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Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions

Replacing Cereal with Ultraprocessed Foods in Pig Diets Does Not Adverse Gut Microbiota, L-glutamate Uptake, or Serum Insulin

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A B S T R A C T

Background: Using ultraprocessed food (UPF) to replace traditional feed ingredients offers a promising strategy for enhancing food production sustainability.

Objective: To analyze the impact of salty and sugary UPF on gut microbiota, amino acids uptake, and serum analytes in growing and finishing pig.

Methods: Thirty-six Swiss Large White male castrated pigs were assigned to 3 experimental diets: *1*) standard (ST), 0% UPF; *2*) 30% conventional ingredients replaced by sugary (SU) UPF; and *3*) 30% conventional ingredients replaced by salty (SA) UPF. The next-generation sequencing was used to characterize the fecal microbiota. Transepithelial electrical resistance and the active uptake of selected amino acids in pig jejuna were also evaluated. Data were enriched with measurements of fecal volatile fatty acids and serum urea, minerals, and insulin. All data analyses were run in R v4.0.3. The packages phyloseq, vegan, microbiome, and microbiomeutilities were used for microbiota data analysis. The remaining data were analyzed by analysis of variance using linear mixed-effects regression models.

Results: The UPF did not affect fecal microbiota abundance or biodiversity. The Firmicutes to Bacteroidetes ratio remained unaffected. SU-induced increase in the Anaerostipes genus suggested altered glucose metabolism, whereas SA increased the abundance of CAG-352 and p-2534-18B. No effects on fecal volatile fatty acids were observed. Assumptions of UPF negatively affecting small intestinal physiology were not supported by the measurements of transepithelial electrical resistance in pigs. Active amino acids uptake tests showed potential decrease in L-glutamate absorption in the SA compared with the SU diet. Blood serum analysis indicated no adverse effects on urea, calcium, magnesium, or potassium concentration but the SU group resulted in a lower blood serum insulin concentration at the time of blood collection.

Conclusions: When incorporated at 30% into a standard growing finishing diet for pigs, UPF does not have detrimental effects on gut microbiota, intestinal integrity, and blood mineral homeostasis.

Keywords: former food products, next-generation sequencing, Ussing chamber, sustainability, dietary intervention

Introduction

Food security is currently addressing the shortage of land, water, and energy and the need to produce more food using fewer natural resources [1]. Quality and quantity and therefore food security depend on the industrial processing of food. In recent years, the society raised the awareness about the impact that processing generates on the nutritional value of food. According to the NOVA classification, food products can be classified into *1*) unprocessed or minimally processed food, *2*) processed culinary ingredients; *3*) processed food, and *4*) ultra-processed food (UPF) [2]. Almost all the food produced is processed to some extent but this study only focuses on UPF, defined as "formulations of ingredients typically created by series of industrial techniques and processes," such as sweet or savory packaged snacks, mass-produced packaged bakery products (bread, cakes, etc.), margarines and other spreads, biscuits, breakfast cereals, and many other products [3]. Usually, UPF contains high levels of refined carbohydrates and fats [3], specifically sugars, starches, oils, and then also proteins. Some of

Abbreviations: AA, amino acid; ASV, amplicon sequence variant; BW, body weight; D-Gluc, D-glucose; F, finishing; FFP, former food product; G, growing; LEfSe, linear discriminant analysis effect size; SA, salty diet; SGLT1, sodium-glucose transporter; ST, standard diet; SU, sugary diet; TEER, transepithelial electrical resistance; TJ, tight junction; UPF, ultraprocessed food; VFA, volatile fatty acid; ΔIsc, delta short-circuit current.

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these nutrients are modified by hydrolysis, hydrogenation, or other physical/chemical/thermal processes. Examples are extrusion, molding, and pre-frying, through which unmodified and modified food substances are assembled with little or no food. Furthermore, the use of high temperature leads to the non-enzymatic production of high levels of advanced glycation end products from proteins and glycated lipids from fats. Preservatives are also used in processed food and UPF to elongate the biological duration, the marketability of the product and to reduce the potential proliferation of microorganisms [3]. Food additives such as coloring and flavoring additives, emulsifiers, sweeteners, thickeners and antifoaming, bulking, carbonating, and glazing agents are used only for UPF to make them more palatable [3].

The human consumption of UPF is positively associated with high glycaemic responses and a low satiety potential [4], and also creates an environment in the gut that selects specific microbes that can potentially activate inflammatory processes at local level [5]. The main outcomes are increased obesity [6], hypertension [7], coronary [8] and cerebrovascular diseases [9,10], dyslipidaemia [11], metabolic syndrome [12], and gastrointestinal disorders [13]. The pathological conditions reported above have been mainly related to the high levels of sugar and sweeteners, partially through the gut microbiota [14,15]. Indeed, an increased consumption of sugars and sweeteners influences the composition of the carbohydrate pool available to the gut microbial community. This can lead to the creation of distinct microbial populations in the gut, which are characterized by the presence of endogenous or exogenous microbes, of which some can be pathogenic [16]. When consumed at high doses, glucose is known to enhance the absorption in the intestinal epithelium [17] by increasing the permeability of the tight junctions (TJs) and changing the distribution of the main proteins in the TJs, as reported only in the Caco-2 cell line, thus suggesting intercellular leakage [18]. It is known that salt in high concentration alters the osmolarity. As like glucose, salt increases the permeability of the intestinal epithelium modulating the action of the TJs [19]. Regarding the effect of salt on microbiota, few data are available. It was observed that high salt concentration increased the abundance of *Lachnospiraceae* and *Ruminococcus* genus, while decreasing the abundance of *Lactobacillus* genus [20]. Also, high salt concentration increased the *Firmicutes/Bacteroidetes* ratio [20], a known marker of intestinal homeostasis that is related to dysbiosis [21]. Other food additives, such as surfactant agents, have been related to increased intestinal permeability and P-glycoprotein inhibition, possibly by decreasing the hydrophobicity of the mucus layer [22]. In light of this, processed food and UPF also contain compounds and nutrients such as glutamine and polyphenols that are known to potentially protect the integrity of the intestinal barrier [22]. The overall effect of UPF on human gut microbiota was confirmed by Atzeni et al. [23], who observed that the high consumption of UPF by senior subjects was positively associated with the abundance of specific taxa, such as *Alloprevotella*, *Negativibacillus*, *Prevotella*, and *Sutterella*, associated with inflammatory gastrointestinal diseases occurrence.

In recent years, the use of UPF as feed ingredients for farm animals is considered an innovative solution for a more sustainable livestock food production. This is because of the high amounts of UPF that is lost and/or wasted by the food industry due to logistical or technical reasons [24]. Given that pigs use of a lot of feed ingredients that could be directly be consumed by humans, the replacement of human-edible unprocessed grains with UPF [in such context also called former food products (FFPs)] in the diet of farm animals could reduce the competition between feed and food and hence reduce the use of natural resources [24]. The hypothesis of this study was that the inclusion of UPF to replace the 30% of traditional ingredients in the pigs' diet would affect the gut microbial community both qualitatively and quantitatively. In addition, the high content of simple sugars and salt could promote a leaky gut condition and an insulin resistance. Thus, this study aimed to clarify if the long-term replacement of slight processed ingredients by sugary or salty UPF in pigs' diet could lead to detrimental effects on gut microbiota, small intestinal physiology, selected serum metabolites of the animals, and insulin secretion.

Methods

Animals, diets, and slaughtering procedure

This study was a continuation of Mazzoleni et al. [25] and details about rearing conditions, diets, and slaughter procedure are reported there. Briefly, 36 Swiss Large White male castrated piglets were reared in a single-group pen equipped with 3 single-space computerized feeders (Mastleistungsprüfung MLP-RAP; Schauer Agrotronic AG), which allowed for recording individual feed intake. The body weight (BW) of all animals was monitored weekly. Three dietary treatments were fed to the pigs when they reached ~20 kg BW (start of the grower period), including: standard (ST), salty (SA), and sugary (SU).

The SA and SU diets were formulated including products such as savory packaged snacks, pasta, bread or candies, chocolate, breakfast cereals, cookies, for salty and sugary diets, respectively. The 3 experimental diets underwent identical processing procedures and both SA and SU diets were sourced from the same foodstuff processing company. The chemical composition of the pure SA and SU FFPs used to formulate the experimental diets was similar to the 2 pure FFPs used for the diets in post-weaned piglets by Luciano et al. [26]. The grower and finisher diets were formulated following the Swiss feeding recommendations for pigs [27] (Table 1). The standard grower diet (ST-G) and the standard finisher diet (ST-F) were formulated considering a reference BW of 40 and 80 kg, respectively. For the SA and SU grower (SA-G and SU-G, respectively) and finisher (SA-F and SU-F, respectively) diets, a portion of conventional ingredients such as cereals and fats included in the ST-G and ST-F diets were replaced by 30% salty and sugary FFPs. During the entire trial and samples collection, the names of the diets were blinded. The pigs had ad libitum access to fresh water and to the grower and finisher diets from 20 to 60 kg BW and from 60 kg BW to slaughter, respectively. The grower and finisher diets were formulated to be isoenergetic and isonitrogenous.

