on the concentration of cortisol and DHEA and their ratio of pig's hair. Diet and sex did not affect the hormones' concentration at any timepoints.

Animal based measurements

Table 7 shows the effect of diet on the behavioural and health indices observed during the study. The

Table 6. Effect of partial replacement of soybean meal with peas and sunflower meal on body weight and average daily gain of growing and finishing Italian heavy pigs and sex on hair' cortisol and dehydroepiandrosterone concentration of growing and finishing Italian heavy pigs.

	Diet[a]			Sex			*p*-value	
Item	CO	TRT	SEM	F	M	SEM	Diet	Sex
d94								
Cortisol	10.60	10.40	2.73	10.80	10.30	2.34	0.95	0.85
DHEA	27.90	33.60	4.26	35.60	26.40	4.39	0.34	0.13
Cortisol/DHEA	0.38	0.31	0.10	0.30	0.38	0.09	0.66	0.42
d181								
Cortisol	12.18	9.55	4.30	12.00	9.67	3.35	0.66	0.40
DHEA	24.60	21.80	5.60	25.30	21.10	5.74	0.73	0.60
Cortisol/DHEA	0.50	0.44	0.22	0.48	0.46	0.19	0.87	0.94

Diet[a]: CO: diet formulated including soybean meal as main protein source; TRT: diet with a reduced content of soy and a decreased CP level in the last feeding phase.
DHEA: Dehydroepiandrosterone

health indices refer to ears and tail's injuries, expressed by the LSI, as mentioned above. At d11, a trend ($p = .10$) for a higher LSI on tails was observed in the TRT group. At d34, the LSI on ear tended to be higher in the TRT group ($p = .06$) while the LSI on tails tended to be lower in the TRT group ($p = .07$). At d102, the dietary treatment had a significant effect on the LSI on tails ($p = .03$), resulting higher in the CO group. No other differences in the LSI, neither for ears nor for tails, were observed between the two groups at the other timepoints.

Regarding the behavioural indices, at d11 a higher percentage of pigs in the CO group showed the behaviour of exploring the enrichments ($p < .01$). At d70, the CO group tended to have a higher percentage of pigs having the other activities behaviour ($p = .08$). At d138, the TRT group had a higher percentage of pigs showing positive social interactions ($p = .04$), while the CO group tended to have a higher percentage of pigs exhibiting exploratory behaviours with enrichments ($p = .08$). No other significant differences in the behavioural were observed between the two groups at the other timepoints.

Environmental parameters

Figure 3 shows the effect of diet, timepoint and their interaction on the environmental parameters recorded in the selected pens. The interaction between diet and timepoints ($p < .0001$), diet ($p < .0001$) and timepoint ($p < .0001$) influenced the carbon dioxide (CO_2) concentration. The contrasts showed that CO_2 was lower in the TRT pens than CO pens at d11, but higher at d34 and d70. The NH_3 was influenced by the interaction between diet and timepoint ($p < .0001$), diet ($p < .0001$) and timepoint ($p < .0001$). The contrasts showed that the NH_3 concentration was higher in the TRT pens than in CO pens at d11, d27, d34, and d70. Both CO_2 and NH_3 had a linear decrease in their concentration with time (linear effect $p < .0001$).

Discussion

The present study showed that the partial replacement of SBM with sunflower and pea meal combined with the CP restriction in the last feeding period did not have a negative impact on the performance and health of fattening pigs. Replacing imported SBM is an important objective to be achieved in Europe as it is well known that its use in pigs' diets can significantly contribute to increasing the environmental impact, both directly and indirectly. This is particularly relevant for Italian pig production, which is largely focused on PDO ham production, where pigs are reared for at least 9 months until an average slaughter BW of 180 kg. In fact, a recent study highlighted that the feed is the first factor that contributes to the environmental impact of heavy pig production and the finishing phase plays the most relevant role (Berton et al. 2024).

