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A novel low-protein diet feeding strategy with reduced amylose/amylopectin ratio: effects on growth performance, gut health, and behaviour in nursery pigs

Juho Lee [a], Federico Correa [a], Luca Laghi [b], Silvia Bencivenni [a], Daniele Bigi [a], Giacomo Biagi [c], Francesco Palumbo [a], Paolo Trevisi [a], Diana Luise [a,*]

[a] Department of Agricultural and Food Sciences, University of Bologna, Viale G. Fanin 44, 40127, Bologna, Italy
[b] Department of Agricultural and Food Sciences, University of Bologna, Piazza Goidanich 60, 47521, Cesena, Italy
[c] Department of Veterinary Medicine, University of Bologna, Via Tolara Di Sopra 50, 40064, Ozzano Dell'Emilia, Italy

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ABSTRACT

Reduction in dietary crude protein (CP) levels is recognized to potentially limit piglet growth. Synchronising blood amino acids and glucose peaks may improve the performance of piglets fed low-CP diets. This study aimed to evaluate the effects of a feeding plan with a low-CP diet and a reduced amylose to amylopectin (AM/AP) ratio, in the second phase, on performance, faecal microbiota and metabolites, and behaviour of nursery pigs. A total of 540 nursery pigs (28 ± 2 d) were fed either a medium-CP (CTR:16.6%, 180 pigs) or low-CP (LCP: 14.9%, 360 pigs) diet during phase 1 (d0 to 21), and then assigned to one of three phase 2 diets (d 21 to 63): medium-CP (CTR: 17.2%;180 pigs), low-CP with normal AM/AP ratio (LP: 15%; 0.17, 180 pigs), or low-CP with reduced AM/AP ratio (LPLA:15%; 0.08, 180 pigs). On d43 and 63, LP and LPLA pigs showed lower body weight than CTR pigs ($P < 0.0001$). No differences in feed to gain (F:G) were observed between CTR and LPLA from d43 to 63. The LPLA and LP diets had lower faecal alanine and proline concentrations than CTR at d48 ($P < 0.05$). The beta diversity of faecal microbiota was affected by the diet at d43 and d63 ($P < 0.01$); at d63, the LPLA diet was characterised by a higher abundance of *Lactobacillus* ($P < 0.05$) and *Limosilactobacillus* ($P < 0.05$). During the late phase of post-weaning, reducing the AM/AP ratio may help improve feed efficiency in pigs fed low-CP diets.

1. Introduction

In pig production, post-weaning diarrhoea (PWD) is one of the most common health issues, causing economic loss. Recently, reducing protein levels of nursery pig diets has garnered considerable attention as a strategy to prevent PWD. Low crude protein (CP) diets have been reported to alleviate gut disorders by decreasing the fermentation of undigested proteins which produce toxic substrates, such as polyamines and phenolic compounds (Luise et al., 2021). Moreover, nursery pigs fed low-CP diets show reduced nitrogen excretion, thereby contributing to a lower environmental footprint of the livestock industry (Wang et al., 2018). Supplementation with feed-grade amino acids (AAs) allows a reduction in dietary CP levels while minimising growth retardation in nursery pigs (Wang et al., 2018).

However, a prolonged lowering in dietary CP level to below 15% for more than three weeks reduces the growth performance of nursery pigs, even when feed-grade AAs are supplemented to meet AAs requirements (Spring et al., 2020). This may be attributed to characteristics of feed-grade AAs. Unlike the AAs from complex protein sources, feed-grade AAs are rapidly absorbed from the intestinal tract to the bloodstream (Eugenio et al., 2022). The absorbed free AAs contribute to protein synthesis; however, to optimise the efficiency of their utilisation, the peak in circulating AAs needs to coincide with an insulin spike induced by increased glucose levels (Eugenio et al., 2023; Liu & Selle, 2017). In pigs, glucose is mainly derived from starch digestion, which generally proceeds more slowly than the absorption of feed-grade AAs (Eugenio et al., 2023). This asynchrony facilitates the catabolism of free AAs through hepatic deamination, thereby resulting in suboptimal AAs utilisation (Eugenio et al., 2023). As a strategy to alleviate the asynchrony between circulating free AAs and insulin concentrations, the inclusion of rapidly digestible starch in the diet has been proposed (Liu & Selle, 2017). From the third week after weaning, when pigs show

* Corresponding author.
E-mail address: diana.luise2@unibo.it (D. Luise).

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rapid growth (Colson et al., 2006), the implementation of this strategy may help alleviate growth retardation associated with low-CP diets by improving the bioavailability of AAs.

Starch consists mainly of two polysaccharides, amylopectin (AP) and amylose (AM). In the small intestine, AP is degraded more rapidly by α-amylase than AM, due to its highly branched structure, which provides more accessible sites for enzymatic activity (Zhou et al., 2022). Because of this property, using starches rich in AP has been suggested to reduce AM/AP ratio and thereby promote a rapid increase in blood glucose levels (Martens et al., 2018). In our companion study, a low-CP diet formulated with waxy corn, characterised by a high level of AP, to lower the AM/AP ratio reduced diarrhoea incidence and promoted faecal microbial diversity in nursery pigs, although this was observed during the first four weeks post-weaning, without adversely affecting growth performance (Correa et al., 2024). These findings indicate the potential of reduced AM/AP ratio in low-CP diets and encourage additional investigation into the physiological and metabolic mechanisms of their beneficial effects. Since low-CP diets are linked to both heightened incidence of abnormal behaviours (McAuley et al., 2022) and decreased nitrogen excretion of pigs (Canh et al., 1998), the comprehensive metabolomic profiling and an investigation of animal behaviour parameters could contribute to a deeper understanding of the mechanism behind this dietary strategy.

The study by Correa et al. (2024) was conducted under experimental conditions; it was not possible to effectively assess the impact of this approach on animal behaviour or on possible lesions resulting from aggressive interactions, which are known to occur under commercial conditions. In addition, in the previous study, the experimental diets included cooked cereals, which could enhance the synchronisation between plasma glucose and AAs. Therefore, further evaluation of the functional potential of waxy corn while simultaneously reducing the inclusion of cooked cereals to isolate its effect is needed.

It is hypothesised that a correctly formulated feeding plan, based on CP reduction and AAs supplementation until the period preceding rapid growth (three weeks post-weaning), followed by a strategy maintaining the same low-CP level but with a reduced AM/AP ratio using waxy corn during the remaining nursery period, can maintain the health and performance of piglets without altering their behaviour.

This study aimed to evaluate a comprehensive feeding plan for the post-weaning phase based on a low-CP diet. The protocol comprised two phases. The first phase (d0 to 21) aimed to improve intestinal health by reducing dietary CP. The second phase (d21 to 63) still involved a low-CP diet while improving the synchronisation between AAs and glucose availability by lowering the AM/AP ratio. The feeding plan's effectiveness was assessed using parameters such as intestinal health (faecal microbiota and metabolome), growth performance, behaviour, and lesion occurrence in pigs.

2. Materials and methods

2.1. Animal care

This study was conducted at a commercial pig farm in Italy from November 2024 to January 2025. The experimental procedures were reviewed and approved by the ethics committee of the University of Bologna (Protocol ID 218339/2024).

2.2. Animals and experimental design

At weaning (28 ± 2d old; d0), a total of 540 pigs {(Landrace × Yorkshire) × Duroc} were transferred from the suckling unit to a weaning commercial farm. The pigs had their tails docked. Immediately after arrival, the pigs were individually weighed and ear-tagged, after which they were subjected to a two-phase feeding plan: phase I from d0 to 21 post-weaning; and phase II from d21 to 63 post-weaning. In phase I, piglets were divided into two groups balanced for body weight (BW) and sex: CTR= *a* standard diet with a medium CP content (16.6% CP) including 180 piglets (5 pens; 36 piglets per pen); LCP= *a* low-CP diet (14.9% CP) including 360 piglets (10 pens; 36 piglets per pen). In phase II, piglets were divided into three groups balanced for BW and sex: CTR= the piglets of group CTR in phase I (180 piglets; 5 pens; 36 piglets per pen) continued to receive a standard diet with a medium CP content (17.2%) and normal AM/AP (0.12); LP= half of the piglets from LCP group in phase I (180 piglets; 5 pens; 36 piglets per pen) received a low-CP diet (15%) with normal AM/AP (0.17) ratio; LPLA= half of the piglets from LCP group in phase I (180 piglets; 5 pens; 36 piglets per pen) received a low-CP diet (15%) with lower AM/AP (0.08) ratio. The LPLA diet was formulated with waxy corn, a corn cultivar approved for use as an animal feed ingredient. A starch of waxy corn used in this study consisted of approximately 98% AP. The ingredient and chemical composition of diets are shown in Table 1. Table 1 also includes the amount of AM, AP, and the AM/AP ratio for the experimental diets. The quantification of amylose and amylopectin contents of the feeds was performed according to the procedure described by Correa et al. (2024).

Each pen was equipped with two feeders and three water cups, and its flooring was partially slatted (45% plastic slatted; 45% metal slatted; and 10% solid). As enrichment material, a metal chain was provided. The temperature of the pigsty was controlled through automated ventilation. During the experimental period, all pigs had ad libitum access to feed and water.

2.3. Data and sample collections

Pigs were individually weighed at d0, 21, 43, and 63 post-weaning. Feed intake was measured through the difference between the total amount of feed supplied and the residual feed in the feeders. From these data, average daily gain (ADG), average daily feed intake (ADFI), and feed to gain ratio (F:G) were calculated. Piglet mortality was checked daily.

At d21, 43, and 63, a rectal swab was taken from 16 pigs per group for each day (144 samples in total) to analyse the microbial composition, dry matter, ammonia concentration, and time-domain nuclear magnetic resonance (NMR)-based metabolomics. In detail, four pens out of the five per group were randomly selected, and within each selected pen, four pigs with medium BW were randomly chosen. The same pigs were sampled at each time-point. These samples were immediately kept in dry ice after collection, transported to the laboratory, and subsequently stored at −80°C in a deep freezer.

2.4. Faecal ammonia and dry matter

The faecal samples were thawed, and 1 g of the faecal samples was then diluted in 9 ml of deionised water and vortexed. The mixture was then centrifuged for 10 min at 7000 RPM and 4°C, and the resulting supernatant was used to analyse the ammonia (NH_3) levels. Faecal NH_3 levels were assessed using an enzymatic colorimetric assay, following the manufacturer's instructions (Urea/BUN-Color; BioSystems S.A., Barcelona, Spain) and presented in μmol/g. In addition, 1 g of faecal sample was freeze-dried for 72 h, and the ratio of dried to wet faecal weight was used to determine the percentage of faecal dry matter (Palumbo et al., 2025).

2.5. Faecal NMR spectroscopic analysis

Metabolomic profiling of faecal samples for proton nuclear magnetic resonance (^{1}H-NMR) analysis following the procedure reported by Palumbo et al. (2025). Briefly, the samples were prepared by centrifuging 80 mg of each sample with 1 mL of deionized water at 18,630 × *g* for 10 min at 4 °C. Subsequently, 700 μL of the resulting supernatant was mixed with 100 μL of a D_2O solution containing 10 mM 2,2,3,3-D_4-3-(trimethylsilyl) propionic acid sodium salt (TSP), used as an internal standard for chemical shift referencing. The solution was buffered

Table 1
Composition of the experimental diets.