Pigs were slaughtered at the Agroscope research slaughterhouse after fasting for 16 h [28] when they reached ~110 kg BW. The animals were stunned with carbon dioxide, after which they were exsanguinated, scalded, mechanically dehaired, and eviscerated.

TABLE 1
Dietary ingredients used for experimental diets in the growing (G) and finishing (F) periods

	Dietary treatments[1]					
	Grower			Finisher		
	SA-G	ST-G	SU-G	SA-F	ST-F	SU-F
Ingredient (%)						
Barley	39.7	41.1	38.0	41.3	46.4	41.8
Wheat	—	30.0	—	—	30.0	—
Salty FFPs[2]	30.0	—	—	30.0	—	—
Sugary FFPs[3]	—	—	30.0	—	—	30.0
Fat	—	2.69	0.79	—	2.22	0.68
Potato protein	5.00	5.00	5.00	5.00	5.00	5.00
Soybean meal	6.16	6.59	7.36	2.77	3.55	4.03
Wheat bran	9.06	4.34	8.76	12.3	3.93	9.87
Dried beet pulp	5.15	5.15	5.15	4.50	4.50	4.50
L-Lysin-HCl	0.26	0.26	0.23	0.12	0.10	0.09
DL-Methionine	0.01	0.02	0.02	—	—	—
L-Threonine	0.03	0.02	0.02	—	—	—
L-Tryptophan	—	—	0.002	—	—	—
MCP	0.45	0.45	0.47	0.11	0.13	0.16
Lime, carbonic acid	1.51	1.53	1.48	1.17	1.20	1.19
Sodium chloride	—	0.16	—	—	0.27	—
Pellan[4]	0.30	0.30	0.30	0.30	0.30	0.30
Celite 545	2.00	2.00	2.00	2.00	2.00	2.00
ALP-S 467 Mast[5]	0.40	0.40	0.40	0.40	0.40	0.40
Natuphos 5000 G	0.01	0.01	0.01	0.01	0.01	0.01
Analyzed nutrient composition (g/kg DM)						
Total ash	74.1	68.4	72.1	65.4	61.7	64.1
Crude fat	53.2	52.2	61.3	53.4	45.3	58.9
Crude protein	174	173	176	151	152	153
Crude fiber	39.5	41.6	38.6	38.9	42.2	39.6
Sodium	3.74	1.25	1.51	3.21	1.66	1.73
SFA	11.7	18.4	17.7	11.7	16.1	20.3
MUFA	26.2	18.5	20.2	29.4	14.5	25.4
PUFA	15.8	17.1	14.1	17.6	16.1	17.9
Calculated						
Digestible P (g/kg DM)	2.87	2.87	2.87	2.19	2.19	2.24
Digestible lysine (g/kg DM)	8.29	8.29	8.29	6.24	6.24	6.24
DE (MJ/kg DM)	13.7	13.7	13.7	13.7	13.7	13.7
ME (MJ/kg DM)	13.2	13.2	13.2	13.3	13.3	13.3

Abbreviations: DE, digestible energy; DM, dry matter; MCP, monocalcium phosphate; ME, metabolizable energy; SA, salty ultraprocessed food-based diet; ST, standard diet; SU, sugary ultraprocessed food-based diet.

[1] All grower diets were formulated for pigs with a body weight (BW) of 40 kg; all finisher diets were formulated for a BW of 80 kg. ST-G, ST-F = standard diet without former foodstuff product (FFP) inclusion for growing (G) and finishing (F) pigs, respectively. SA-G and SA-F = grower and finisher diets where a part of the cereals and fats were replaced with 30% salty FFPs. SU-G and SU-F = diets where a part of cereals and fats were replaced with 30% sugary FFPs for growing (G) and finishing (F) pigs, respectively.

[2] Pure salty former foodstuff products.

[3] Pure sugary former foodstuff products.

[4] Binder that aids in pellet formation.

[5] Mineral-vitamin premix that supplied the following nutrients per kg of diet: 20,000 IU vitamin A, 200 IU vitamin D3, 39 IU vitamin E, 2.9 mg riboflavin, 2.4 mg vitamin B6, 0.010 mg vitamin B12, 0.2 mg vitamin K3, 10 mg pantothenic acid, 1.4 mg niacin, 0.48 mg folic acid, 199 g choline, 0.052 mg biotin, 52 mg Fe as $FeSO_4$, 0.16 mg I as $Ca(IO)_3$, 0.15 mg Se as Na_2Se, 5.5 mg Cu as $CuSO_4$, 81 mg Zn as ZnO_2, and 15 mg Mn as MnO_2.

Sample collection, DNA extraction, and sequencing

The collection of fecal samples from the rectal ampulla occurred at 3 different time points: before starting feeding the experimental diets (T1); 1 d before the end of the growing period (T2, 47.4 ± 0.6 d on feed), and 1 d before the slaughter (T3, 94.5 ± 1.2 d on feed). Samples were immediately snap-frozen in liquid nitrogen and stored at −80°C until analysis. Starting with 200 mg feces, the DNA was extracted with the QIAamp Fast DNA Stool Mini Kit (QIAGEN) following the manufacturer's instructions and quantified with Nanodrop ND2000. The universal primers for prokaryotic (341F/802R:CCTACGGGNGGCWGCAG/GACTACHVGGGTATCTAATCC, respectively) were used to amplify by PCR the V3 and V4 regions of the 16S rRNA gene. The amplicons were sequenced by BMR Genomics through the Illumina MiSeq platform and a v2 500 cycle kit. The paired-end reads obtained were tested for chastity and subjected to demultiplexing and trimming by Illumina real-time analysis software v2.6. The read quality was checked by FastQC v0.11.8. USEARCH v11.0.667 was used to trim forward and reverse reads of the paired-end reads.

Tissue recovery for ex vivo analysis

At the slaughterhouse, intestinal segments from the third-meter distal to the pylorus were removed within 15 min after exsanguination. A cold (4°C) saline solution (Phosphate Buffered Saline pH 7.4, Bioconcept Ltd) was used to remove the intestinal content, then tissues were stored in a serosal buffer solution (see the following). Before mounting in the Ussing chamber device (Physiologic Instruments) equipped with8 chambers, the outer muscle layers have been removed from the tissues. Each experiment started within 30 min from the tissue recovery. A minimum of 6 independent Ussing chamber experiments per each group were performed. Each experiment was carried out using intestinal tissues from 2 pigs mounted in 4 different chambers per pig. Thus, a minimum of 6 biological and 24 technical replicates per experimental group were used.

Ussing chamber experimental procedure

The jejunum tissue (exposed area of 1 cm^2) was mounted on an Ussing chamber for the evaluation of D-glucose (D-Gluc) and amino acid (AA) transport across intestinal epithelial cells. The chambers were filled with 4 mL Krebs–Ringer mucosal buffer (115 mmol/L NaCl, 2.4 mmol/L K_2HPO_4, 0.4 mmol/L KH_2PO_4, 1.2 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, and 25 mmol/L $NaHCO_3$−aHCO 25 mmol/mucosal buffer 4) also contained 10 mmol/L glucose as an energy source that was osmotically balanced with 10 mmol/L mannitol in the mucosal buffer (pH 7.4). Indomethacin was added in both the mucosal and serosal buffers at a final concentration of 0.01 mmol/L. Buffers were continuously perfused with a 95% oxygen and 5% carbon dioxide gas mixture. The temperature was kept constant at 37°C by a circulating water bath. After a 30–40-min equilibration period, baseline short-circuit current (Isc) (in mV) values were measured. The transepithelial electrical resistance (TEER) was also measured at 2-min intervals under current clamped conditions. The TEER was determined at an applied current of 100 mA, and the Isc was calculated using Ohm's law ($R = V/I$). Furthermore, D-Gluc and AA uptake was performed according to the following protocol: after the stabilization period (10–15 min), 10 mmol/L D-Gluc was added to the mucosal buffer, followed by the addition of the same concentration of L-Arg, L-Meth, and L-Glut. The substrates were added in the aforementioned order at intervals of 15 min. D-Gluc or each AA addition was kept in an equilibrated osmotic condition by the addition of equimolar (10 mmol/L) mannitol on the serosal side. Forskolin (10 μmol/L) was added to the serosal compartment at the end of the experiment to test tissue viability. Active uptake was evaluated according to electrical changes in the short circuit. The total active transport through the tissue was verified by monitoring the change in short-circuit current (ΔIsc), which was representative of ion flux, and thus active transport within the jejunal tissues. Only tissues showing a change in the Isc generated by the addition of forskolin were considered for the data analysis.

Blood collection and serum urea, calcium, magnesium, and potassium analysis

Blood was sampled directly during bleeding after carbon dioxide stunning using blood collection tubes with serum clot activator (Vacuette; Greiner Bio-One GmbH), which were stored upside down at room temperature for 1 h before processing. The Vacuette serum tubes were then centrifuged for 15 min at 3000 g and subsequently for 2 min at 4000 g. Two aliquots of serum were stored at −20°C in Eppendorf tubes. Levels of blood urea (UV Urease-GLDH), calcium (Calcium O-Cresolftalein Complexone), and magnesium (Magnesium Xylidyl Blue) were measured in the serum using commercial kits provided by Biotecnica Instruments Spa following manufacturers' procedure using an autoanalyzer BT 1500 (Biotecnica instruments Ltd), whereas potassium was quantified by using the Stat Profile PrimeVet ES electrolyte analyzer (Nova Biomedical).