One possible strategy to address this issue is to increase the use of local feeds like sunflower and pea meal, both of which are interesting protein sources for the EU and are authorised for feeding the pigs reared for the Italian PDO production. However, little data is available on the combination of these protein sources in pig diets. With regard to peas, a number of studies observed no negative effect on the growth performance of growing-finishing pigs up to 30% inclusion, when the diet was balanced for AAs (Tuśnio et al. 2017; Degola and Jonkus 2018; Lombardi et al. 2020; Argemí-Armengol et al. 2022). On the contrary, other studies reported that the complete replacement of SBM with peas and faba beans improved the weight gain of pigs and did not affect the palatability of the diet (White et al. 2015; Argemí-Armengol et al. 2022). On the other hand, literature regarding the effect of sunflower meal

Table 7. Effect of partial replacement of soybean meal with peas and sunflower meal on body weight and average daily gain of growing and finishing Italian heavy pigs on behaviours and injuries' prevalence of rowing and finishing Italian heavy pigs.

	Diet[a]			
Item	CO	TRT	SEM	*p*-value Diet
d11				
Lesions				
LSI ears	56.30	60.80	8.44	0.72
LSI tail	34.10	68.20	13.50	0.10
d34				
Behaviour, %				
Rest	40.60	53.80	5.89	0.15
Suckling	0.00	0.00	0.00	1.00
Positive interaction	5.58	4.38	0.96	0.40
Negative interaction	1.44	1.62	0.44	0.78
Enrichment investigation	7.56	1.99	1.22	<0.01
Pen exploration	43.80	34.30	7.02	0.36
Other activities	0.97	3.84	1.21	0.12
Lesions				
LSI ears	59.20	91.10	10.90	0.06
LSI tail	55.30	22.90	11.60	0.07
d70				
Behaviour, %				
Rest	58.90	68.70	6.14	0.29
Suckling	0.16	0.88	0.39	0.22
Positive interaction	2.54	3.81	1.45	0.55
Negative interaction	3.13	0.99	0.98	0.16
Enrichment investigation	1.21	1.21	0.47	0.99
Pen exploration	23.40	18.40	4.13	0.42
Other activities	10.72	6.03	1.72	0.08
Lesions				
LSI ears	69.30	74.60	6.95	0.60
LSI tail	5.74	6.03	2.56	0.93
d102				
Behaviour, %				
Rest	74.90	82.60	3.42	0.14
Suckling	0.17	0.79	0.46	0.35
Positive interaction	4.92	3.12	1.04	0.25
Negative interaction	0.89	0.89	0.52	0.99
Enrichment investigation	2.16	1.63	0.88	0.68
Pen exploration	12.20	7.50	2.48	0.21
Other activities	4.79	3.51	0.97	0.37
Lesions				
LSI ears	56.50	41.70	7.24	0.17
LSI tail	29.90	15.80	3.89	0.03
d138				
Behaviour, %				
Rest	69.90	73.90	4.73	0.56
Suckling	0.16	0.00	0.11	0.34
Positive interaction	0.79	5.26	1.36	0.04
Negative interaction	1.33	2.03	1.40	0.73

Diet[a]: CO: diet formulated including soybean meal as main protein source; TRT: diet with a reduced content of soy and a decreased CP level in the last feeding phase.
LSI: Lesion Score Index

inclusion is rather controversial. Indeed, there are studies in which a decrease in ADG and G:F was observed in pigs fed with 22%–59% of sunflower meal compared to those fed with SBM (Shelton et al. 2001; Povod et al. 2022). On the contrary, other studies found no difference in growth performance in growing and heavy pigs fed diets including sunflower meal (Cortamira et al. 2000; Mordenti et al. 2012; Trombetta and Mattii 2005; Povod et al. 2021).