Item	Phase I: d0 to 21		Phase II: d21 to 63		
	CTR	LCP	CTR	LP	LPLA
Ingredients, %					
Wheat	18	20.5	12.5	14.2	20
Barley	18	19.3	18	24	20
Mixed wheat and oat flaked	17	17	13	13	5
Corn	15	15	25	25	-
Waxy corn	-	-	-	-	30.00
Wheat bran	4	4	5	5	5
Milk	4	4	4	4	4
Soybean meal (44% CP)	2.5	-	11	2.8	3.7
Soybean oil	2.5	3.2	2.5	2.8	3.1
Potato protein	2	2	2	2	2
Fish meal	2	15	2	2.2	2.2
Premix 1[1)]	15	-	-	-	-
Premix 2[2)]	-	-	5	-	-
Premix3[3)]	-	15	-	-	-
Premix 4[4)]	-	-	-	5	5
Nutrient composition, %					
Dry matter	88.94	88.94	88.44	88.4	88.38
Water	11.06	11.06	11.56	11.6	11.62
Crude protein	16.63	14.87	17.15	15.03	15.07
Crude lipid	5.54	6.04	5.49	5.61	5.83
Crude fiber	3.29	3.21	3.37	3.05	2.99
Starch	46.4	58.4	47.1	47.1	47.7
Amylose	6.8	7.8	5	6.7	3.6
Amylopectin	39.6	50.6	42.1	40.4	43.1
Amylose/amylopectin ratio	0.17	0.16	0.12	0.17	0.08
Sugar	6.48	6.46	4.79	4.66	4.8
Metabolic energy, kcal/kg	3298	3300	3295	3295	3300
Ash	4.8	4.65	5.13	4.72	4.72
Ca	0.55	0.55	0.6	0.58	0.58
P	0.59	0.58	0.6	0.58	0.58
Available P	0.55	0.54	0.53	0.52	0.52
Lysine	1.37	1.35	1.37	1.36	1.36
Lysine SID	1.21	1.21	1.21	1.21	1.22
Met + Cyst	0.84	0.84	0.85	0.85	0.84
Met + Cyst SID	0.73	0.72	0.73	0.73	0.73
Threonine	0.93	0.91	0.96	0.95	0.95
Threonine SID	0.78	0.78	0.8	0.81	0.81
Tryptophane	0.28	0.27	0.26	0.26	0.26
Tryptophane SID	0.23	0.23	0.22	0.22	0.22
Valine	0.92	0.91	0.92	0.9	0.9
Valine SID	0.77	0.77	0.76	0.76	0.76

Abbreviations: CTR= control group fed a standard diet with medium CP content and a normal AM/AP ratio (d0 to 21: 16.6% CP, 0.17 AM/AP; d21 to 63: 17.2% CP, 0.12 AM/AP), LCP= group fed a low-CP diet with a normal AM/AP ratio (d0 to 21; 14.9% CP, 0.16 AM/AP), LP= group fed a low-CP diet with a normal AM/AP ratio (d21 to 63: 15.0% CP, 0.17 AM/AP), and LPLA= fed a low-CP diet with a low AM/AP ratio (d21 to 63: 15.1% CP, 0.08 AM/AP).

[1)] Premix provided the following per kilogram of premix: 12.8% of lactose, 2.79% of Ca, 2.72% of P, 0.09% of Mg, 0.79% of K, 1.12% of Na, 2.16% of Cl, 4.22% of L-Lysine, 2.01% of L-Methionine, 0.53% of L-Cystine, 3.09% of Threonine, 0.91% of L-Tryptophan, 0.87% of L-Isoleucine, 0.58% of Histidine, 1.82% of L-Leucine, 2.00% of L-Valine, 6700 OTU of Phytase, 9999.95 UV of B-glucanase Rovabio, and 7333.3 UV of B-xylanase Rovabio.

[2)] Premix provided the following per kilogram of premix: 8.88% of Ca, 7.47% of P, 0.04% of Mg, 0.08% of K, 2.96% of Na, 6.9% of Cl, 7.93% of L-Lysine, 5.13% of L-Methionine, 0.04% of L-Cystine, 6.54% of L-Threonine, 1.40% of L-Tryptophan, 0.06% of L-Isoleucine, 0.04% of Histidine, 0.11% of L-Leucine, 2.04% of L-Valine, 20,000 OTU of Phytase, 30,000 UV of B-glucanase Rovabio, and 22,000 UV of B-xylanase Rovabio.

[3)] Premix provided the following per kilogram of premix: 12.8% of lactose, 3.13% of Ca, 2.89% of P, 0.08% of Mg, 0.77% of K, 1.12% of Na, 2.52% of Cl, 5.16% of L-Lysine, 2.35% of L-Methionine, 0.52% of L-Cystine, 3.57% of L-Threonine, 0.97% of L-Tryptophan, 0.86% of L-Isoleucine, 0.57% of Histidine, 1.79% of L-Leucine, 2.64% of L-Valine, 6700 OTU of Phytase, 9999.95 UV of B-glucanase Rovabio, and 7333.3 UV of B-xylanase Rovabio.

[4)] Premix provided the following per kilogram of premix: 8.86% of Ca, 7.47% of P, 0.03% of Mg, 0.06% of K, 2.95% of Na, 8.11% of Cl, 11.20% of L-Lysine, 6.3% of L-Methionine, 0.03% of L-Cystine, 8.48% of Threonine, 1.98% of L-Tryptophan, 0.04% of L-Isoleucine, 0.03% of Histidine, 0.08% of L-Leucine, 4.17% of L-Valine, 20,000 OTU of Phytase, 30,000 UV of B-glucanase Rovabio, and 22,000 UV of B-xylanase Rovabio.

to pH 7.00 ± 0.02 using 1 M phosphate buffer.10 μL of sodium azide (NaN_3, 2 mmol/L) was added to inhibit microbial growth. The mixture was then subjected to a second centrifugation under the same conditions.

^{1}H-NMR spectra were acquired at 298 K using a Bruker AVANCE™ III spectrometer (600.13 MHz, Bruker, Milan, Italy). To correct for baseline variations, the ^{1}H-NMR spectra were processed using the rolling ball algorithm, which is implemented in the R package 'baseline', followed by a linear adjustment to ensure that the baseline points were randomly distributed around zero. Variations in sample water content were addressed using probabilistic quotient normalisation (PQN). Resonance signals were assigned by comparing chemical shifts and multiplicities using the Chenomx software (Chenomx Inc., Canada, ver 8.3) by means of comparisons with Chenomx's (ver. 10) and HMDB's (release 2) databases. The absolute quantification of the molecules was carried out in a single reference sample, using TSP as the internal standard. The concentration of each molecule was determined from the area of one of its signals, obtained through global spectra deconvolution implemented in MestReNova software (Mestrelab Research S.L., Santiago de Compostela, Spain; version 14.2.0-26256). This was performed after applying a line broadening of 0.3 and adjusting the baseline using the Whittaker smoother procedure.

2.6. Faecal microbial composition

From a total of 144 faecal samples, bacterial DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, Ca, USA), according to the manufacturer's instructions. The concentration and purity of the isolated DNA were assessed using NanoDrop spectrophotometry (Fisher Scientific, 13 Schwerte, Germany) with absorbance ratios of 260/280 and 260/230. The V3-V4 region of the 16S rRNA gene was amplified using universal primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′), and this amplification was conducted with Platinum™ Taq DNA polymerase High Fidelity (Thermo Fisher Scientific, Italy). The amplicons were sequenced on the Miseq platform 300 dry mat platform (Illumina, San Diego, CA, USA). The 16S rRNA gene library was constructed and sequenced using the Miseq® Reagent kit V3-V4 on the MiSeq-Illumina® platform. The DADA2 pipeline was used to analyse microbiota, and taxonomy was assigned using the Silva database (release 138.1) as a reference. Primers were trimmed to a consistent length: forward reads were truncated at position 290 and reverse reads at position 220, in order to remove low-quality sequences. Alpha diversity indices (Chao1, Shannon, Simpson) and beta diversity index (Bray-Curtis distance matrix) were calculated using "phyloseq", "Vegan", "Adonis" and "DESeq2" packages.

2.7. Tail and ear lesions and behaviour observation

Tail and ear lesions were evaluated when the pigs were individually weighed. Lesion scoring and behavioural observations were performed by trained personnel following the criteria from Welfare Quality (2009) protocol. The same observers conducted all assessments using predefined criteria to ensure consistency in evaluation and to minimise the observer's bias. The scoring system is described below:

Tail lesion scores:

i. Score 0: no lesion
ii. Score 1: superficial scratch, but no swelling detected
iii. Score 2: a visible open lesion on the tail, evidence of scar, swelling or partial tail loss

Ear lesion scores:

i. Score 0: fewer than 4 lesions
ii. Score 1: 5 to 10 lesions
iii. Score 2: >11 lesions

To further clarify ear lesion scores, '1 lesion' indicated specific conditions such as a single scratch longer than 2 cm, a pair of parallel scratches with <0.5 cm between them, or a lesion smaller than 2 cm. '5 lesions' referred to cases with a bleeding lesion between 2 and 5 cm or a healed lesion exceeding 5 cm. 16 lesions indicated a deep and open lesion of longer than 5 cm.

The lesion score index (LSI) was calculated, considering both the frequency and severity of tail and ear lesions. The LSI ranged from 0 (no lesion) to 200 (all pigs with severe lesions), based on the following formula:

$$LSI = (\textit{percentage of pigs scored as } 1) + (2 \times \textit{percentage of pigs scored as } 2)$$

The behaviour of pigs in each pen was assessed directly between 10:00 a.m. and 12:00 pm. at d7, 14, 21, 28, 35, 43, 53, and 63 post-weaning. The observation was performed five times per pen at two-minute intervals, following the Welfare Quality (2009) protocol. Detailed criteria for the behaviour, including resting, abnormal, positive and negative interaction, enrichment investigation, pen exploration, and other activities are presented in Supplementary Table S1. The frequency of each behaviour was expressed as a percentage of the average number of animals exhibiting the behaviour within the pen across five observations.

2.8. Environmental parameters

Lux, oxygen, carbon dioxide, ammonia, hydrogen sulfide, and carbon monoxide were measured at d7, 14, 21, 28, 35, 43, 53, and 63 post-weaning at pig height (approximately 50 cm) at two distinct points located one meter to either side of the centre of each pen. The average level of the two measurements was calculated. All measurements were performed using the Dräger X-am® 8000 multi-gas detector (Drägerwerk AG & Co, Lübeck, Germany).

2.9. Statistical analysis

The statistical analysis of the data was performed using R Studio (v4. 3. 3) using the "car", "lsmeans", and "lme4" packages. To test the normality of the data, normal Q-Q plots were used for visual analysis of the distribution of the model residuals and to ensure adherence to the normality assumption. The Shapiro-Wilk test was also performed for quantitative analysis of the normality of the data.