Insulin secretion test

Eight pigs were randomly selected from each treatment at the beginning of the experiment to undergo the insulin secretion test. Once these pigs reached a BW of 40 kg ($n = 4$) and 80 kg ($n = 4$), they were transferred to clean individual pens for a 2-h period after fasting overnight. Then, 1 kg of feed (SA, SU, or ST growing and finishing diets at 40 and 80 kg BW, respectively) was offered to each pig and 1 h later a blood sample was collected by the jugular vein. This specific time point was selected to ensure that all the pigs could consume the entire kilogram of feed and to standardize blood sampling. To minimize stress for the animals, only one blood sample was taken per pig.

Plasma samples were further obtained as described above. The commercial Porcine Insulin ELISA kit (10-1200-01, Mercodia AB) was used to quantify insulin concentration according to manufacturer's protocol. The detection limit was 1.15 mU/L as determined with the methodology described in the manufacturer's manual.

Intestinal volatile fatty acids quantification

The volatile fatty acid (VFA) profile in feces was determined by HPLC Briefly, feces samples previously weighed and frozen at −20°C with 1 mL of phosphoric acid (25%, w/v) were thawed. After defrosting, 1 mL of internal standard (pivalic acid at 1%, w/v) and 18 mL of distilled water were added into the tube. This preparation was shaken for 3 h at room temperature before being centrifuged for 5 min at 4000 g. The supernatants were filtered and analyzed for VFA using a liquid chromatography (Ultimate 3000, Thermo Fisher Scientific) with an exchange ion column (Nucleogel ION 300 OA 300 × 7.8 mm) and equipped with a refractive index detector (RefractoMax 521, Thermo Fisher Scientific).

Statistical analysis

All microbiota data analyses were run in R v4.0.3. The R packages used were phyloseq v1.26.1, vegan v2.5–5, microbiome v1.12.0, and microbiomeutilities. v1.00.14. The alpha diversity indexes used were the number of amplicon sequence variant (ASVs) and Chao1, Simpson, and Shannon indexes (microbiome package, v.1.12.0). Both the weighted and unweighted Unifrac distances were calculated on rarefied ASVs. Both the variance (permutational multivariate analysis of variance, PERMANOVA) and similarities (analysis of similarity, ANOSIM) of the tested groups were also calculated. The linear discriminant analysis effect size (LEfSe) between groups was calculated using the following conditions: alpha value <0.05 for the Kruskal–Wallis sum-rank test among the classes; threshold >3.0 on the logarithmic linear discriminant analysis score [29]. To estimate the common core microbiota, the "microbiome"

library was used (detection threshold: 0.001, prevalence: 80/100).

Multivariate analysis was conducted using MaAsLin to investigate associations between microbial abundances (from the domain to genus taxonomic level) and fecal VFAs and blood serum measurements. Default settings were used for this analysis, specifically: maximum false discovery rate (significance threshold) = 0.05. Minimum for feature relative abundance filtering = 0.0001. Minimum for feature prevalence filtering = 0.01.

Data about fecal VFAs were analyzed by repeated measures analysis of variance using linear mixed-effects regression models (Lmer) [30] implemented in R (version 4.0.5). The model contained the treatment and the time point as fixed effects, whereas the pig was considered as random effect. The model for Ussing chamber, blood serum and measurement, speed of food consumption, and insulin secretion test did not include the time effect. For pairwise comparisons, a modified Tukey test for multiple comparisons of means, the Sidak function was used. Statistical means and SEM were calculated with the lsmeans function from the package emmeans [31]. Residuals of Lmer models were checked for normality and homoscedasticity. Differences were considered significant for $P < 0.05$.

Results

Performance

Detailed information about the effect of UPF inclusion on growth performance and feeding behavior can be found in

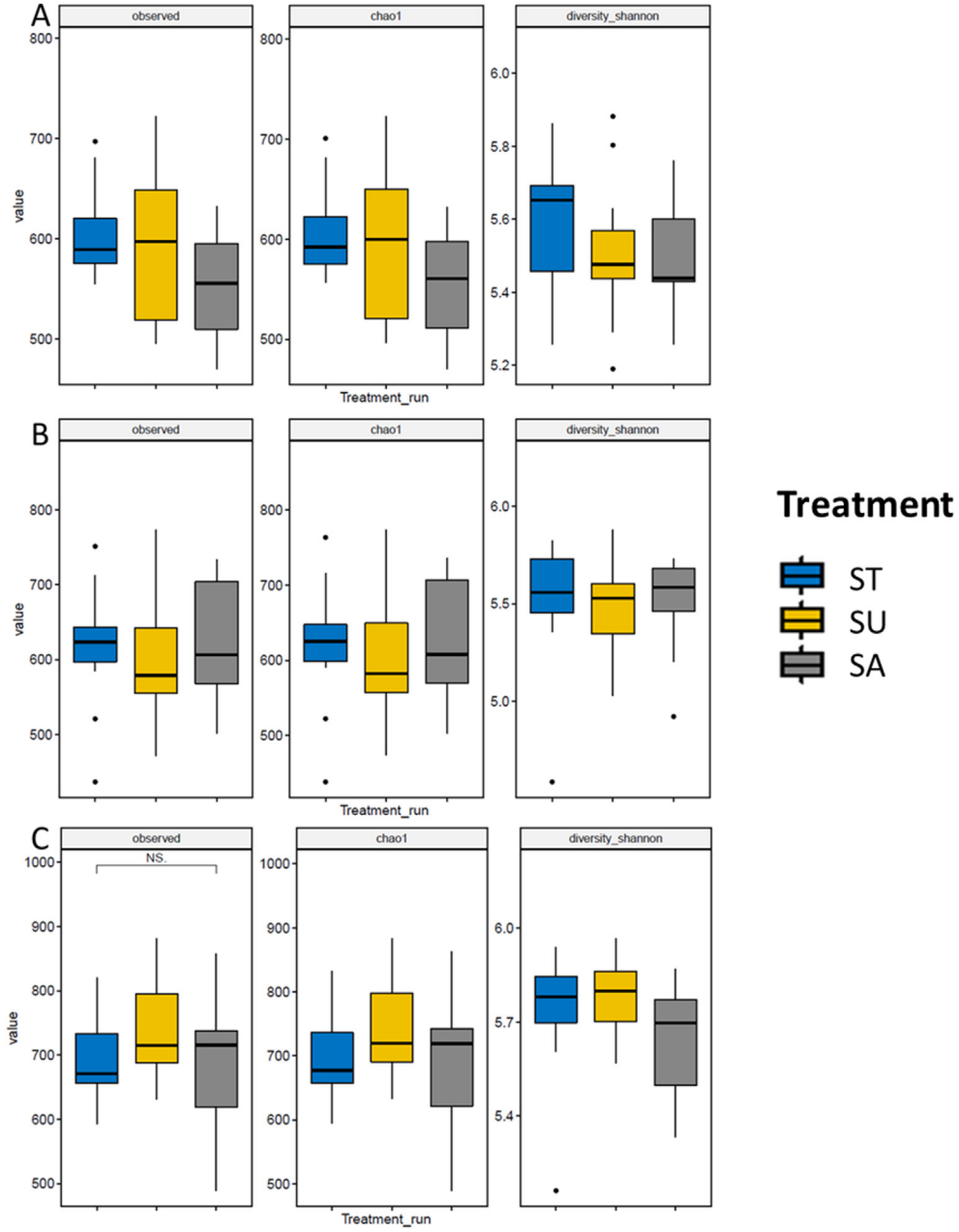

FIGURE 1. Nonphylogenetic diversities at the (A) T1 (20 kg BW), (B) T2 (60 kg BW), and (C) T3 (100 kg BW) of fecal microbiota from pigs fed either a basal grower-finisher diet or the basal diet with 30% salty (SA) or sugary (SU) UPF. BW, body weight; UPF, ultraprocessed food.

Mazzoleni et al. [25]. Briefly, UPF did not influence the average daily gain, average daily feed intake, feed conversion ratio, or BW of the pigs at slaughter. The average daily fat intake was higher ($P < 0.05$) in pigs fed the SU diet, even though both categories of UPF had no effects on the parameters related to the pigs' body composition (for example, average daily fat weight gain).

Microbiota analysis

Fecal samples were obtained from 36 pigs at T1, T2, and T3. At T1, from 1 SA and 1 ST piglets, it was not possible to obtain fecal samples. Therefore, a total of 106 samples have been analyzed. Because of the low number of sequences obtained in 2 samples (1 from ST treatment at T1 and 1 from SU treatment at T3), they have been removed from the dataset. The rarefaction curve showing the sequencing depth is reported in Supplemental Figure 1.