In the present study, the replacement of 31.4% of SBM led to a decrease in ADG and G:F and an increase in ADFI during the growing phase (from d11 to d94). In the fattening period (reduction of SBM by approximately 68%) and during the whole experiment (56% of the average reduction of SBM) the ADG and the G:F of the growing and fattening pigs were improved by SBM reduction.

During the second feeding phase, the diets of the two experimental groups differed not only in their protein sources but also in their energy sources for economic issue: specifically, the CO diet included maize, whereas the TRT diet comprised sorghum and wheat meals. Taking this into account it could be possible that from d29 to d94 the presence of sorghum in the diet may have masked the beneficial effects on pigs' performances of the TRT diet observed in the subsequent period. In fact, Shelton et al. (2004) reported a significant decrease in feed conversion efficiency of growing pigs of fed a sorghum based diet relative to corn based diet. On the other hand, Pan and An (2020) shown no significant difference in ADG of growing pigs fed with a corn or sorghum based diet in their trial. Probably because the diets were balanced according to standard ileal digestible AAs and net energy, the negative effect was not observed on the growth performance of the pigs.

Furthermore, the contradictory results observed in the two periods could be related to the production period and the different digestive efficiency of the pigs. Indeed, the stress related to the transport and housing in the growing unit as well as the adaptation to the sunflower-pea diet, may have limited the growth capacity of the pigs (Kerr et al. 2005). It is largely reported that sunflower meal has greater insoluble fibre contents and thus lower digestibility compared with SBM, while pea has a comparable digestibility to SBM, especially for the protein fraction (Nørgaardet al. 2012; Lannuzel et al. 2022). However, as mentioned above, these raw materials are not clearly associated with a reduction in performance and results may depend on the inclusion rate used in the different studies, genetic factors and the growing phase.

Apart for digestibility and growth, modification of the diet can affect the animal behaviour, due to the different digestion kinetics that can affect the sense of satiety. Anyway, in this study, despite the higher level of insoluble fibre in the TRT group due to the inclusion of the sunflower meal, the diet only slightly affected the animal behaviour. An effect has been observed just in the last feeding phase, with lower tail LSI and high positive behaviour characterising the TRT