For the analysis of the data on BW, ADG, faecal dry matter and ammonia levels, and ear and tail lesion scores and LSI, a linear mixed model followed by an ANOVA model was used, in which the diet (phase I: LCP vs CTR; phase II: CTR vs LP vs LPLA) was included as a fixed factor and the pen was included as a random factor. A linear model followed by an ANOVA model was fitted to the data for ADFI, F:G, the ear and tail lesion scores and LSI, and behavioural and environmental parameters, with the pen as a fixed effect. Piglet mortality, calculated at pen level, was analysed using a generalised linear model followed by an ANOVA model with a binomial distribution. For individual performance, pigs were the experimental unit, while for group performance, the pen was used as the experimental unit. 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. A post-hoc test was conducted with the Tukey-Kramer method to compare the means among diets.

The statistical analysis of data on metabolites was performed through MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/MetaboAnalyst/). The metabolomic datasets were normalised by sum, mean-centred, and scaled to unit variance by dividing each variable by its standard deviation to account for systematic differences among samples. After data normalisation, partial least squares-discriminant analysis (PLS-DA) was conducted at each time point, and variable importance in projection (VIP) scores were calculated for dietary effects. Metabolites with VIP scores >1.8 were further analysed using a linear mixed-effects model followed by the ANOVA model, applying the same fixed (diets) and random (pen) factors as those used for individual piglet performance (BW and ADG).

For the microbiota data, analyses of alpha diversity, beta diversity, and taxonomic differences were conducted in R (v 4. 3. 3) using the following packages: "phyloseq" v1.38, "vegan" v2.6, and the microbiomeutilities package v1.0. Data analysis on alpha diversity indices (Chao1, Shannon, and Simpson diversity) was performed using a linear mixed model followed by an ANOVA model, with diets as a fixed factor and the pen as a random factor. In addition, the depth of sequencing was included as a covariate in the model. For beta diversity, a dissimilarity matrix was constructed with a Bray-Curtis distance of centred log-ratio transformed data, and its results were presented using a Principal Coordinates Analysis plot. Differences were assessed through a PERMANOVA (Adonis) model with 9999 permutations, including diets as a factor. Taxonomic differences were expressed at the genus level with the LEfSe algorithm (LDA score > 3 and $P < 0.05$).

Data on BW, ADG, ADFI, F:G, faecal dry matter, ammonia concentrations, and lesion scores were analysed by dividing the treatments into two groups (CTR and LCP) for phase I, and into three groups (CTR, LP, and LPLA) for phase II. In contrast, faecal microbial and metabolite data were analysed in both phase I and II, considering three dietary groups (CTR, LP, and LPLA).

3. Results

3.1. Growth performance

Results on growth performance are shown in Table 2. At d21, the LCP diet (LP + LPLA) tended to have a lower BW compared to the CTR ($P = 0.054$). At d43 and 63, LP and LPLA pigs had significantly lower BW than CTR pigs ($P < 0.001$).

The LCP diet did not results in lower ADG compared to the CTR from d0 to d21 ($P = 0.160$), however, both LP and LPLA diets had a decreased ADG during the following periods: d21 to 43, 43 to 63, 21 to 63, and 0 to 63 ($P < 0.001$ for all).

The LCP diet did not have a different ADFI compared to the CTR from d0 to d21 ($P = 0.49$). The diet influenced the ADFI during the periods from d43 to 63 ($P < 0.01$), 21 to 63 ($P < 0.05$), and 0 to 63 ($P < 0.05$). The pairwise comparison showed that both LP and LPLA diets had lower ADFI during the periods d21 to 43 ($P < 0.01$) compared to the CTR diet, while during the periods from d43 to d63 and from d0 to 63, the LPLA had a lower ADFI than CTR ($P < 0.05$), while the LP diet had intermediate values.

The LCP diet tended to increase the F:G from d0 to d21 ($P = 0.051$). Both the LP and LPLA diets had an increased F:G during the period d21 to 43 ($P < 0.001$), 21 to 63 ($P < 0.001$), and 0 to 63 ($P < 0.001$) compared to the CTR diet. During the period from d43 to 63, only LP pigs showed higher F:G than CTR pigs ($P < 0.05$), while no differences were observed between LPLA and CTR and between LPLA and LP.

There was also no difference in the piglet mortality rate among groups throughout the entire experimental period.

3.2. Faecal dry matter and ammonia concentrations

Table 3 presents results for faecal dry matter and ammonia concentrations. At d21, the LCP diet increased faecal dry matter ($P < 0.05$) compared to the CTR diet. However, the diet had no significant effect on faecal dry matter at d43 and 63, irrespective of the CP level and AM/AP

Table 2
Effects of low-protein diets with different amylose/amylopectin ratios on growth performance of nursery pigs.

Items	Diet			SEM	*P*-value
	CTR	LP[1)]	LPLA[1)]		
BW, g[2)]					
d0	5878	5879		82.18	1.000
d21	8781^{x}	8490^{y}		132.87	0.054
d43	16111^{a}	14516^{b}	14235^{b}	325.86	<0.0001
d63	27983^{a}	23430^{b}	23090^{b}	613.99	<0.0001
ADG, g[2)]					
d0 to 21	138	124		6.02	0.160
d21 to 43	332^{a}	272^{b}	263^{b}	11.21	<0.0001
d43 to 63	577^{a}	427^{b}	432^{b}	20.89	<0.0001
d21 to 63	452^{a}	349^{b}	345^{b}	13.19	<0.0001
d0 to 63	349^{a}	276^{b}	271^{b}	9.75	<0.0001
ADFI, g[3)]					
d0 to 21	247	242		6.02	0.4983
d21 to 43	441	436	413	19.63	0.571
d43 to 63	932^{a}	780^{b}	764^{b}	29.92	0.003
d21 to 63	650^{a}	581^{ab}	563^{b}	20.06	0.028
d0 to 63	509^{a}	467^{ab}	454^{b}	14.26	0.044
F:G[3)]					
d0 to 21	1.80	1.97		0.06	0.051
d21 to 43	1.33^{b}	1.60^{a}	1.57^{a}	0.02	<0.0001
d43 to 63	1.63^{b}	1.83^{a}	1.77^{ab}	0.05	0.025
d21 to 63	1.44^{b}	1.66^{a}	1.63^{a}	0.03	0.001
d0 to 63	1.47^{b}	1.69^{a}	1.68^{a}	0.03	<0.0001
Cumulative mortality, %[3)]					
d21	1.67	2.22		0.02	0.661
d43	7.78	3.89	3.89	0.08	0.175
d63	11.67	8.89	6.67	0.03	0.254

Abbreviations: CTR= control group fed a standard diet with medium CP content and a normal AM/AP ratio (d0 to 21: 16.6% CP, 0.17 AM/AP; d21 to 63: 17.2% CP, 0.12 AM/AP), LP= group fed a low-CP diet with a normal AM/AP ratio (d0 to 21: 14.9% CP, 0.16 AM/AP; d21 to 63: 15.0% CP, 0.17 AM/AP), LPLA= fed a low-CP diet with a low AM/AP ratio (d0 to 21: 14.9% CP, 0.16 AM/AP; d21 to 63: 15.1% CP, 0.08 AM/AP), BW= body weight, ADG= average daily gain, and ADFI= average daily feed intake.
a, b Different superscripts within a row indicate significant differences among the values ($P < 0.05$).
x, y Different superscripts within a row indicate a tendency for differences among values ($P < 0.10$).
[1)] The piglets were fed the same diet (14.9% CP, 0.16 AM/AP) from d0 to 21. In Phase 2 (d 21 to 63), pigs assigned to the LP and LPLA treatments were provided with their respective diets: LP (15.0% CP; 0.17 AM/AP) and LPLA (15.1% CP; 0.08 AM/AP).
[2)] The number of pigs per treatment group at each sampling time point was as follows: d 0, 180 pigs for all treatments; d 21, CTR = 177 pigs, LP = 178 pigs, LPLA = 174 pigs; d 43, CTR = 166 pigs, LP = 173 pigs, LPLA = 173 pigs; d 63, CTR = 159 pigs, LP = 164 pigs, LPLA = 168 pigs.
[3)] Each treatment consisted of 5 pens.

ratio. No effect of the diet on the faecal ammonia concentrations was observed at d21, 43, and 63.

3.3. Faecal metabolites

A total of 71 metabolites were analysed in the faecal samples. To assess differences in metabolomic profiles among dietary groups, a PLS-DA was performed. At d21, the first two principal components (PC1 and PC2) explained 14.2% and 18.2% of the variance, respectively (Fig. 1A); at d43, 20.0% and 12.6% (Fig. 1C); and at d63, 28.2% and 9.3% (Fig. 1E). Although some overlap among the three groups was observed, a clearer separation of the CTR group from the low-CP groups along PC1 was evident at d43 and 63. Fig. 1B, D, and F, representing d21, 43, and 63, respectively, show the top 15 metabolites with VIP > 1 scores in each comparison. At d21, the CTR group was characterised by a greater faecal acetate concentration (VIP score= 2.33), carnitine (VIP score= 1.73) and xylose (VIP score = 1.63), whereas the LP group was characterized by a higher concentration of glutamate (VIP score= 1.97),

Table 3
Effects of low-protein diets with different amylose/amylopectin ratios on faecal dry matter and ammonia concentrations of nursery pigs.

Items	Diet			SEM	*P*-value
	CTR	LP[1)]	LPLA[1)]		
Dry matter, %[2)]					
d21	23.7^{b}	26.1^{a}		0.80	0.011
d43	25.6	26.9	25.6	0.82	0.406
d63	26.4	26.5	27.4	1.11	0.785
Ammonia, μmol/g[2)]					
d21	15.6	17.5		1.96	0.407
d43	18.6	17.2	19.0	1.64	0.711
d63	21.2	20.3	21.2	1.89	0.918

Abbreviations: CTR= control group fed a standard diet with medium CP content and a normal AM/AP ratio (d0 to 21: 16.6% CP, 0.17 AM/AP; d21 to 63: 17.2% CP, 0.12 AM/AP), LP= group fed a low-CP diet with a normal AM/AP ratio (d0 to 21: 14.9% CP, 0.16 AM/AP; d21 to 63: 15.0% CP, 0.17 AM/AP), and LPLA= fed a low-CP diet with a low AM/AP ratio (d0 to 21: 14.9% CP, 0.16 AM/AP; d21 to 63: 15.1% CP, 0.08 AM/AP).
a, b Different superscripts within a row indicate significant differences among the values ($P < 0.05$).
[1)] The piglets were fed the same diet (14.9% CP, 0.16 AM/AP) from d0 to 21. In Phase 2 (d 21 to 63), pigs assigned to the LP and LPLA treatments were provided with their respective diets: LP (15.0% CP; 0.17 AM/AP) and LPLA (15.1% CP; 0.08 AM/AP).
[2)] At each sampling day, 16 samples per treatment were included.

phenylalanine (VIP score= 1.92), and valerate (VIP score= 1.93). At d43, higher concentrations of faecal proline (VIP score= 2.89), lactate (VIP score= 1.96), alanine (VIP score= 1.95), and uracil (VIP score= 1.90) characterized the CTR group; the LP group showed higher faecal glucose (VIP score= 1.60), galactose (VIP score= 1.61), and dimethylamine (VIP score= 1.51). At d63, the LP group was characterised by a higher concentration of aspartate (VIP score= 2.22), valerate (VIP score= 1.88), glutamate (VIP score= 1.75), 2-oxocaproate (VIP score= 1.73) and dimethylamine (VIP score= 1.69); the LPLA group was characterised by a higher concentration of betaine (VIP score= 1.80) and lysine (VIP score= 1.78)

Fig. 2 presents the ANOVA results for the metabolites with a VIP score greater than 1.8. At d21 (Fig. 2A) and d63 (Fig. 2B), no significant differences in overall metabolite concentrations were observed among the dietary groups. Nevertheless, at d21, pigs fed the LP diet tended to show a lower concentration of acetate ($P < 0.10$) and a higher concentration of phenylalanine ($P < 0.10$) compared with the CTR pigs (Fig. 2A). At d63, the LP pigs also tended to exhibit a higher concentration of aspartate relative to CTR pigs (Fig. 2C). In contrast, at d43 (Fig. 2B), pigs receiving the LP and LPLA diets had significantly lower concentrations of alanine ($P < 0.05$ for both) and proline ($P < 0.01$ for both) compared with CTR pigs. Furthermore, lactate and uracil concentrations were significantly reduced in the LPLA pigs ($P < 0.05$), whereas the LP pigs showed only a tendency toward lower levels of these metabolites compared with CTR pigs ($P < 0.10$).