Nonphylogenetic diversities and composition

Considering the overall period, the diets did not influence the observed ASV, the Chao1, and the Shannon indexes (data not showed). Similarly, over time no effect of UPF inclusion on the bacterial abundance or biodiversity was found (Figure 1).

As expected, statistically significant differences were found when considering the effect of the pig's age, with increasing abundance and biodiversity with increasing age (Figure 2).

The composition plots at family level of the fecal microbiota of pigs at the 3 different time points are reported in Figure 3. No differences can be observed between the 3 dietary treatments in each time point.

An effect of the time can be observed at family level, in particular regarding the abundance of the Prevotellaceae family that linearly decreased ($P < 0.05$) with time (Figure 4). The Firmicutes/Bacteroidetes ratio was similar between the treatments in each time point (data now showed).

Beta-diversities and core microbiota

The diet did not affect the unweighted or the weighted beta-diversity. Specifically, for the unweighted beta-diversity, the PERMANOVA showed no differences between the treatment groups at T1 ($P = 0.141$, $R_2 = 0.06$), at T2 ($P = 0.202$, $R_2 = 0.06$), and at T3 ($P = 0.068$, $R_2 = 0.06$). Similarly, the weighted beta-diversity was similar among treatment groups at T1 ($P = 0.612$, $R_2 = 0.05$), T2 ($P = 0.775$, $R_2 = 0.04$), and T3 ($P = 0.178$, $R_2 = 0.06$). As expected, the time point strongly influenced the beta-diversity. Both the unweighted (Figure 5A) and the weighted (Figure 5B) Unifrac beta-diversity showed a clear cluster ($P < 0.001$) of the fecal microbial community between T1, T2, and T3. Specifically, the unweighted beta-diversity determined at T1 differed from T2 ($P = 0.048$) and tended to differ from T3 ($P = 0.058$). No differences were observed between T2 and T3 ($P = 0.684$). The weighted beta-diversity differed between T1 and T3 ($P = 0.032$), but not from T2 ($P = 0.838$). The weighted beta-diversity tended to differ between T2 and T3 ($P = 0.055$).

The core microbiota composition at T2 and T3 was similar between the 3 dietary groups. The core microbiota of ST and SU pigs exhibited greater similarity, with 9 and 8 ASVs, respectively. In contrast, the core microbiota of the SA group at T3 consisted of 12 ASVs (see Figure 6).

Linear discriminant analysis of effect size

We conducted a LEfSe to identify potential biomarkers among the 3 dietary groups. At T1, no significant differences in taxa were observed between the groups (data not shown). However, at the genus level, biomarkers were detected between the ST, SU, and SA groups at T2 and T3 (as shown in Figure 7A and B, respectively). Both SU and SA diets had a higher number of biomarkers compared with ST at both T2 and T3. For both the time points, the main biomarker of the SU group was the Anaerostipes genus, whereas for ST group was an unclassified genus of the Ruminococcaceae family at T2 and an unclassified

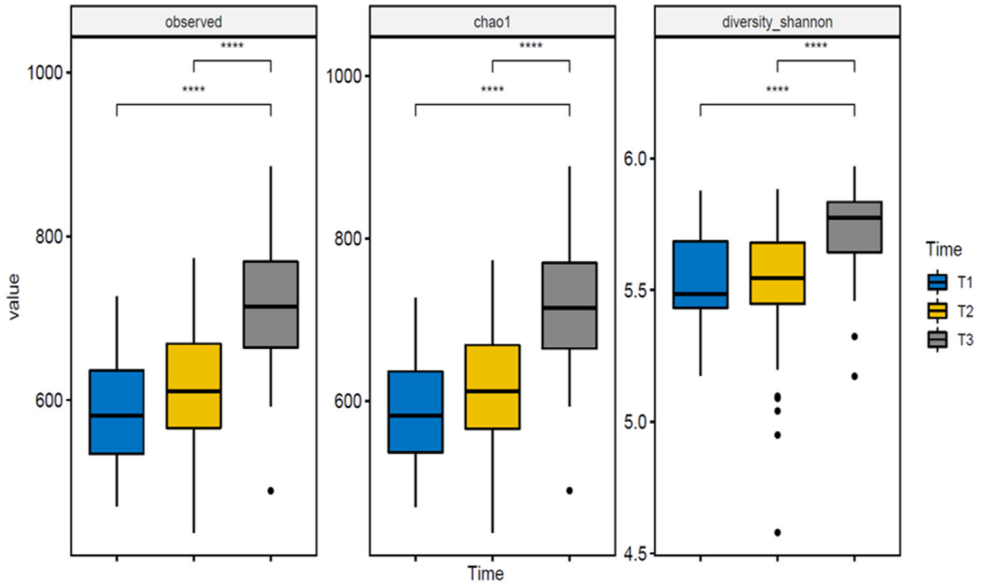

FIGURE 2. Independent of the diet, nonphylogenetic diversities at T1 (20 kg BW), T2 (60 kg BW), and T3 (100 kg BW). ****P value <0.001. BW, body weight.

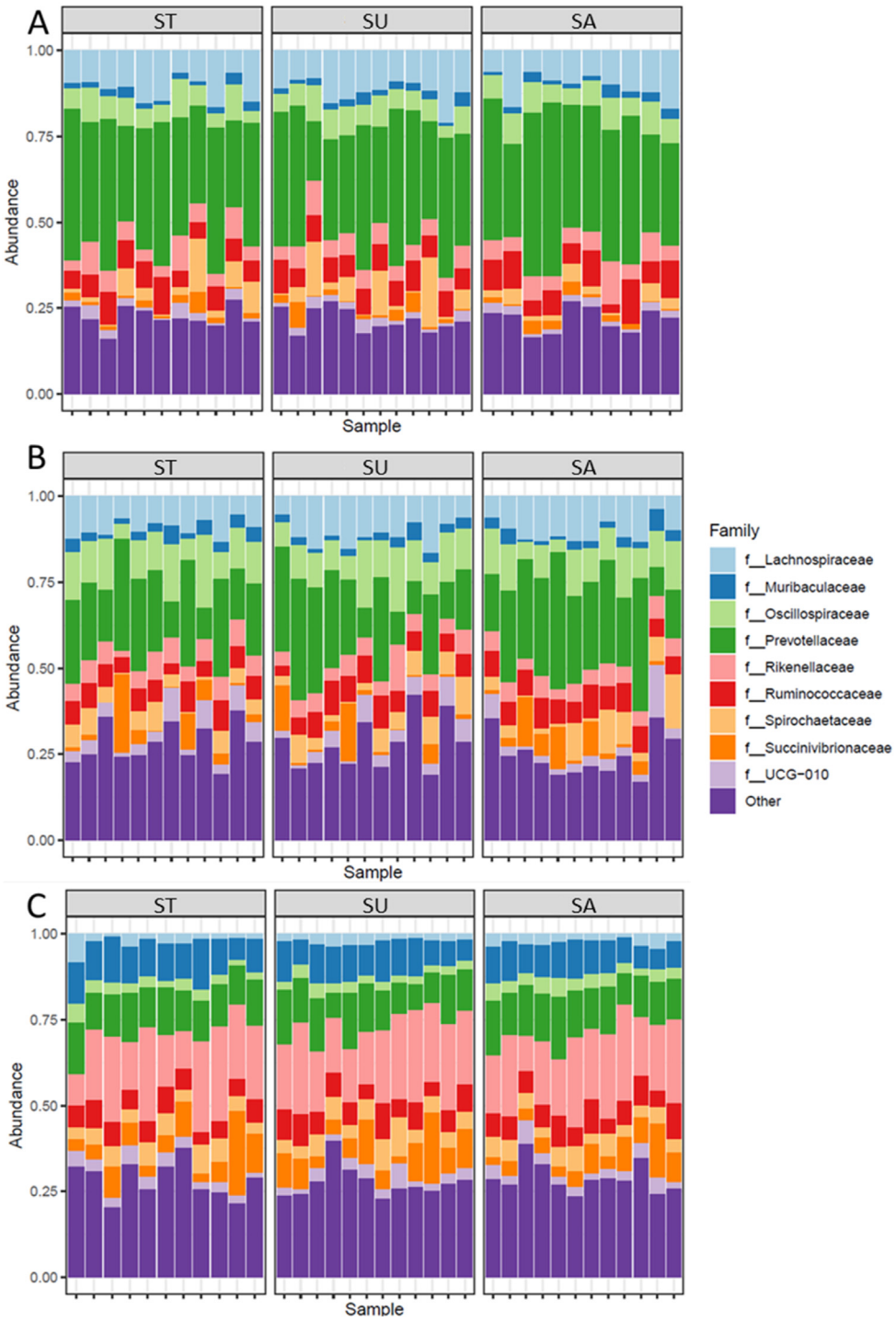

FIGURE 3. Relative abundance of fecal microbiota families at (A) T1 (20 kg BW), (B) T2 (60 kg BW), and (C) T3 (100 kg BW) from pigs fed either a basal grower-finisher diet or the basal diet with 30% salty (SA) or sugary (SU) UPF. BW, body weight; UPF, ultraprocessed food.

genus of the Lachnospiraceae family at T3. The main biomarkers for the SA group were the genera CAG-352 and p-2534-18B5 gut group at T2 and T3, respectively (Figure 6A and B).