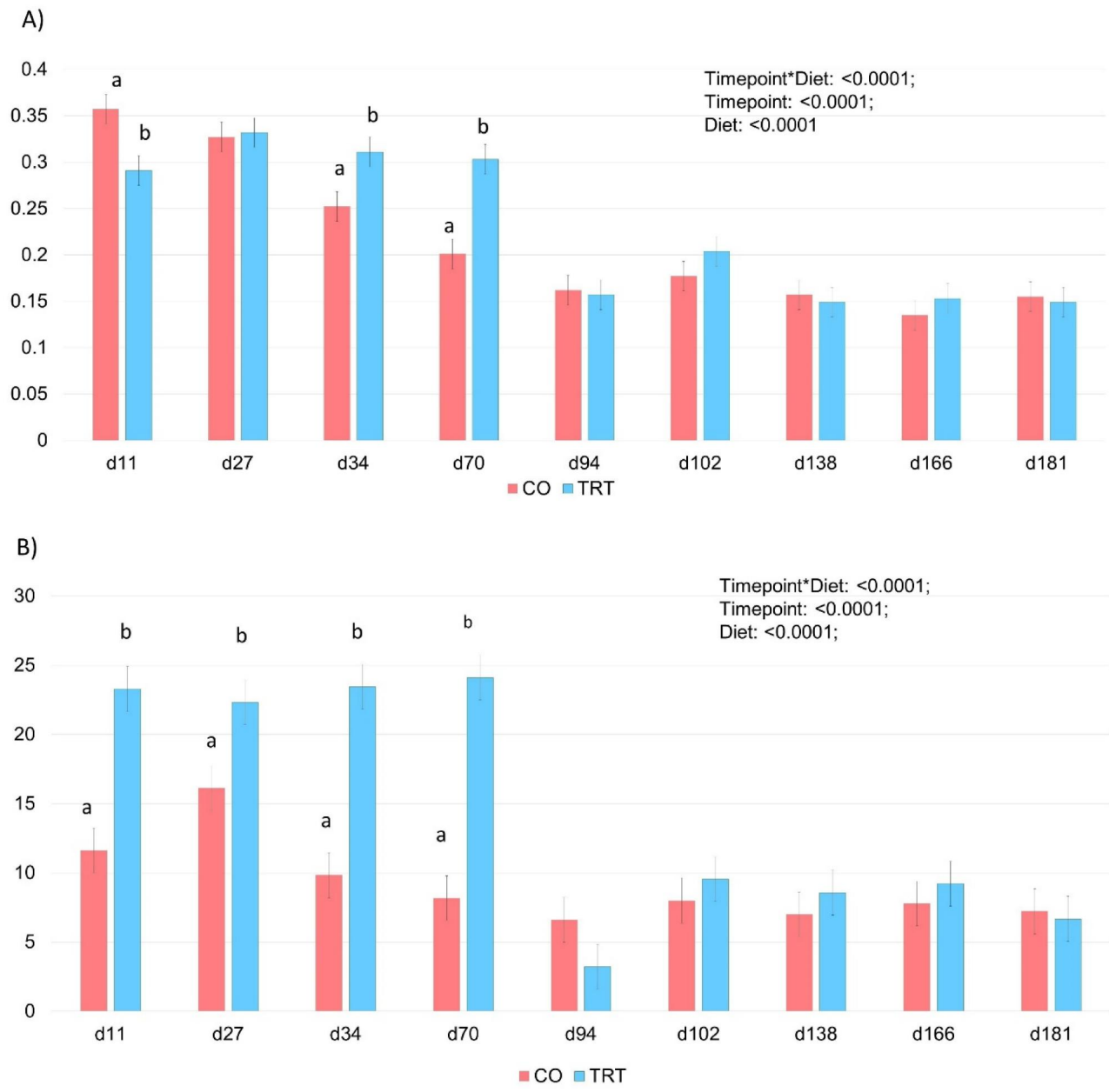

Figure 3. Effect of partial replacement of soybean meal with peas and sunflower meal on carbon dioxide (A) and ammonia (B) concentration in pens of growing and finishing Italian heavy pigs. Statistics and relative means of the environmental parameters recorded in the pens where the ear-tagged animals were housed as a function of diet and time. The superscript letters next to the result represent the statistically significant difference ($p < .05$) between the CO and TRT groups at the same timepoint. Diet: CO: diet formulated including soybean meal as main protein source; TRT: diet with a reduced content of soy and a decreased CP level in the last feeding phase.

group animals. To our knowledge, no data are reported from other authors on these parameters. In addition to the modification of the protein sources, in this study, in the last feeding phase (120–180 kg), the diets were differentiated for the CP level but not for the Lys level (CO: CP = 12.5% – Lys = 0.7%; TRT: 11.3% – Lys = 0.68%) and no detrimental effect was observed on the performance. These data confirm those of Gallo et al. (2014) for the Italian heavy pigs, where they suggested a CP and Lys level of 10.8% and 4.8 g/kg, respectively, for the same growing phase, highlighting the possibility of a higher restriction of dietary nitrogen in the finishing diet, even in diets with a high restriction in SBM. Finally, the lack of difference in cortisol and DHEA concentrations, which are considered sensitive biomarkers for animal welfare (Pollock et al. 2021), allows the exclusion of a long-term distress caused by the dietary changes.

Despite the manipulation of the protein sources as well as the reduction of the CP level, the faecal NH_3

did not differ between the groups. It has been reported also that nitrogen emission from the manure of pigs is affected by the use of cereal types (Kaufmann 2015). In a study of growing pigs, conducted by Pan et al. (2017), nitrogen utilisation of sorghum based diets was decreased by increasing manure nitrogen output compared with that of corn based diets. However, it has not been demonstrated if the disadvantages observed in sorghum-based diets could be reversed in wheat-based diets. However, this difference does not appear to have influenced the NH_3 content in the faeces of the animals under examination.