3.4. Faecal microbial composition

Bacterial DNA from faecal samples was successfully extracted and amplified from a total of 144 samples. Through quality control, a total of 8,552,955 sequences were retained; each sample had an average of 59,395 sequences. After bioinformatic analysis, a total of 4829 Amplicon Sequence Variants (ASVs) were generated. Rarefaction curves are presented in Supplementary Figure S1., which shows the number of different species observed as a function of the number of sequences. The trend of the rarefaction curves to a plateau indicates that the sequencing procedure was able to capture all the variability present in the samples.

Among the 4829 ASVs recovered, 21 phyla, 90 families, and 248 genera were identified. The most abundant phyla were *Bacillota* 82.0 ± 7.3%, *Bacteroidota* 12.7 ± 6.4%, and *Methanobacteriota* 2.2 ± 1.7%. The

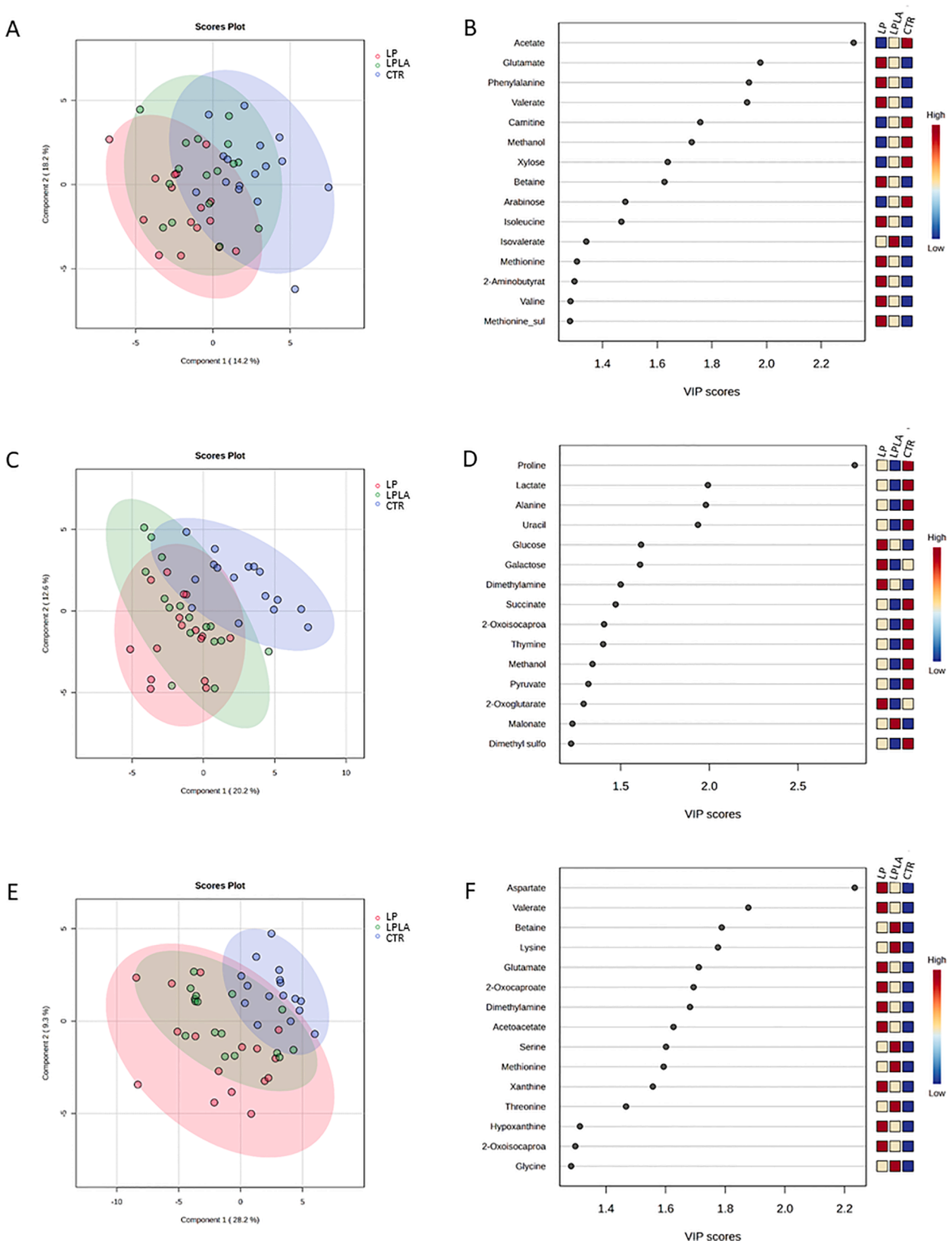

Fig. 1. PLS-DA score plots between components 1 and 2 of metabolites in faecal samples of pigs collected at d21 (A), 43 (C), and 63 (E) post-weaning. The top 15 metabolites with VIP > 1 for each time point (B, D, and F).

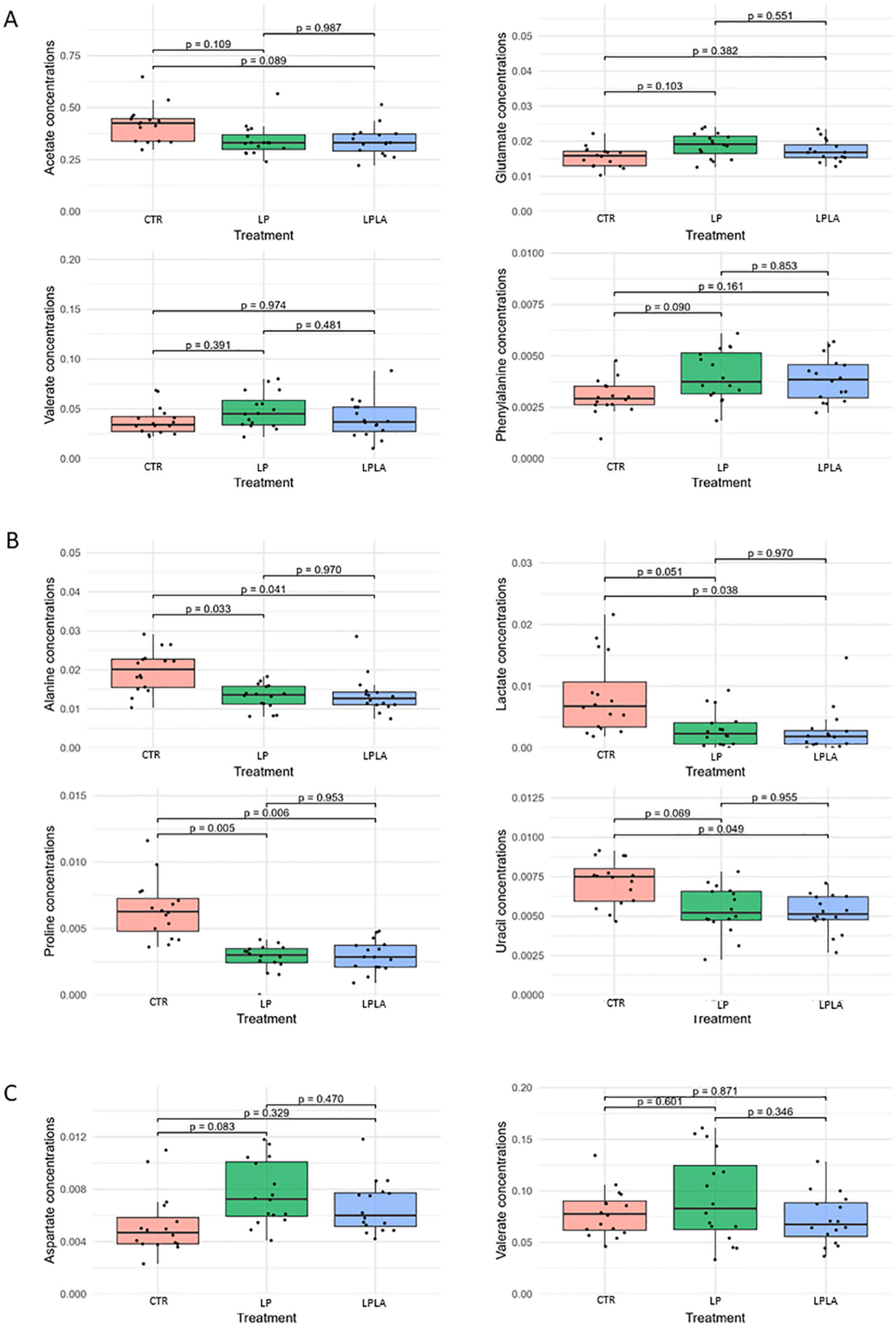

Fig. 2. Effects of low-protein diets with different amylose/amylopectin ratios on faecal metabolite concentrations of pigs at d21 (A), 43 (B), and 63 (C) post-weaning. Abbreviations: CTR= control group fed a standard diet with medium CP content and a normal AM/AP ratio (d0 to 21: 16.6% CP, 0.17 AM/AP; d21 to 63: 17.2% CP, 0.12 AM/AP), LP= group fed a low-CP diet with a normal AM/AP ratio (d0 to 21: 14.9% CP, 0.16 AM/AP; d21 to 63: 15.0% CP, 0.17 AM/AP), and LPLA= fed a low-CP diet with a low AM/AP ratio (d0 to 21: 14.9% CP, 0.16 AM/AP; d21 to 63: 15.1% CP, 0.08 AM/AP).

most abundant families were *Lactobacillaceae* 23.8 ± 13.2%, *Lachnospiraceae* 15.7 ± 6.4%, and *Prevotellaceae* 8.8 ± 5.8%. The most represented genera were *Lactobacillus* 22.4 ± 12.7%, *Clostridium* 8.6 ± 8.4%, and *Gemmiger* 3.7 ± 2.5%.