Fecal VFAs

VFAs were quantified in the feces at T1, T2, and T3 (Table 2). The diet did not affect any of the VFAs analyzed during the overall period. Propionate and valerate were affected by the time point, but not acetate and butyrate. Also, propionate and butyrate level was lower in T1 than T2 and T3 in ST.

Values of VFAs in feces were also combined with NGS data to investigate correlations between bacterial taxa and VFAs level in feces though the MaAsLin analysis. Several positive and negative associations between specific bacterial taxa and VFAs level were found (Figure 8). Only the top 50 associations with a *P* value

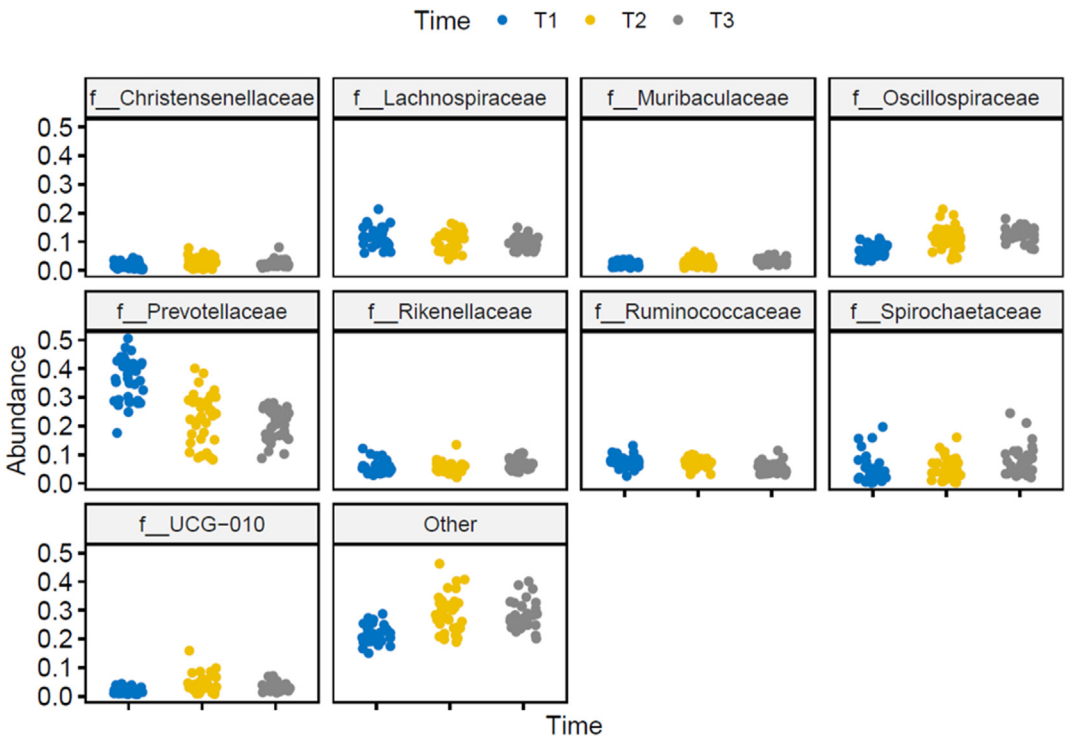

FIGURE 4. Composition plots of pigs' fecal microbiota families at the T1 (20 kg BW), T2 (60 kg BW), and T3 (100 kg BW). BW, body weight.

<0.05 are reported. Among all the correlations found between the microbiota data and VFAs, only few taxa correlated with both VFAs and a specific dietary treatment. Specifically, the Anaerostipes genus was a biomarker of the SU group and at the same time positively correlated with propionate and negatively correlated with butyrate. Similarly, unclassified ASV301, mycoplasma genus, an uncultured Ruminococcaceae family, clostridium sensu stricto, and an uncultured prokaryote specie belonging to the Christensenellaceae family negatively correlated with SU but positively correlated with propionate and negatively with butyrate, valerate and, with the exception of the unclassified ASV301, also with acetate. Only the unclassified ASV301 negatively correlated with SA group (Figure 8).

Jejunum nutrients active uptake and transepithelial integrity

The ex vivo trial was performed to further investigate the effect of SU and SA diets on the small intestinal physiology. The use of UPF in pigs' diets did not affect the active D-Gluc uptake in the jejunum nor the active uptake of the AAs L-arginine and L-methionine (Table 3). However, the active uptake of the L-glutamate was lower in the SA group compared with the SU group. The intestinal integrity, represented by the TEER, was also similar between the 3 experimental groups (Table 3).

Effect of salty and sugary processed food on serum urea, minerals, and insulin

The SA and SU diets had no effect ($P > 0.05$) on serum urea, calcium, magnesium, and potassium concentrations compared with pigs fed the ST diet (Table 4).

All the pigs completely consumed the kilogram of feed during the insulin test. The average speed of feed consumption was 36.4 ± 2.23 and 39.1 ± 1.85 g/min at BW40 and BW80 pigs, respectively. In each time point, the speed of feed consumption was similar between groups (Supplemental Table 1).

Despite the distinct characteristics of SA and SU products compared with the conventional feed ingredients utilized in the ST diet, the dietary treatment did not impact the release of serum insulin at a BW of 40 kg. However, it significantly ($P = 0.011$) reduced the insulin concentration in SU (20.7 ± 10.1 milliunit/L) compared with the ST (144.5 ± 25.2 milliunit/L) diet after 1 h of feeding at a BW of 80 kg (see Figure 9). No significant relationship between the microbiota and the serum parameters analyzed has been found.

Discussion

UPF and fecal microbiota

To our knowledge there are very few studies that used the pig as model to investigate the effects of UPF on gut microbiota and physiology. One study examined the effects of a maternal Western diet during gestation and lactation [32]. The authors demonstrated that the Western diet modified offspring's microbiota activity in Yucatan pigs [32]. However, the ingredients used in the Western diets were not ultraprocessed and diets differed mainly in sugar and fat content and not for their processing levels. It is therefore difficult to attribute the effects observed to the processing of the ingredients used. In this study, the standard and experimental diets were similar in energy, protein, and fiber content and the observed effects can be related to the 30% replacement of traditional ingredients by UPF. The gut microbiota refers to the complex community of microorganisms, including bacteria, fungi, and viruses that reside in the digestive tract. In this study, by microbiota we refer only to the bacterial community characterized in the pigs' feces. Surprisingly, the use of UPF did not affect the abundance or the

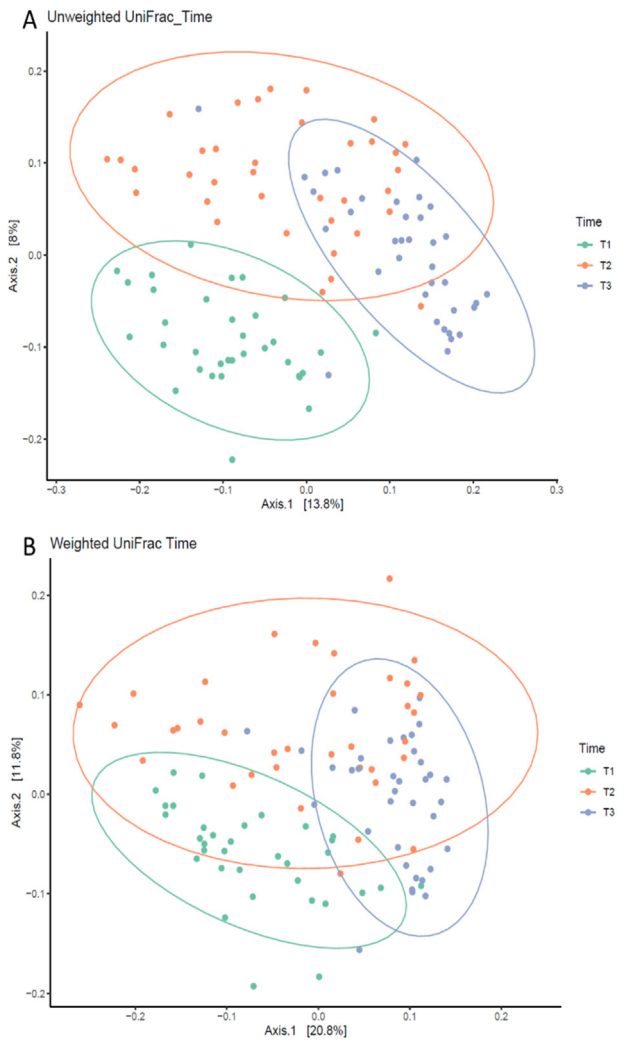

FIGURE 5. (A) Unweighted and (B) weighted UniFrac beta-diversity distances of the pigs' fecal microbiota at the T1 (20 kg BW), T2 (60 kg BW), and T3 (100 kg BW). BW, body weight.

biodiversity indexes in the fecal microbiota of pigs, independently of the age of the pigs and the sugar and salt content of the diets. This is in contrast to what has been observed in human studies where a Western diet is normally associated with lower bacterial richness and biodiversity [9]. A reason could be that although human studies often associate the UPF consumption with a lower consumption of fiber and complex carbohydrates, in our study the 3 experimental diets were similar in fiber and also energy content [9]. Even if a comparison between human and pig is not possible because of the different physiology, exposure time to UPF, and different chemical composition of the diets, our study suggests that in pigs, the food processing alone do not impair the abundance and the biodiversity of the fecal microbiota when UPF replace 30% of the standard ingredients. The Firmicutes to Bacteroidetes ratio was also unaffected by the UPF. The literature reports that a higher Firmicutes to Bacteroidetes ratio is related to a decrease in diarrhea in pigs, with a strong negative correlation between Firmicutes and pathogenic bacterial population in the intestine [33].