However, faecal NH_3 alone may not provide a comprehensive assessment of nitrogen retention efficiency in pigs. Other direct or indirect measures, such as urinary NH_3 and blood urea (Kohn et al. 2005), should be considered in further studies to assess protein synthesis efficiency accurately.

In this study, we measured the concentration of some gases at the pen level to assess the possible implication of the diet on the farm environment. During the initial phase of the trial, the pens housing the pigs from the TRT group exhibited higher NH_3 concentrations compared to those of the CO group. The reduced feed efficiency of the TRT group during the initial phase could be correlated with this observation (Monteiro et al. 2021). However, this negative aspect diminished over time, and no further differences were observed between the TRT and CO groups in the subsequent phases when feed efficiency was higher in the TRT group than in the CO group. The lack of difference in the latter phase could be ascribed to the general reduction of NH_3 detected in the latter phases. In fact, the environment's NH_3 and CO_2 concentration exhibit a linear reduction over time for both groups. This improvement in air quality for both groups is likely to be due to a reduction in the dietary CP levels from the first to the last feeding phase, and to the fact that the facility's ventilation was strictly natural, and with the onset of warmer months, there was increased air circulation.

Feeding different diets is very likely to affect the gut microbiota of pigs (Trevisi et al. 2021). Nevertheless, a clear difference in clustering samples based on bacterial composition did not indicate distinct patterns or groups of samples associated with different experimental groups, the samples primarily clustered according to sampling time, confirming that time is an important factor in modulating the gut microbiota (Trevisi et al. 2021). Anyway, the effect of time in modulating the gut microbiota is not the target of this manuscript. Taking into account the effect of the diet, the modulation exerted by different protein sources and dietary level of CP can have a selective effect on the gut microbial taxa. In fact, in this study, the diet influenced the abundance of some specific taxa which can play an important role on pigs' health but it did not influence the alpha and beta diversity indices. The LDA effect size analysis revealed that at d11, the CO group was characterised by higher abundance of *Prevotella*, while the TRT group was characterised by higher abundance of *Treponema* and *Butyricicoccus*. This latter result would suggest an increase in butyrate in the TRT group (Trachsel et al. 2018), while an increase in butyrate was observed in the CO group. However, the results in terms of VFAs can also be attributed to other genera. In fact, based on the literature, both *Prevotella* and *Treponema* are two of the main genera that characterise pigs' microbial enterotypes, which can affect the VFAs production (Ke et al. 2019; Amat et al. 2020; Chen et al. 2021). Furthermore, results from the present study agree with the literature, which reports that *Prevotella* is known to co-exclude the *Treponema* enterotype (Ramayo-Caldas et al. 2016; Yang et al. 2018). Moreover, *Prevotella* is positively associated with the production of propionic acid, which was significantly higher in the CO group (Sebastià et al. 2023). Furthermore, our findings confirm previous observations linking members of the *Prevotella* genus to favourable outcomes in pig farming, such as improved growth performance and immune response (Amat et al. 2020). On the other hand, the predominance of *Treponema* in the TRT group could be associated with the different fibre sources present in the diets as suggested by studies in human beings (De Filippo et al. 2010) and pigs (He et al. 2023). In addition, at d94, the end of the second feeding phase, where the inclusion of pea and sunflower meals was higher than in the other feeding periods, differences also emerged between the groups. In the CO group, the genus *Romboutsia* become predominant, while in the TRT group *Methanobrevibacter*, *Mitsuokella* and Family_XIII_AD3011 (Anaerovoracaceae family) were the most abundant. Among the aforementioned genera, even if *Methanobrevibacter* abundance is common in finishing pigs, it seems to be promoted by dietary fibre like thus from pea in pigs (Luo et al. 2017; Mi et al. 2019). Methanogens play an important role in maintaining the balance of the gut microbiome (Mi et al. 2019) and are also a significant contributor to energy production (Camara et al. 2021). Indeed, dietary fibre is a complex and heterogenous nutrient that, depending on its characteristics can affect microbiota composition