Fig. 3 shows the values for Chao1, Shannon, and InvSimpson indices for each diet at d21, 43, and 63. No significant differences were observed in all periods considered. For beta diversity, principal coordinates analysis (PCoA) plots were generated using a Bray–Curtis distance matrix for the three sampling points (d21, 43, and 63). There was no clear separation among the three sampling points (Supplementary Figure S2). However, at d43 and 63, the Adonis test confirmed that the beta diversity was significantly affected by diets ($P < 0.001$; $R^2 = 0.11$ and $P < 0.01$; $R^2 = 0.11$, Fig. 4B and C, respectively). At d43 and 63, the beta diversity of the CTR group is significantly different from that of the LP ($P < 0.001$; $R^2 = 0.10$ and $P < 0.001$; $R^2 = 0.13$, respectively) and LPLA groups ($P < 0.01$; $R^2 = 0.07$ and $P < 0.01$; $R^2 = 0.10$, respectively). Moreover, at d63, the beta diversity tended to be different between LP and LPLA ($P < 0.10$; $R^2 = 0.05$).

To identify specific bacterial markers for each diet, LEfSe analysis was performed at d21, 43, and 63 (Fig. 5A, B, and C, respectively). At d21, the LPLA group was characterised by a greater abundance of *UCG-005* (LDA score= 3.74, $P < 0.05$), *Anaerovibrio* (LDA score= 3.73, $P < 0.05$), *Lachnospiraceae NK4A136* group (LDA score= 3.51, $P < 0.05$), and *Xylanophilum* group (LDA score= 3.46, $P < 0.05$).

At d43, the CTR group was characterised by a higher abundance of *Lactobacillus* (LDA score= 4.96, $P < 0.05$), *Blautia* (LDA score= 4.19, $P < 0.05$), [*Ruminococcus*] *gauvreauii* group (LDA score= 3.61, $P < 0.05$), *Prevotella* (LDA score= 3.4, $P < 0.05$), *Dorea* (LDA score= 3.32, $P < 0.05$), and *Lachnospira* (LDA score= 3.18, $P < 0.05$), the LP group by a greater abundance of *Clostridium* (LDA score= 4.70, $P < 0.05$), *Turicibacter* (LDA score= 3.88, $P < 0.05$), *UCG-005* (LDA score= 3.86, $P < 0.05$), *Parabacteroides* (LDA score= 3.47, $P < 0.05$), *Romboutsia* (LDA score= 3.44, $P < 0.05$), *Intestinimonas* (LDA score= 3.03, $P < 0.05$), and *Desulfovibrio* (LDA score= 3.01, $P < 0.05$) and the LPLA group by a greater abundance of *Terrisporobacter* (LDA score= 4.25, $P < 0.05$), *Succinivibrio* (LDA score= 3.80, $P < 0.05$), and *Oscillospira* (LDA score= 3.14, $P < 0.05$).

At d63, the CTR group was characterised by a higher abundance of *Clostridium* (LDA score= 4.94, $P < 0.05$), *Terrisporobacter* (LDA score= 4.45, $P < 0.05$), *UCG-005* (LDA score= 3.98, $P < 0.05$), *Methanosphaera* (LDA score= 3.60, $P < 0.05$), *Oscillospira* (LDA score= 3.37, $P < 0.05$), and *Xylanibacter* (LDA score= 3.22, $P < 0.05$), the LP group by a greater abundance of *Shuttleworthia* (LDA score= 3.95, $P < 0.05$), [*Eubacterium*] *ruminantium* group (LDA score= 3.47, $P < 0.05$), *Erysipelotrichaceae UCG-006* (LDA score= 3.33, $P < 0.05$), *Holdemanella* (LDA score= 3.25, $P < 0.05$), *Collinsella* (LDA score= 3.16, $P < 0.05$), and *Erysipelotrichaceae UCG-009* (LDA score= 3.07, $P < 0.05$) and the LPLA group by a greater abundance of *Lactobacillus* (LDA score= 5.01, $P < 0.05$), *Falcatimonas* (LDA score= 3.51, $P < 0.05$), and *Limosilactobacillus* (LDA score= 3.20, $P < 0.05$).

3.5. Ear and tail lesion scores, behaviour observations, and environmental parameters

The results of tail and ear lesion scores are shown in Table 4. During phase I of feeding (d0 to 21) no effect of the diet was observed on both ear and tail lesions. In phase II, no effect of the diet was observed on both ear and tail lesions at d43, while at d63, the pigs in the LP group tended to have a higher proportion of individuals with an ear lesion score of 2, as well as greater ear LSI, compared to those in the CTR group by d63 ($P < 0.10$ for both), while the pigs in the LPLA groups had intermediate value.

Table 5 and Supplementary Table S2 show the results of behavioural observation and environmental parameters, respectively. No significant differences among groups were observed in behaviour observation and environmental parameters throughout the experimental period.

4. Discussion

Long-term feeding (more than three weeks) of a diet containing ≤ 15% CP to nursery pigs can result in growth retardation (Spring et al., 2020), even when supplemented with synthetic AAs. We hypothesised that this adverse effect could be mitigated by a reduced AM/AP ratio during the second phase post-weaning, a period characterised by rapid growth. This study demonstrates the potential of a reduced AM/AP ratio in improving feed efficiency of pigs fed low-CP diets during the second phase post-weaning. Dietary treatments differed not only in the AM/AP ratio but also in ingredient composition, including the inclusion levels of wheat, barley, oat flakes, and waxy corn. Therefore, the observed responses cannot be attributed solely to the AM/AP ratio, and possible effects of ingredient composition or their interaction, such as palatability or digestive characteristics, cannot be excluded.

In phase 1, we found that a reduction in dietary CP levels from 16.6% to 14.8%, while maintaining a similar AM/AP ratio and supplementing with feed-grade AAs, tended to negatively affect the growth performance of nursery pigs. This suggests that diets containing <15% CP cannot maintain growth performance even if feed-grade AAs are supplemented and even during the early post-weaning period. Although the results of Spring et al. (2020) showed pigs fed a diet with 14.3% of CP and feed-grade AAs had comparable growth performance to pigs fed a standard diet for up to 3 weeks post-weaning, the pigs were individually housed, unlike commercial conditions. Some previous studies also suggested that a reduction in CP level coupled with the integration of synthetic AAs does not compromise piglets' performance in the immediate post-weaning period (Heo et al., 2008); however, their CP levels were not <15%.

Later in phase II (d43 to 63), the LPLA diet, characterised by a reduced AM/AP ratio, demonstrated potential advantages in feed efficiency, microbiota modulation, and welfare indicators compared to the LP diet. These findings support the hypothesis that synchronising AAs absorption with insulin peaks through a reduced AM/AP ratio may mitigate the negative consequences of low-CP diets in nursery pigs. In addition, no significant differences in F:G were observed between the CTR and LPLA groups, whereas the LP group exhibited a higher F:G than the CTR group from d43 to 63. This pattern may be related to the numerically higher feed intake recorded in the LP group compared with the LPLA diet. As a result, the LP group achieved a feed intake comparable to that of the CTR group from d21 to 63. Based on the findings of Castaing et al. (1987), who reported greater feed intake in piglets fed flaked versus raw cereals, the higher feed intake in the LP group may be attributed to the greater proportion of flaked cereals in the LP diet compared with the LPLA diet. Feed-grade AAs have the characteristic of being absorbed faster than glucose from starch, leading to a mismatch between circulating AAs and insulin peaks (Eugenio et al., 2022 and 2023). This asynchrony promotes hepatic deamination of AAs, potentially compromising growth (Eugenio et al., 2023). Based on the comparable F:G between CTR and LPLA pigs, we suggest that an AM/AP ratio of 0.08 may help synchronise postprandial spikes in circulating free AAs and insulin concentrations during the late part of phase II. However, F:G, as an indirect indicator, does not provide sufficient evidence to determine the potential effects of reduced dietary AM/AP ratios. Therefore, further studies are needed to analyse glucose and insulin concentrations in the blood during the post-prandial time.

Moreover, both LP and LPLA pigs showed reduced feed consumption compared to CTR pigs during the late part of phase II, possibly due to the lower palatability of low-CP diets. In this study, the reduction in dietary protein content was achieved by decreasing the amount of soybean meal, which may have reduced palatability of the low-CP diets, given that soybean meal is highly palatable (Guo & Wang, 2025). Nevertheless, both LP and LPLA pigs consumed feed similar to CTR pigs during the early part of phase II. This might be because pigs tend to consume more feed when the diet is changed (Kyriazakis & Emmans, 1990).