The absence of adverse impacts on the Firmicutes to Bacteroidetes ratio is promising for the potential use of UPF without affecting the gut health in pigs.

As expected, both the abundance and the diversity of the bacterial community increased with the age of the pigs, in accordance with the literature [34]. The core microbiota was moderately influenced by the presence of UPF in pig diets. Slight effects could be observed in the finishing period, in particular in the core microbiota of pigs fed the SA diets compared with the ST and SU diets. Given that the primary distinction between the SU and SA UPF lies in their salt and sugar content, one might hypothesize that the salt exerts a more significant influence than refined sugar in modulating the core microbiota, as reported by Hamad et al. [35] and Smiljanec and Lennon [36]. The core microbiota of the ST and SU groups were characterized by 9 and 8 ASVs, respectively, whereas the one of the SA group was composed by 12 ASVs. The intestinal core microbiota is defined as the number and the identity of bacteria that are shared among different individuals. The core microbiota therefore focuses only on the stable and permanent members of the bacterial community [37].

It is hypothesized that these shared taxa represent the most ecologically and functionally significant microbial associates of the host or environment under the sampled conditions. Indeed, it has been suggested that identifying core microbiome components may aid in addressing various topics, including the maintenance of gut health [38]. In this study, the ASVs that constitute the core microbiota of the pigs fed the ST diet are also present in the core microbiota of the SU and SA groups, and given that the experimental diets did not reduce the size of the core microbiota, we can conclude that the UPF did not lead to any detrimental effect on the pig gut core microbiota.

The LEfSe analysis performed at the genus level showed that the dietary treatment only influenced a few taxa during the trial. In fact, although at the beginning of the dietary treatment no taxa abundance was found to be significantly different between the 3 treatment groups, differences were found at T2 and T3. At T2, the SU diet was the one influencing the highest number of bacteria, with 6 ASVs being more abundant compared with the ST and SA diets. Contrastingly, in the finishing period (T3), the SA diet showed the highest number of significant differences, with 6 ASVs being more abundant compared with the other groups. The SU diet increased the abundance of the Anaerostipes genus both in the T2 and T3, compared with the other groups. Members of the Anaerostipes genus, within the phylum Firmicutes, are strictly anaerobic microrganisms with a strong glucose fermentation metabolism, resulting in the production of mainly butyrate, acetate, and lactate [39]. In fact, sugar is the main source of carbon and energy for such bacteria [39,40]. This genus represents >2 % of total colonic microbiota in the healthy human colon [41]. This finding suggests a higher presence of rapidly fermentable carbohydrates, such as sugar residues, in the large intestine of SU diet-fed pigs. It is known that the glucose uptake from the intestinal lumen to the systemic circulation takes place mainly in the small intestine and it is mediated by active [sodium-glucose transporter (SGLT1)] and passive transporters (glucose transporters) [42]. However, SGLT1 expression in the large intestine remains controversial, because SGLT1 mRNA in the proximal colon has been detected by in situ hybridization but not by PCR [43]. Therefore, taking into account the similar fiber content between the 3 experimental diets, the higher abundance of sugar-utilizing bacteria in the feces of SU-fed pigs suggests a higher amount of unabsorbed sugars

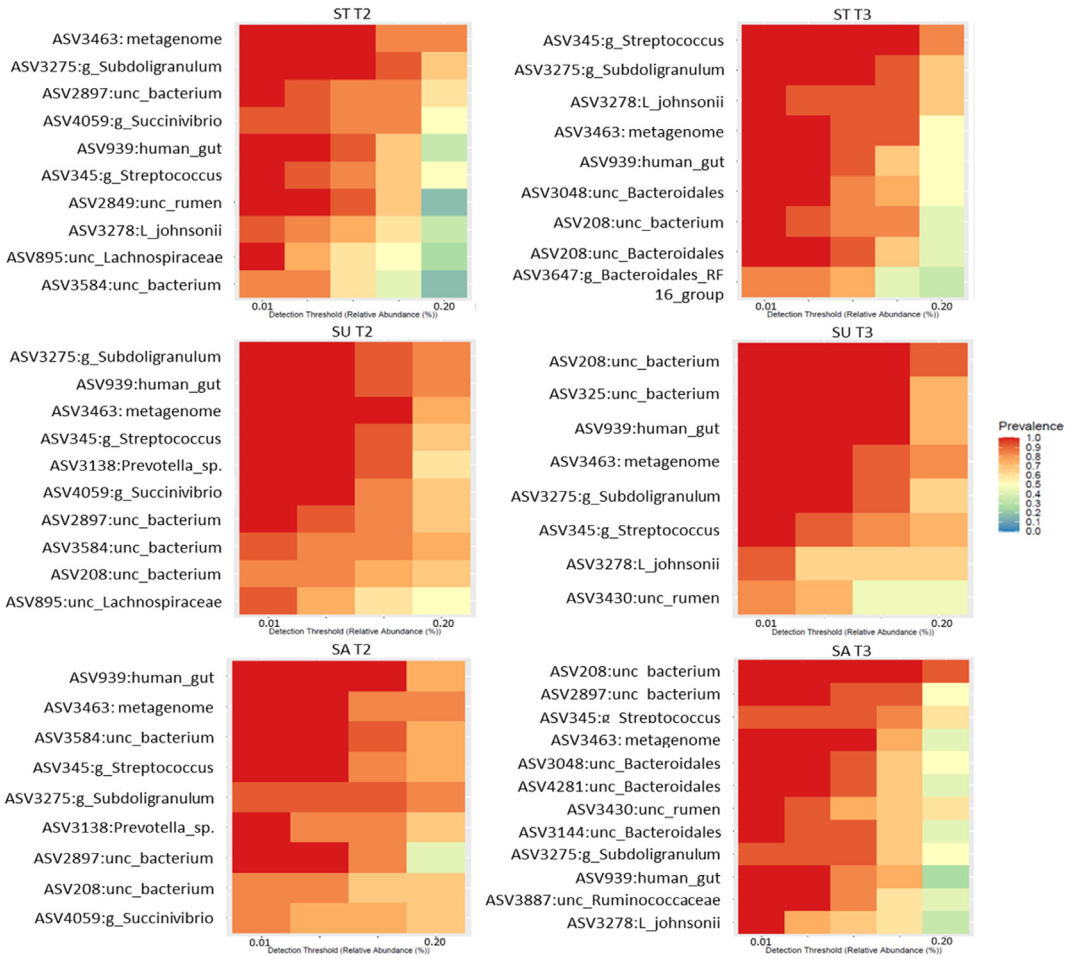

FIGURE 6. Heatmaps of the fecal core microbiota at T2 (60 kg BW) and T3 (100 kg BW) from pigs fed either a basal grower-finisher diet or the basal diet with 30% salty (SA) or sugary (SU) UPF. BW, body weight; UPF, ultraprocessed food.

reaching the large intestine in both the growing and finishing periods, compared with the ST and SA pigs. Such hypothesis is encouraged by the higher abundance of the gauvreauii group belonging to the *Ruminococcus* genus in the SU group at T2 and T3. Similarly to the *Anaerostipes* genus, also *Ruminococcus gauvreauii* produces acetic acid as major end-product of glucose metabolism and mainly utilizes D-Gluc, D-galactose, D-fructose, D-ribose, D-sorbitol, D-mannitol, inositol, and sucrose as substrate [44]. Readily fermentable carbohydrates such as starch, sugar residues, mucus, and soluble nonstarch polysaccharides have been considered substrate for bacterial growth in caecum and proximal colon of pigs also by Knudsen et al. [45]. Several of these substrates are also part of soluble fiber (for example, nonstarch polysaccharides), that can probably reach the large intestine and induce microbiota changes in this group. However, starch polysaccharides were not quantified in the large intestine content and the hypothesis cannot be confirmed by this study.

Among the most affected taxa by the dietary treatment, the genus bacteroidales p2534-18B5 and members of the Muribaculaceae family were increased by the SA diet. No information was found about the p2534-18B5 genus, but the literature reports that Muribaculaceae family regulates the community composition and metabolites of the gut microbial population and that participates in the degradation of polysaccharides, leading to the production of succinate, acetate, and propionate [46]. The increase in the Prevotellaceae UCG-003, belonging to the Prevotella genus, was already observed by our research about the use of UPF as replacement of traditional ingredients in post-weaning piglets' diets and it is probably correlated with the fermentation of non-structural carbohydrates [47].