due to its different degradability (He et al. 2023). Finally, at d181, the faecal samples from the TRT group had a higher concentration of propionic and butyric acids. In particular, in our study, females in the CO group had significantly lower butyric acid faecal concentrations than females in the TRT group and males in both groups. Other studies conducted in humans have shown higher butyric acid concentrations in human males than in human females (Cui et al. 2021). These gender differences were associated with a stronger, possibly masking, effect from differential intake of macronutrients, expecially carbohydrates (Teixeira et al. 2013). On the basis of this finding, it could be assumed that females in the TRT group utilised nutrients more efficiently than females receiving the CO diet. Butyric acid has health-promoting physiological functions in the gut and both propionic and butyric acid serve as an energy source for the gut epithelium and thus play a significant role in gut health (Liang et al. 2021). At the same time, it cannot be concluded that the TRT diet improved the intestinal health of the pigs. Firstly because, once again at d181, the CO group had a high abundance of *Bifidobacterium*, which is known to be an antagonist of pathogenic micro-organisms and therefore helps to preserve intestinal health (Homma, 1988), while the TRT group was characterised by a high abundance of *Clostridium* sensu stricto. Zhu et al. (2018) found that *Clostridium* sensu stricto had a positive correlation with diarrhoea in piglets, but at the same time it was closely related to the animals' ADFI as in our study. In growing pigs, the presence of this genera can be related with the highest fermentative capacity in the hindgut and not forcely is no evidence highlighted an association with impairment in adult pigs. In this study, the results do not allow us to determine if the observed effects are truly and exclusively related to the replacement of soybean meal. In fact, as previously mentioned, during the second feeding phase, SBM was not the only ingredient that was replaced. Nonetheless, studies demonstrated that the replacement of maize by the sorghum in diets did not influence the microbiota community based on alpha and beta diversity in caecal and colonal digesta of piglets (González-Ortiz et al. 2020; Pan et al. 2021).

Conclusions

In conclusion, the present study showed that replacing an average of 56% of SBM with sunflower and pea meals, while reducing CP, did not adversely affect the performance and health of fattening pigs. This finding is particularly important given the importance of Italian pig production, which is oriented on PDO ham production, and the need to reduce the environmental impact associated with pig diets. The pig industry can reduce its environmental footprint by adopting feeding strategies that include locally sourced proteins. An LCA analysis could be carried out to accompany this kind of research and to combine the findings with the impact of feeding strategies on pig performance and health.

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Ethical 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 (Trial ID 4525, Prot. n. 130114 – 5th May 2023).

Disclosure statement

No potential conflict of interest was reported by the Authors.

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ORCID

Diana Luise http://orcid.org/0000-0001-7864-7822
Roxana Elena Amarie http://orcid.org/0000-0002-0534-1803
Federico Correa http://orcid.org/0000-0001-8558-6503
Alberto Elmi http://orcid.org/0000-0002-7827-5034
Sara Virdis http://orcid.org/0000-0002-4251-3328
Clara Negrini http://orcid.org/0000-0003-0895-0070
Francesco Palumbo http://orcid.org/0000-0001-7817-2361
Giacomo Biagi http://orcid.org/0000-0001-9078-5224
Maria Laura Bacci http://orcid.org/0000-0001-6702-5868
Nadia Govoni http://orcid.org/0000-0003-1639-7121
Andrea Serra http://orcid.org/0000-0001-7728-3430
Paolo Trevisi http://orcid.org/0000-0001-7019-6828

Data availability statement

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

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