In addition to its impact on growth performance, a reduction in

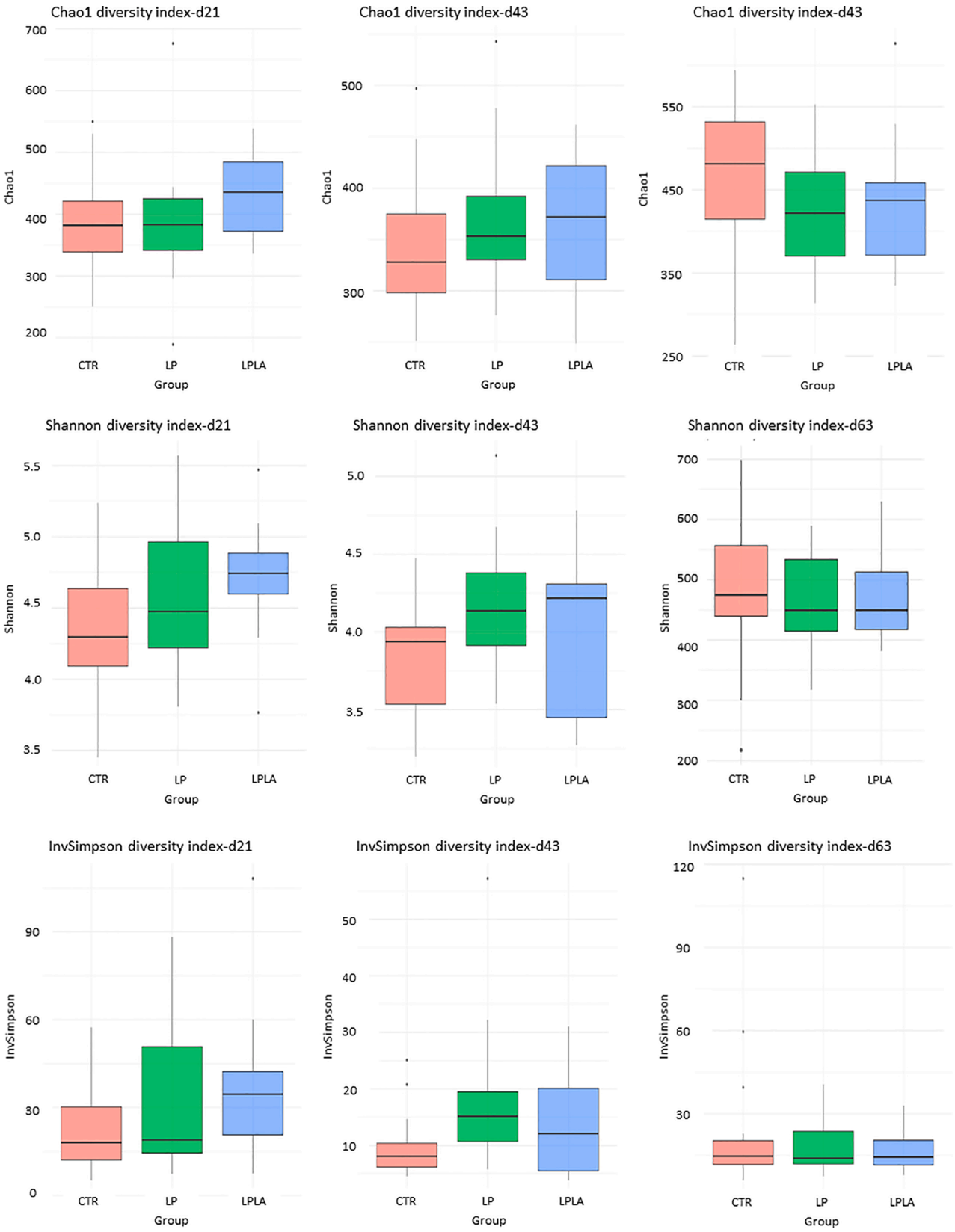

Fig. 3. Effects of low-protein diets with different amylose/amylopectin ratios on faecal alpha diversity of pigs at d21, 43, and 63 post-weaning. Abbreviations: CTR= control group fed a standard diet with medium CP content and a normal AM/AP ratio (d0 to 21: 16.6% CP, 0.17 AM/AP; d21 to 63: 17.2% CP, 0.12 AM/AP), LP= group fed a low-CP diet with a normal AM/AP ratio (d0 to 21: 14.9% CP, 0.16 AM/AP; d21 to 63: 15.0% CP, 0.17 AM/AP), and LPLA= fed a low-CP diet with a low AM/AP ratio (d0 to 21: 14.9% CP, 0.16 AM/AP; d21 to 63: 15.1% CP, 0.08 AM/AP).

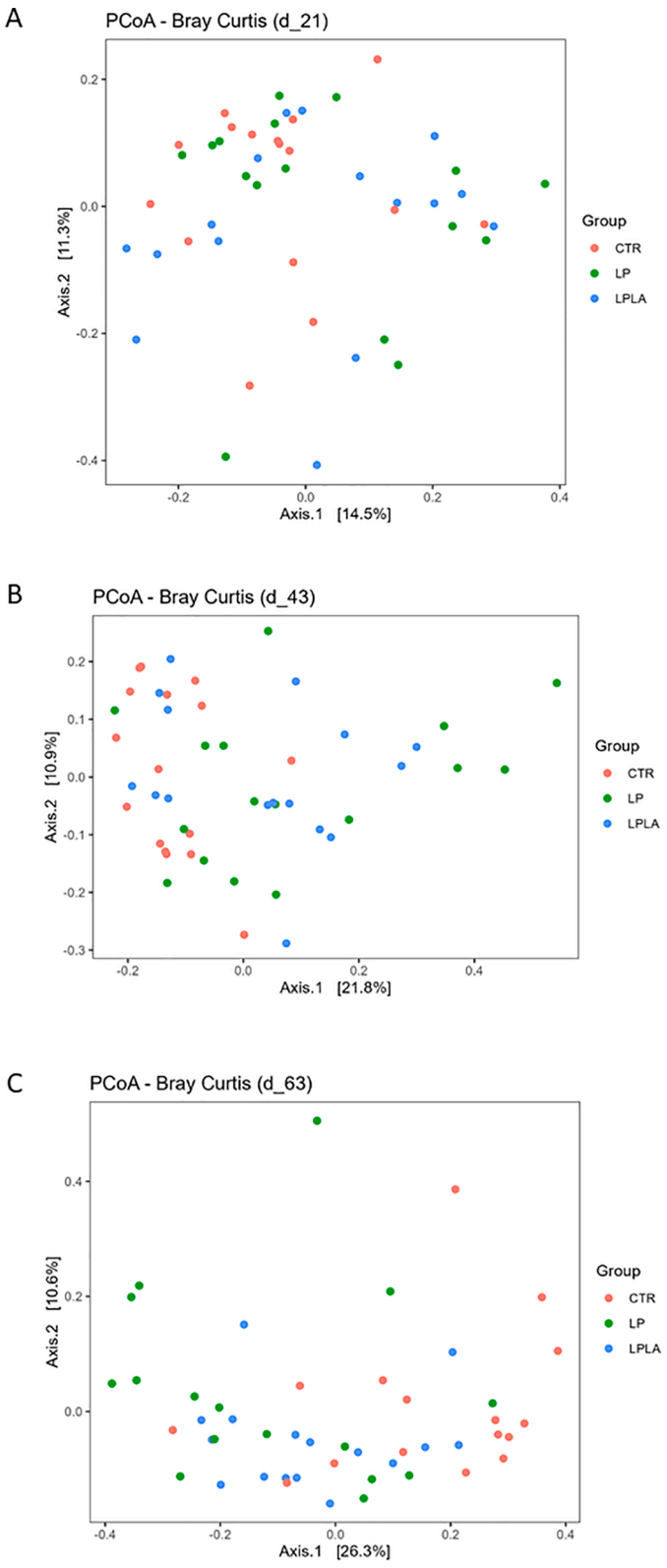

Fig. 4. Effects of low-protein diets with different amylose/amylopectin ratios on faecal beta diversity of pigs at d21 (A; $P = 0.31$, $R^2 = 0.06$), 43 (B; $P < 0.011$, $R^2 = 0.11$), and 63 (C; $P < 0.01$, $R^2 = 0.11$) post-weaning. Abbreviations: CTR= control group fed a standard diet with medium CP content and a normal AM/AP ratio (d0 to 21: 16.6% CP, 0.17 AM/AP; d21 to 63: 17.2% CP, 0.12 AM/AP), LP= group fed a low-CP diet with a normal AM/AP ratio (d0 to 21: 14.9% CP, 0.16 AM/AP; d21 to 63: 15.0% CP, 0.17 AM/AP), and LPLA= fed a low-CP diet with a low AM/AP ratio (d0 to 21: 14.9% CP, 0.16 AM/AP; d21 to 63: 15.1% CP, 0.08 AM/AP).

dietary CP may also be associated with alterations in the substrates available for bacterial fermentation (Luise et al., 2021) and have some consequences on the gut health of the piglets. Fermentation of incompletely digested proteins in the hindgut produces toxic substances, such as ammonia and biogenic amines, which have been implicated in gut disorders (Luise et al., 2021). In the present study, the faecal dry matter was used as a specific indicator for diarrhoea severity, as it reflects water content in the intestine (Eriksen et al., 2023). At d21, LCP pigs showed increased faecal dry matter, which may be attributed to the better digestibility and water absorption of the diet containing <15% CP compared with the 16.6% CP diet during phase I, since piglets have a less developed gastrointestinal tract during this period (Pluske et al., 2018). Numerous studies have demonstrated that piglets fed low-CP diets (16 to 19% CP) show a lower incidence of diarrhoea compared to those fed high-protein diets (20 to 26% CP) (García et al., 2014; Heo et al., 2010, 2009). On the other hand, at d43 and 63, the diets had no impact on faecal dry matter. Considering the more developed gastrointestinal tract of piglets during phase II compared with phase I (Pluske et al., 2018), we suggest that the dietary CP levels (15 to 17%) used in the present study, together with the more developed gastrointestinal tract of piglets in phase II, are unlikely to cause gut disorders associated with the fermentation of undigested protein.

In order to monitor microbial fermentation, the amount of ammonia was also analysed in this study, as well as the metabolomic profile. According to our results, the reduction in the CP level of the diet did not affect the faecal NH_3 concentration, suggesting no differences in the nitrogen retention among the groups. However, the evaluation of nitrogen retention efficiency in pigs may not be adequately addressed by the analysis of faecal NH_3 alone. In the interest of achieving a more accurate assessment of protein synthesis efficiency, further studies should consider the implementation of additional direct or indirect measures, including urinary NH_3 and blood urea.

Metabolomic profiling based on PLS-DA indicated that the effects of the dietary treatments on the faecal metabolic profile were dependent on sampling time. At d21, no evident separation among the dietary groups was observed, suggesting that the early post-weaning phase was not characterised by marked metabolic divergence. In contrast, at d43 and d63, the control diet tended to separate from pigs receiving low-CP diets (LP and LPLA groups) along the first component, indicating that prolonged dietary CP reduction gradually shaped the faecal metabolomic differences. However, the LP and LPLA groups overlapped, implying that modification of the AM/AP ratio had a comparatively limited influence on the overall metabolomic profile when contrasted with the effect of CP level.

Despite the moderate group discrimination observed in the score plots, the multivariate analysis identified metabolites contributing to group separation, as reflected by the VIP scores. In detail, at d43, the control diet was characterised by a higher abundance of several AA and derivates, including proline, lactate, alanine, and uracil, as identified by the VIP scores derived from the PLS-DA. This multivariate discrimination was consistent with the subsequent univariate analysis of metabolites with high VIP values, which revealed specific metabolic alterations in pigs fed the low-CP diets, particularly reduced concentrations of alanine and proline. As these metabolites are closely related to amino acid metabolism and microbial protein fermentation, their decreased levels in low CP diets likely reflect reduced nitrogen availability in the hindgut resulting from limited dietary protein supply. Thus, the reduced availability of protein could have resulted in low levels of faecal alanine and proline in pigs fed low-CP diets. In parallel, faecal lactate levels were lower in LPLA pigs and showed a decreasing trend in LP pigs, compared to CTR. Given that lactate is the end-product of carbohydrate fermentation by *Lactobacillus* (Zhao & Gänzle, 2018), this reduction could be related to the lower abundance of *Lactobacillus* in the hindgut of pigs fed low-CP diets, as supported by microbiota data showing that *Lactobacillus* was not a dominant genus in these groups at d43, while it was a discriminant taxon for the CTR group. Furthermore, faecal uracil