Fecal VFAs and microbial community

The gut microbiota plays a crucial role in the production of VFAs in the intestine. Acetate, propionate, butyrate, and valerate are the main VFAs produced by the microbial fermentation of dietary fibers and complex carbohydrates that escape digestion in the small intestine and serve as substrate for microbial growth [48]. The UPF undergoes processing procedures aimed to increase mainly their digestibility. Given this characteristic, and because of our previous studies where we observed a high in vitro digestibility of the UPF-based diets for pigs [49], our hypothesis was that different amount or type of feed material would have escaped the digestion process in pigs fed the SU and SA diets, resulting in different amount of substrate for the large intestine bacterial fermentation and subsequent different VFAs production. By contrast, we did not find differences between treatments in the fecal VFAs production. The majority of the bacterial taxa that correlated (positively or negatively) with

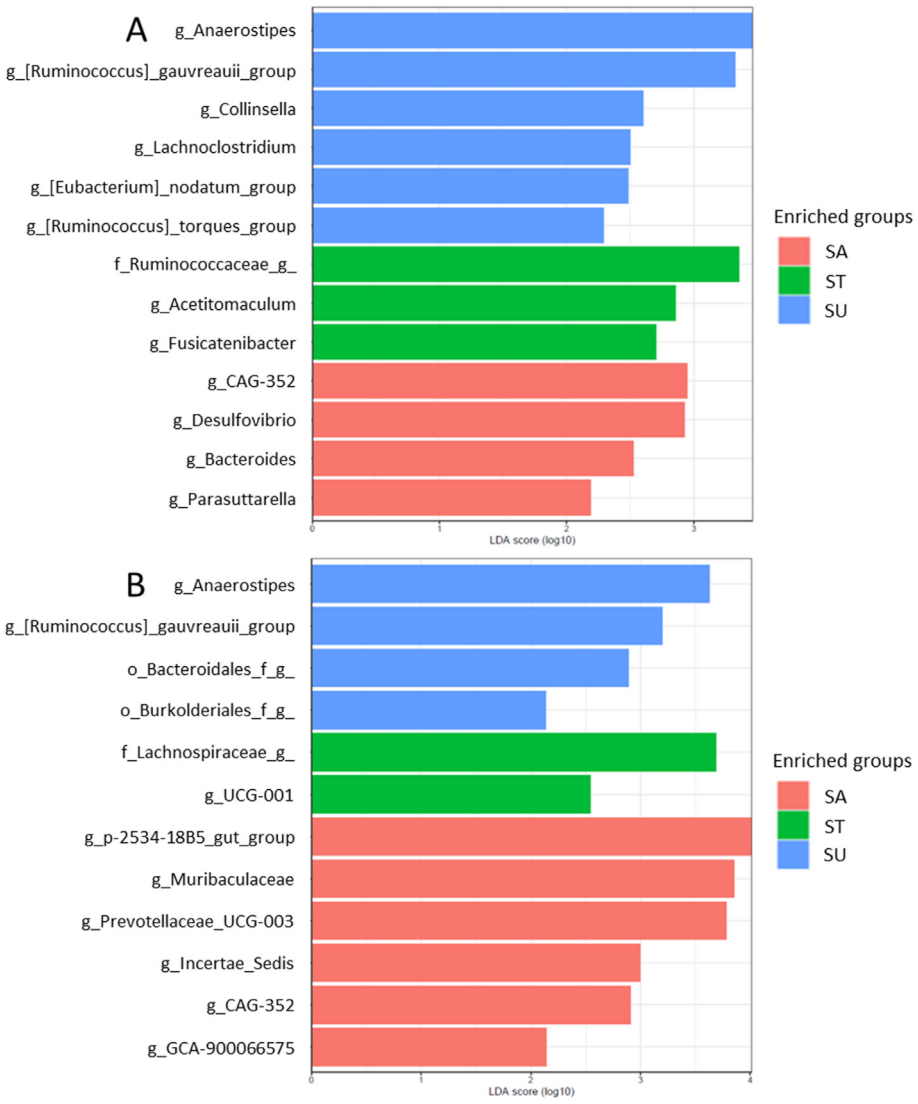

FIGURE 7. Biomarker taxa analysis conducted on the fecal microbiome at the genus level at (A) T2 (60 kg BW) and (B) T3 (100 kg BW) from pigs fed either a basal grower-finisher diet or the basal diet with 30% salty (SA) or sugary (SU) UPF. The outcomes were derived from linear discriminant analysis effect size (LEfSe). BW, body weight; UPF, ultraprocessed food.

TABLE 2
Volatile fatty acids (VFAs, g/kg) quantified in pig feces ($n = 12$ per each group) at T1, T2, and T3

	SA			ST			SU				*P*		
	T1	T2	T3	T1	T2	T3	T1	T2	T3	SEM	Diet	Time	D × T
Acetate	1.35	1.45	1.46	1.29	1.51	1.47	1.35	1.4	1.33	0.057	0.730	0.205	0.251
Propionate	1.75^{ab}	2.17^{bc}	2.39^{bc}	1.54^{a}	2.53^{c}	2.36^{c}	1.91^{abc}	2.27^{bc}	2.21^{bc}	0.164	0.143	0.001	0.035
Butyrate	1.37^{ab}	1.58^{ab}	1.49^{ab}	1.13^{a}	1.92^{b}	1.58^{ab}	1.53^{ab}	1.59^{ab}	1.33^{ab}	0.154	0.133	0.525	0.042
Valerate	0.23	0.37	0.35	0.24	0.45	0.31	0.26	0.41	0.32	0.036	0.757	0.001	0.602

Abbreviations: SA, salty ultraprocessed food-based diet; ST, standard diet; SU, sugary ultraprocessed food-based diet.
Values are least square means with the SEM. *P* values for the diet (D), time point (T), and their interaction (D × T) were calculated by using repeated measures analysis of variance using linear mixed-effects regression models (Lmer). For pairwise comparisons, a modified Tukey test for multiple comparisons of means, the Sidak function was used. Least square means with different superscript letters differ ($P < 0.05$).

specific VFAs were equally expressed in SU and SA groups compared with the ST. Only the *Anaerostipes* taxa, positively correlated with the propionate production and negatively correlated with the butyrate production, was more abundant in the SU group. Among the top 50 taxa that correlated with the VFAs production, only 5 were less abundant in SU and only 1 in the SA group, compared with the ST. This confirms the lack of detrimental effects of UPF on VFAs production in pigs.