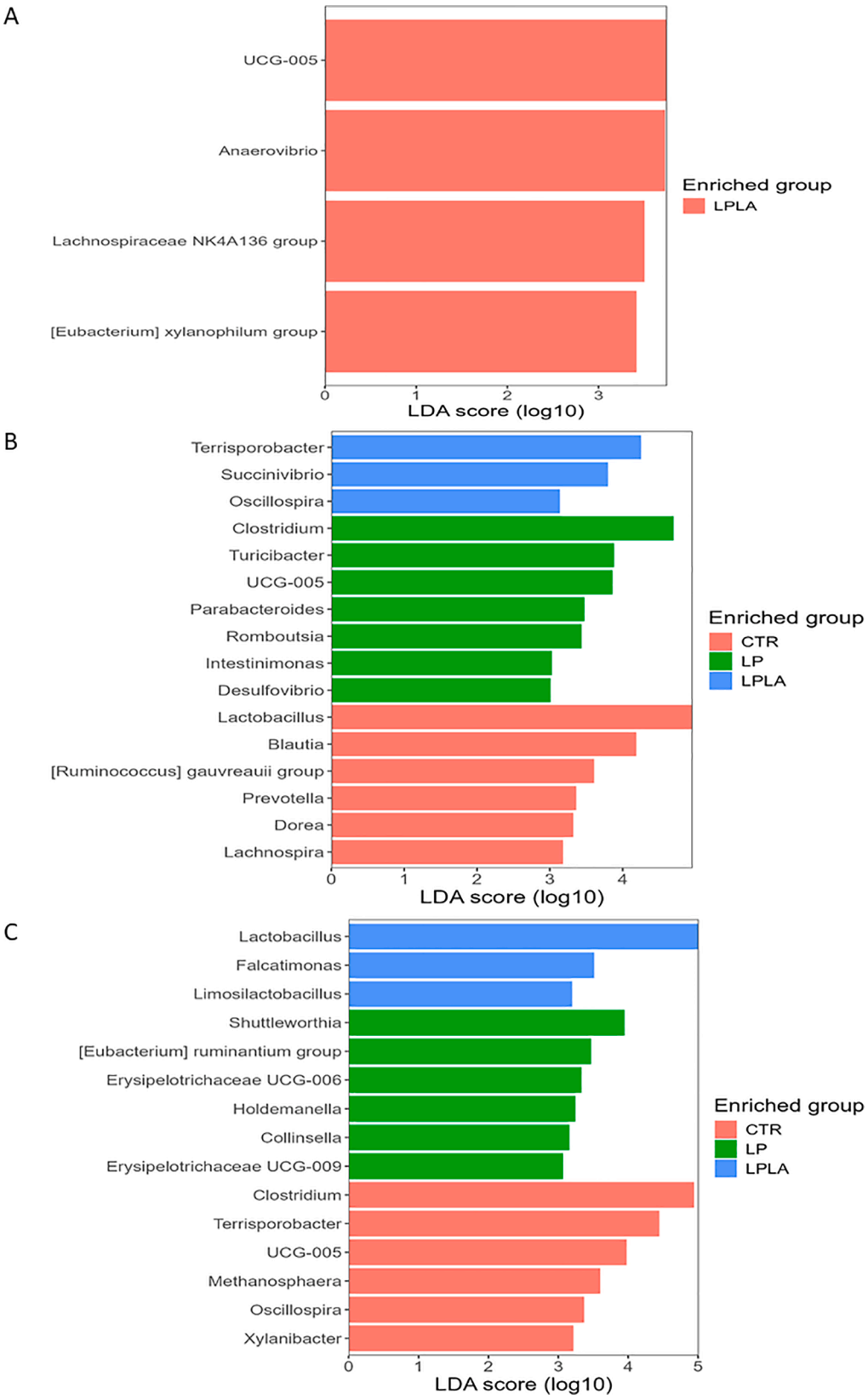

Fig. 5. Effects of low-protein diets with different amylose/amylopectin ratios on faecal microbial biomarkers of pigs at genus level at d21 (A), 43 (B), and 63 (C) post-weaning. Abbreviations: CTR= control group fed a standard diet with medium CP content and a normal AM/AP ratio (d0 to 21: 16.6% CP, 0.17 AM/AP; d21 to 63: 17.2% CP, 0.12 AM/AP), LP= group fed a low-CP diet with a normal AM/AP ratio (d0 to 21: 14.9% CP, 0.16 AM/AP; d21 to 63: 15.0% CP, 0.17 AM/AP), and LPLA= fed a low-CP diet with a low AM/AP ratio (d0 to 21: 14.9% CP, 0.16 AM/AP; d21 to 63: 15.1% CP, 0.08 AM/AP).

Table 4
Effects of low-protein diets with different amylose/amylopectin ratios on the proportion of ear and tail lesions in nursery pigs.

Item	Diet			SEM	*P*-value
	CTR	LP[1)]	LPLA[1)]		
Phase I: d0 to 21					
d0					
Ear lesion, %					
0	36.1	45.6		13.14	0.567
1	42.8	39.2		10.84	0.790
2	21.1	15.3		5.70	0.418
LSI	85.0	69.7		17.11	0.479
Tail lesion, %					
0	97.2	98.3		1.56	0.570
1	2.2	1.67		1.51	0.769
2	0.6	0		-	-
LSI	3.3	1.67		1.66	0.427
d21					
Ear lesion, %					
0	38.3	35.3		5.90	0.677
1	58.9	59.9		6.71	0.905
2	2.8	4.9		2.50	0.508
LSI	64.4	64.4		6.08	0.500
Tail lesion, %					
0	90.0	91.1		4.05	0.824
1	10.0	8.33		4.01	0.735
2	0.0	0.58		0.45	0.317
LSI	10.0	2.92		4.13	0.916
Phase II: d21 to 63					
d43					
Ear lesion, %					
0	41.3	47.7	34.7	8.62	0.580
1	54.6	45.3	47.5	7.01	0.632
2	4.2	7.0	17.8	4.76	0.146
LSI	62.9	83.0	59.2	12.02	0.354
Tail lesion, %					
0	81.1	86.1	81.3	6.95	0.761
1	17.7	13.9	16.9	6.00	0.896
2	1.2	0.0	1.8	1.24	0.600
LSI	20.1	13.9	20.4	7.98	0.812
d63					
Ear lesion, %					
0	36.8	7.5	12.3	9.45	0.104
1	35.4	13.8	24.1	8.51	0.238
2	27.8^{x}	78.7^{y}	63.6^{xy}	13.38	0.051
LSI	91.0^{x}	171.0^{y}	151.0^{xy}	21.55	0.054
Tail lesion, %					
0	92.0	87.7	80.4	6.31	0.761
1	6.1	12.3	9.6	3.61	0.498
2	2.6	0.0	10.0	5.96	0.490
LSI	11.2	12.3	29.6	11.67	0.480

Abbreviations: CTR= control group fed a standard diet with medium CP content and a normal AM/AP ratio (d0 to 21: 16.6% CP, 0.17 AM/AP; d21 to 63: 17.2% CP, 0.12 AM/AP), LP= group fed a low-CP diet with a normal AM/AP ratio (d0 to 21: 14.9% CP, 0.16 AM/AP; d21 to 63: 15.0% CP, 0.17 AM/AP), and LPLA= fed a low-CP diet with a low AM/AP ratio (d0 to 21: 14.9% CP, 0.16 AM/AP; d21 to 63: 15.1% CP, 0.08 AM/AP), LSI= lesion score index.
x, y Different superscripts within a row indicate a tendency for differences among values ($P < 0.10$).
[1)] The piglets were fed the same diet (14.9% CP, 0.16 AM/AP) from d0 to 21. In Phase 2 (d 21 to 63), pigs assigned to the LP and LPLA treatments were provided with their respective diets: LP (15.0% CP; 0.17 AM/AP) and LPLA (15.1% CP; 0.08 AM/AP).

concentration was lower in LPLA pigs and tended to be lower in LP pigs compared to CTR. As uracil is known to be secreted by non-commensal pathogenic bacteria, such as *Pseudomonas aeruginosa* and *Enterococcus faecalis* (Lee et al., 2013), the lower faecal uracil concentrations observed in pigs fed low-CP diets may imply a relatively reduced abundance or activity of such pathogenic microbes, although this cannot be demonstrated by 16S rRNA sequencing. One possible explanation is that the reduced dietary CP levels limited the amount of undigested protein available for intestinal fermentation, thereby decreasing the production of potentially harmful metabolites and attenuating the

Table 5
Effects of low-protein diets with different amylose/amylopectin ratios on the proportion of behavioural patterns of nursery pigs.

Item	Diet			SEM	*P*-value
	CTR	LP[1)]	LPLA[1)]		
Phase I: d0 to 21					
d7					
Resting, %	19.20	26.30		11.92	0.635
Abnormal, %	1.90	2.24		0.55	0.624
Positive interaction, %	10.30	9.05		2.47	0.689
Negative interaction, %	5.57	4.47		0.80	0.282
Enrichment investigation, %	0.91	1.29		0.62	0.624
Pen exploration, %	36.30	30.4		5.61	0.407
Other activities, %	25.80	26.2		4.08	0.934
d14					
Resting, %	16.90	14.80		3.75	0.646
Abnormal, %	4.49	5.13		1.20	0.668
Positive interaction, %	8.91	9.13		1.68	0.919
Negative interaction, %	5.45	5.62		0.86	0.870
Enrichment investigation, %	1.38	1.19		0.48	0.745
Pen exploration, %	35.90	35.60		2.48	0.927
Other activities, %	26.90	28.50		2.22	0.565
d21					
Resting, %	13.82	9.33		4.44	0.424
Abnormal, %	2.68	3.34		0.73	0.472
Positive interaction, %	9.31	8.58		1.51	0.702
Negative interaction, %	4.51	4.06		0.45	0.430
Enrichment investigation, %	1.37	1.27		0.66	0.904
Pen exploration, %	40.80^{b}	47.90^{a}		2.49	0.037
Other activities, %	27.50	25.5		2.21	0.476
Phase II: d21 to 63					
d28					
Resting, %	23.50	16.70	22.10	4.99	0.6130
Abnormal, %	3.93	5.42	3.16	1.55	0.5914
Positive interaction, %	6.93	6.76	6.69	0.59	0.9577
Negative interaction, %	6.73	5.55	5.31	1.32	0.7231
Enrichment investigation, %	3.44	2.16	3.03	0.85	0.5719
Pen exploration, %	28.70	38.00	38.60	3.62	0.1372
Other activities, %	26.80	25.40	21.00	1.82	0.1048
d35					
Resting, %	25.00	25.00	19.60	5.63	0.7419
Abnormal, %	1.21	1.63	2.19	0.39	0.2506
Positive interaction, %	9.24	8.30	8.43	1.70	0.9151
Negative interaction, %	5.78	4.89	4.48	1.27	0.7664
Enrichment investigation, %	2.16	3.12	4.19	0.83	0.2673
Pen exploration, %	31.10	33.90	37.20	5.59	0.7490
Other activities, %	25.60	23.10	23.90	1.84	0.6456
d43					
Resting, %	17.00	10.80	11.90	4.16	0.5452
Abnormal, %	1.09	2.10	1.62	0.70	0.6056
Positive interaction, %	4.70	5.92	6.11	1.00	0.5762
Negative interaction, %	3.38	3.98	3.54	0.97	0.9050
Enrichment investigation, %	2.41	3.30	3.01	1.12	0.8494
Pen exploration, %	43.80	46.00	48.90	4.36	0.7168
Other activities, %	27.60	27.90	24.90	1.65	0.4028
d53					
Resting, %	20.60	19.20	26.90	4.97	0.5306
Abnormal, %	2.25	2.86	1.65	0.53	0.3041
Positive interaction, %	8.35	6.89	7.61	2.07	0.8840
Negative interaction, %	7.30	7.45	7.22	1.15	0.9895
Enrichment investigation, %	5.21	3.67	5.94	1.30	0.4747
Pen exploration, %	31.90	36.20	28.70	6.00	0.6798
Other activities, %	24.30	23.70	22.10	1.35	0.4959
d63					
Resting, %	42.10	35.00	28.90	5.25	0.2454
Abnormal, %	3.19	3.29	2.52	0.67	0.6814
Positive interaction, %	5.61	5.62	5.11	0.81	0.8783
Negative interaction, %	2.79	2.04	2.89	0.56	0.5256
Enrichment investigation, %	0.74^{y}	2.72^{x}	2.50^{xy}	0.57	0.0580
Pen exploration, %	24.20	31.20	36.10	3.61	0.1030
Other activities, %	21.40	20.20	21.90	0.94	0.4455

Abbreviations: CTR= control group fed a standard diet with medium CP content and a normal AM/AP ratio (d0 to 21: 16.6% CP, 0.17 AM/AP; d21 to 63: 17.2% CP, 0.12 AM/AP), LP= group fed a low-CP diet with a normal AM/AP ratio (d0 to 21: 14.9% CP, 0.16 AM/AP; d21 to 63: 15.0% CP, 0.17 AM/AP), and LPLA= fed a low-CP diet with a low AM/AP ratio (d0 to 21: 14.9% CP, 0.16 AM/AP; d21 to 63: 15.1% CP, 0.08 AM/AP).

a, b Different superscripts within a row indicate significant differences among the values ($P < 0.05$).
x, y Different superscripts within a row indicate a tendency for differences among values ($P < 0.10$).
[1)] The piglets were fed the same diet (14.9% CP, 0.16 AM/AP) from d0 to 21. In Phase 2 (d 21 to 63), pigs assigned to the LP and LPLA treatments were provided with their respective diets: LP (15.0% CP; 0.17 AM/AP) and LPLA (15.1% CP; 0.08 AM/AP).

associated inflammatory responses (Luise et al., 2021). Such an intestinal environment may have favoured the proliferation of commensal bacteria while constraining the growth of opportunistic or pathogenic species (Pickard et al., 2014). Taken together, these findings suggest that lower faecal uracil levels could indicate a shift towards a more stable and beneficial microbial ecosystem under reduced-CP feeding strategies.