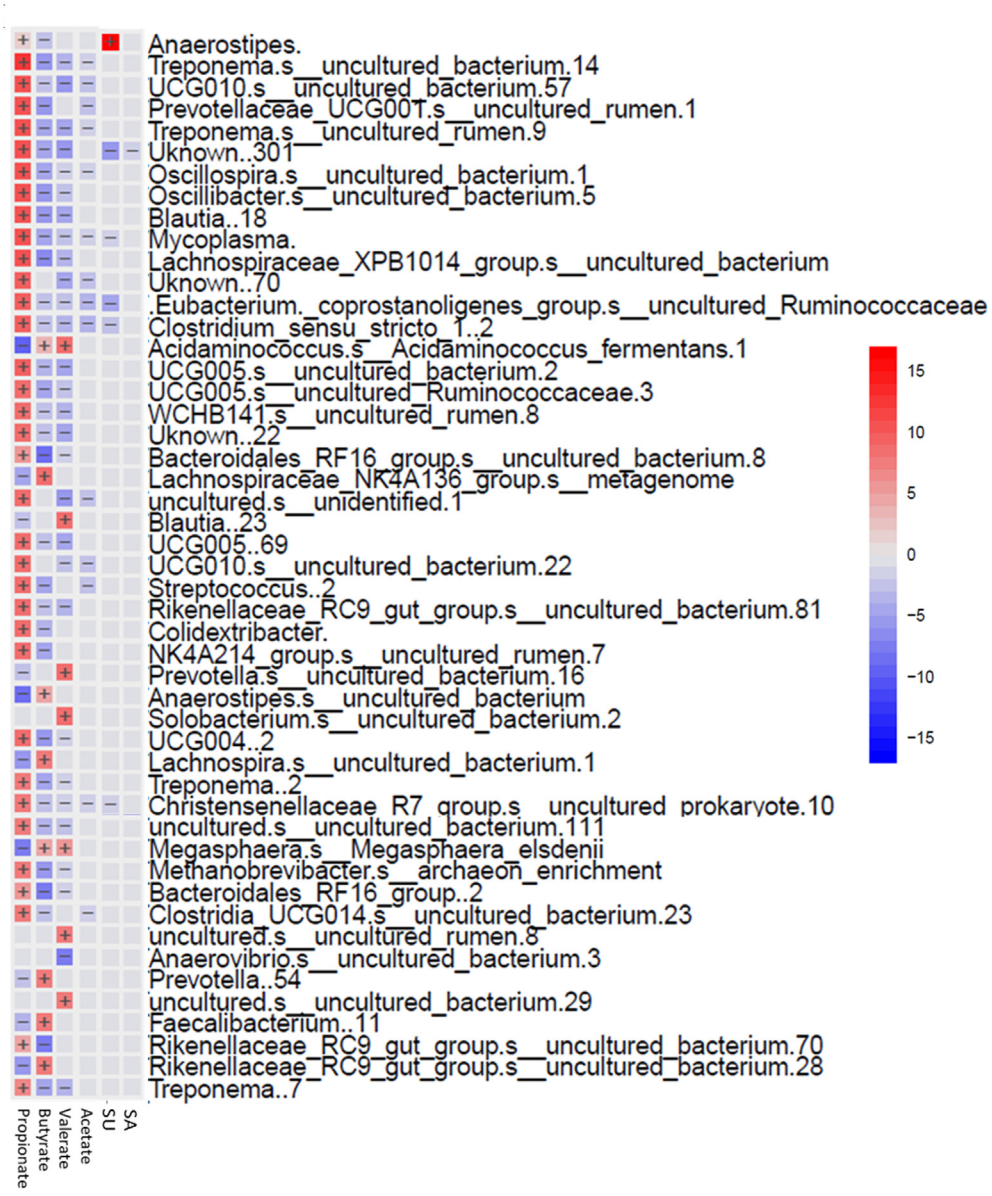

FIGURE 8. Top 50 significant associations between volatile fatty acids (acetate, valerate, butyrate, and propionate) and gut microbiota composition in pigs, irrespective of time point of fecal collection, fed the growing finishing diets supplemented with sugary or salty ultraprocessed food. The MaAsLin2 analysis incorporated dietary treatment effects as fixed factors. The color scale-bar represents positive relationships (red) and negative ones (blue) between taxa and factors derived from normalized significant results. Presented are the top 50 correlations, all exhibiting a *P* value <0.05.

Jejunum physiology and blood serum measurements

In this study we assumed that the long-term consumption of the UPF by pigs could lead to detrimental effects on the small intestinal physiology. Specifically, our hypothesis was that UPF could have impaired the integrity of the intestinal barrier function and lead to a lower transepithelial electric resistance, an indicator of a condition known as "leaky gut," characterized by an increased intestinal permeability [50]. In this environment, toxins, bacteria, and other unwanted molecules are allowed to enter the systemic circulation triggering inflammation and other health issues [50]. However, our theory was not confirmed by the Ussing chamber measurements. In addition, no differences were found in performance traits and health status, as better described in Mazzoleni et al. [25]. The TEER was in fact similar between pigs fed the UPF-based diets compared with the ones fed the standard diet, indicating that the UPF did not promote a leaky gut condition in pigs.

The high content of SFAs, added sugars, and sodium in UPF may interfere with nutrient absorption, including AAs. In our experiment on jejunum tissues, we considered the L-glutamate, L-arginine, and L-methionine to test the activity of different classes of AA transporters, specifically anionic, cationic, and neutral AA transporters, respectively. The jejunum of pigs fed the

TABLE 3
D-glucose and amino acid-induced change in short-circuit current (ΔIsc, μA) in mid-jejunum of pigs ($n = 6$ in ST and $n = 7$ in SA and SU groups)

	SA	ST	SU	SEM	*P*
D-Glucose	2.54	4.49	4.46	1.235	0.327
L-Glutamate	0.33[a]	0.81[ab]	1.54[b]	0.251	0.006
L-Arginine	4.25	3.58	4.89	1.431	0.769
L-Methionine	3.52	4.54	4.84	0.951	0.504
TEER	34.2	31.6	34.1	2.902	0.666

Abbreviations: SA, salty ultraprocessed food-based diet; ST, standard diet; SU, sugary ultraprocessed food-based diet; TEER, transepithelial electrical resistance.
P values were obtained by 1-way analysis of variance statistical analysis. For pairwise comparisons, a modified Tukey test for multiple comparisons of means, the Sidak function was used. Least square means with different superscript letters differ ($P < 0.05$).

TABLE 4
Serum concentration (mmol/L) of urea, calcium, magnesium, and potassium of pigs ($n = 8$ per each group) fed a standard growing finishing diet (ST) or a growing finishing diet supplemented with 30% sugary (SU) or salty (SA) ultraprocessed food

	SA	ST	SU	SEM	*P*
Urea	6.31	5.79	6.35	0.151	0.231
Calcium	2.55	2.51	2.51	0.010	0.390
Magnesium	0.98	0.99	1.01	0.022	0.872
Potassium	3.38	3.39	3.26	0.051	0.481

Abbreviations: SA, salty ultraprocessed food-based diet; ST, standard diet; SU, sugary ultraprocessed food-based diet.
P values were obtained by 1-way analysis of variance statistical analysis.

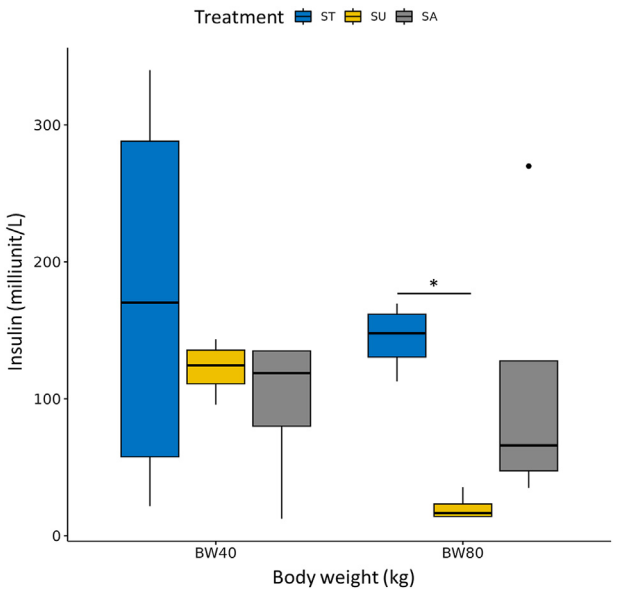

FIGURE 9. Comparison of insulin concentration (milliunit/L) between pigs fed a standard growing finishing diet (ST) or a growing finishing diet supplemented with 30% sugary (SU) or salty (SA) ultraprocessed food at body weight (BW) of 40 or 80 kg. Boxplots with SDs. BW, body weight.

SA diet showed a lower ability to actively absorb L-glutamate, compared with the SU diet, and tended to absorb less L-glutamate compared with the ST pigs. It has been observed in mice that a high salt content diet created a high local concentration of sodium in the colon, despite the fact that sodium levels from food are rapidly normalized in the small intestine [51]. Therefore, we believe that also in our study, the SA diets could have created a high luminal salt concentration at the jejunum level. How such sodium chloride concentration could modulate the physiology of the L-glutamate uptake is unclear. What is known is that the intestinal L-glutamate uptake is mainly mediated by the sodium-dependent excitatory amino acid transporter-3 [52]. Therefore, further studies should focus on the effects of UPF on the activation status of the excitatory amino acid transporter-3 transporter and related L-glutamate uptake.

The consumption of UPF has been associated also with an excessive sodium chloride intake that could disrupt the balance of certain minerals in the body such as potassium, calcium, and magnesium, essential for the animal health [53]. Blood analysis performed on blood serum showed that in our study, no effect of the SU or SA diets was observed on urea, calcium, magnesium, and potassium concentration. This suggests that when used to partially replace traditional ingredients in a balanced diet, UPF did not lead to severe deficiency in pig.

At a BW of 80 kg, pigs fed a SU diet exhibited significantly lower blood insulin concentrations 1 h after meal compared with the ST and SA groups. This could be due to the higher simple sugar content in UPF compared with standard feed ingredients [49], leading to a quicker decline in insulin secretion. However, because this study measured insulin at only one time point, this hypothesis cannot be confirmed. Also, when translating the results of UPF studies on insulin secretion from pigs to humans, it is essential to consider significant differences in glucose and insulin metabolism. Pigs are known to be resistant to the spontaneous development of type 2 diabetes mellitus, even after intervention with high-fat, high-fructose, and high-carbohydrate diets [54, 55]. The resistance of pigs to type 2 diabetes is likely attributed to variations in the composition of their bile acid pool, particularly the high concentration of hyocholic acid and its derivatives. These hyocholic acids play a crucial role in improving glucose homeostasis by modulating the activity of the cell membrane G-protein-coupled bile acid receptor TGR5 and the nuclear farnesoid X receptor signaling mechanism, as described by Zheng et al. [56].

In conclusion, the partial replacement of traditional feed ingredients with UPF has no detrimental effects on gut microbiota, intestinal integrity, and mineral homeostasis when included in a balanced diet for pigs. More targeted studies should be performed to better investigate the effect of sodium chloride intestinal accumulation and its effect on specific intestinal transporter's activity, in particular the excitatory amino acid transporters (EAAT) and the related L-glutamate intestinal uptake.

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Author contributions

The authors' responsibilities were as follows – MT: conducted research, performed statistical analysis, and wrote the article; SM: conducted research and drafted the article; GB: designed research and provided essential reagents, or provided essential materials; PS: provided essential reagents, or provided essential materials; LP: drafted the article and had primary responsibility for final content; and all authors: read and approved the final manuscript.

Conflict of interest

The authors report no conflicts of interest.

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Data availability

Data described in the manuscript will be made available upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2024.07.032.

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