Looking at the main metabolites characterising the LP diet, a higher abundance of faecal glucose, galactose, and dimethylamine (both d43 and 63) was observed compared to the LPLA diet (VIP score of the PLS-DA). The LP diet, characterised by a higher AM/AP ratio, may have slowed starch hydrolysis and small intestinal digestion, thereby increasing the flow of starch-derived substrates to the hindgut (Tan & Zijlstra, 2021). Amylose-rich starch is known to be less rapidly digestible and more prone to escape small intestinal digestion compared with low-amylose starch (Birt et al., 2013). The greater availability of fermentable carbohydrate in the large intestine could have promoted the release of simple sugars through microbial enzymatic activity (Jiang et al., 2024). However, under conditions of limited nitrogen availability associated with the low-CP levels, microbial utilisation of these substrates may have been constrained, potentially resulting in the accumulation of glucose and galactose in faeces. The concomitant increase in dimethylamine, a microbial metabolite derived from methylated nitrogen-containing compounds (Asatoor & Simeshoff, 1965), may further support the hypothesis of altered microbial nitrogen metabolism under protein-restricted conditions. Without measurements of pH or short-chain fatty profiles in the hind gut, these interpretations remain speculative but consistent with an imbalance between rapidly fermentable carbohydrates and nitrogen supply in the hindgut.

We observed differences in beta diversity during phase II between the CTR and low-CP diet groups, whereas no such differences were observed in phase I. According to previous studies, the early post-weaning phase (1 to 2 weeks post-weaning) is a period of microbial instability, during which the composition of the gut microbiota shifts rapidly, and stabilisation tends to begin gradually after the second week (Chen et al., 2017). We, thus, speculate that the lack of group-level separation in beta diversity at d21 can be attributed to the still-unstable composition of the gut microbiota during the early post-weaning period, which may have attenuated the effects of dietary interventions as well as to the smaller difference in AM/AP ratio between the diets. Conversely, during the overall phase II, the prolonged reduction in nitrogenous substrates in low-CP diets may have limited microbial metabolic activity in the large intestine, thereby leading to shifts in microbial composition. However, interestingly, we found a tendency for beta diversity to be different between the LP and LPLA groups at d63. This result could be linked to the low AM/AP ratio of the diet since intermediate products generated during the enzymatic hydrolysis of AP can promote prebiotic effects, enhancing both the abundance and diversity of the gut microbiome (Zhang et al., 2020), despite the limited fermentability of AP by gut microbiota. The intermediate products could contribute to the increased relative abundance of *Succinivibrio*, which metabolises rapidly fermentable carbohydrates such as starch (Patterson & Hespell, 1985). However, a limitation of this study is the absence of measurements related to intestinal fermentation, such as starch digestion kinetics, luminal pH, or short-chain fatty acid production, which would help clarify the mechanisms underlying diet-induced changes in gut microbiota.

Unlike d43, when Lactobacillus was discriminating the CTR group, at d63, the LPLA pigs showed the higher abundance of *Lactobacillus*. As the gastrointestinal tract matured, a decline in intestinal pH could foster a more favourable environment for *Lactobacillus*, which is known for its acid tolerance. Additionally, the depletion of dominant fermentative taxa, driven by the persistent lack of fermentable substrates, might have resulted in a microbial niche, allowing *Lactobacillus* to establish dominance. Similar to *Lactobacillus*, the acid-tolerant *Limosilactobacillus* also seems to become dominant in the LPLA diet. The findings suggest that the use of a low AM/AP ratio diet may beneficially modulate the intestinal microbiome during the late post-weaning period by suppressing competition from dominant fermentative taxa and promoting the expansion of acid-tolerant beneficial microbes, such as *Lactobacillus*. During the same period, the CTR pigs were characterised by bacterial taxa typically regarded as commensal members of the pig gut microbiota during the growing phase, and often associated with favourable performance or intestinal health. This was the case, for instance, for *Blautia, Dorea* and *Lachnospira*, which are commonly recognized as producers of key volatile fatty acids, as well as *Prevotella*, which is frequently reported to increase in pigs showing good growth performance, as also observed in the present study. Notably, the superior performance of the CTR pigs became evident from d43 onwards. Thus, the microbiota findings are consistent with the productive outcomes recorded for this group.

Furthermore, it should be noted that at d63, the LP pigs were characterised by the presence of two taxa belonging to the *Erysipelotrichaceae* family (*UCG-009* and *UCG-006*). This family has recently received considerable attention in both humans and pigs. In humans, members of *Erysipelotrichaceae* have been associated with and immunomodulation (Palm et al., 2014). In pigs, they have been implicated in reduced growth performance (Kiros et al., 2019), which is consistent with our observations when comparing the LP and CTR pigs. It has been reported that an increase in intestinal N-acetylgalactosamine (GalNAc), a simple sugar derived from the shedding of intestinal mucosa (mucins) or linked to the blood groups of pigs, has been associated with the expansion of *Erysipelotrichaceae* (Yang et al., 2022). Moreover, strains belonging to the family *Erysipelotrichaceae* are able to metabolise complex carbohydrates, and their abundance can vary depending on certain AAs (Luise et al., 2022). In our case, their discrimination in the LP pigs and not in the LPLA pigs could reflect a lack of synchronism between the absorption of glucose and AAs: this imbalance may have increased the amount of fermentable substrate available in the intestinal lumen, thus favouring the growth of *Erysipelotrichaceae* only in the LP pigs. This hypothesis will have to be tested in future studies.

Regarding the effect of the diet on the piglet behaviour and LSI scoring, at the end of the experiment, the LP diet increased the ear LSI of pigs, compared to the CTR diet, while the LPLA group did not differ from the others. Low-CP diets can result in a relative deficiency of non-essential AAs, thereby impairing biological function, which in turn has been associated with abnormal behaviours in pigs (Martínez-Miró et al., 2016). Thus, we speculate that long-term feeding of a low-CP diet might have tended to increase ear biting of the LP pigs. Interestingly, LPLA pigs did not differ from CTR pigs in ear LSI at this time point, despite low-CP levels of the LPLA diet. This effect could be attributed to the synchronisation between AAs and insulin peaks induced by the reduced AM/AP diet. Large neutral amino acids (LNAA), including tryptophan, tyrosine, phenylalanine, leucine, isoleucine, and valine, are transported across the blood-brain barrier (BBB) via the L-type amino acid transporter (LAT1) (Huttunen et al., 2019; Van Vliet et al., 2018). Because this transporter operates on a competitive basis (Van Vliet et al., 2018), the relative plasma concentrations of these AAs determine their rate of brain uptake (Shulkin et al., 1995). Following insulin secretion, LNAA are rapidly taken up by muscle, thereby lowering their plasma concentrations (Tessari, 2023). In contrast, tryptophan is largely bound to albumin and thus less affected by insulin, resulting in an increased tryptophan/LNAA ratio (Hood et al., 2005; Richard et al., 2009). A higher tryptophan/LNAA ratio enhances the transport of tryptophan, the precursor of serotonin, across the BBB via LAT1, leading to increased

serotonin synthesis (Shen et al., 2012). Elevated serotonin levels, in turn, are associated with reduced aggressiveness and attenuation of stress responses (Shen et al., 2012). We therefore suggest that reducing the AM/AP ratio in low-CP diets can help to alleviate the adverse behavioural effects associated with low-CP feeding in nursery pigs, possibly through enhanced serotonin synthesis, however this hypothesis still remains to be validated.

According to Jensen et al. (1993), pigs fed low-CP diets showed increased activity, which may be related to poor satiety induced by a low-CP diet, as protein stimulates the release of gut hormones, including glucagon-like peptide-1 and peptide YY, signalling satiety to the brain (Belza et al., 2013). However, in this study, the reduction in dietary CP did not reduce the concentration of environmental gases. This could be explained by the relatively small difference in CP levels (15–17%) of the experimental diets used in the present study.

5. Conclusions

Even in short-term early post-weaning feeding, reducing dietary CP to ≤15% can compromise the growth performance of nursery pigs, even with AAs supplementation. During the later period of post-weaning (d43 to 63), reducing the AM/AP ratio within low-CP diets showed improved feed efficiency and provided additional benefits for gut health and animal welfare. However, comprehensive studies incorporating blood glucose and insulin levels, video-recorded behavioural evaluation and omics approaches, including shotgun metagenomics and transcriptomics, are needed to further clarify degree of synchronisation and how synchronisation between AA and insulin peaks influences gut microbial composition, host metabolic pathways, and central serotonin signalling, thereby providing mechanistic insight into how low-AM/AP diets enhance pig performance and welfare.

Ethical approval

This study was conducted at a commercial pig farm in Italy from November 2024 to January 2025. The experimental procedures were reviewed and approved by the ethics committee of the University of Bologna (Protocol ID 218339/2024).

Declaration of generative AI

During the preparation of this work, ChatGPT was used for the purpose of improving linguistic clarity and refining the readability of the manuscript. After using this tool, the manuscript was reviewed and edited as needed and authors will have full responsibility for the present article.

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CRediT authorship contribution statement

Juho Lee: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Federico Correa:** Writing – review & editing, Supervision, Software, Methodology, Data curation, Conceptualization. **Luca Laghi:** Writing – review & editing, Resources, Methodology, Investigation, Data curation. **Silvia Bencivenni:** Writing – review & editing, Methodology, Investigation, Data curation. **Daniele Bigi:** Writing – review & editing, Formal analysis, Data curation. **Giacomo Biagi:** Writing – review & editing, Resources, Formal analysis. **Francesco Palumbo:** Writing – review & editing, Software, Methodology, Investigation, Formal analysis, Data curation. **Paolo Trevisi:** Writing – review & editing, Validation, Supervision, Resources, Data curation, Conceptualization. **Diana Luise:** Writing – review & editing, Validation, Supervision, Software, Resources, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Supplementary materials

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