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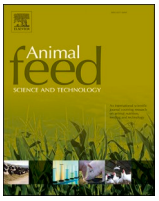

Inclusion of a fish oil processing fraction as additive in diets for weaning piglets

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ABSTRACT

The weaning process in pig production is commonly associated to low feed intake, slow growth rate and increased morbidity of piglets. This study evaluates a nutritional intervention consisting of supplementing with a fish oil product rich in mono- and diglycerides (FOMG) and containing 0.069 g/g of ω-3, to improve animal health and productive performance during the post-weaning period. In a preliminary experiment (experiment 1), a total of 136 piglets were randomly distributed in two groups (4 pens per group) to evaluate the productive effects of dietary supplementation with FOMG at 15 g/kg (T15) in substitution of lard (T0) as fat source during the post-weaning period in a commercial farm. Besides, in experiment 2 a total of 72 weaned piglets were fed on a control diet (T0) or supplemented with 15 (T15) or 30 g FOMG/kg (T30) in substitution of sunflower oil, with 6 pens of 4 piglets per treatment. In experiment 2 growth and intake were weekly controlled, and blood was sampled on days 14 and 34. At day 35 post-weaning, 6 piglets per treatment were euthanized to study the microbial fermentation and the ileal and caecal bacterial community by 16 S amplicon sequencing. Results indicated that piglets fed T15 diet tended to have a higher growth gain during the post-weaning period in experiment 1 ($P = 0.067$). This increased growth was partially explained by a greater feed intake (0.14 higher) but also due to improved animal health as showed by the lower proportion of neutrophils ($P = 0.006$), blood cortisol ($P = 0.098$) and morbidity ($P < 0.05$) in experiment 2. Treatment T15 also tended ($P = 0.064$) to promote a higher volatile fatty acids (VFA) concentration at the ileum, which could be compatible with a higher nutrient absorption and a subsequent lower VFA concentration in the hindgut ($P < 0.001$). Moreover, FOMG supplementation at T15 exerted modulatory effects on the gut microbiota promoting a shift in the bacterial community structure, lower diversity (Richness index, $P < 0.05$) and a trend ($P = 0.076$) for a higher butyrate proportion at the ileum, together with a lower ($P < 0.05$) and most favourable Firmicutes-to-Bacteroidetes ratio at the caecum. On the contrary, T30 diet promoted less beneficial effects than T15. These findings indicated that supplementation of piglets with FOMG at a level of 15 g/

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; ALT, alanine aminotransferase; ALP, alkaline phosphatase; aNDFom, amylase-treated neutral detergent fibre organic matter basis; AST, aspartate aminotransferase; BCFA, branched-chain volatile fatty acids; CP, crude protein; DHA, docosahexaenoic acid; DM, dry matter; EPA, eicosapentaenoic acid; FOMG, fish oil product rich in mono- and diglycerides; G:F, gain to feed ratio; MCH, mean corpuscular haemoglobin; OM, organic matter; RBC, red blood cells; SEM, standard error of means; VFA, volatile fatty acids; WBC, white blood cells.

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kg represents a suitable strategy to improve pig performance and gut health during the post-weaning period.

1. Introduction

Nutritional and social changes at weaning makes transition a critical stage in pig production, which is commonly associated to a reduction of feed intake and growth, and increases the risk of microbial disorders that often lead to diarrhoea episodes (Campbell et al., 2013; Heo et al., 2013). In particular, the abrupt shift from a milk-based feeding regime to a solid diet, together with the social stress derived from the end of maternal interaction and rearrangement of piglets in larger groups, challenges the development of their digestive tract and immune system. As a result, the weaning process commonly implies alterations in the small intestine architecture, insufficient enzymatic activity, increase in mucosal permeability, disturbed absorptive-secretory electrolyte balance and altered local inflammatory cytokine patterns and intestinal microbiota (Lallès et al., 2007). Moreover, health problems such as leukocytopenia, increased acute phase proteins, neutrophilia and alterations of white blood cells (WBC) often appear after weaning, making piglets more susceptible to digestive and respiratory infections and low weight gain and feed efficiency (Cabrera et al., 2010). Having into account the importance of the gut-associated lymphoid tissue, new feeding strategies are needed to overcome this situation while minimising the use of antibiotic therapy. In this sense, the dietary supplementation with bioactive compounds such as vitamins or amino acids have gained interest for supporting the protective response (Kiczorowska et al., 2017), despite the dietary supplementation with immunostimulants has not shown conclusive results to date (Cheng et al., 2014).

Fish oil has been also suggested as immunostimulant because of its low ω-6:ω-3 ratio and high proportion of long chain poly-unsaturated fatty acids, mainly eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids (Korver and Klasing, 1997; Rossi et al., 2010). It has been suggested that these fatty acids down regulate activation of lymphocytes (Liu et al., 2003), reduce the secretion of pro-inflammatory cytokines and enhance the expression of peroxisome proliferator receptor activator gamma (PPAR-γ), which promotes the differentiation of immune cells to anti-inflammatory phenotypes (Marion-Letellier et al., 2008). Thus, adding diets with 50 g/kg DM fish oil to immunologically challenged piglets reduced cortisol, tumour necrosis factor-α and interferon-γ, suggesting a down-regulation of the immune system activation without depressing growth (Gaines et al., 2003; Li et al., 2014).

From the 21.7 million tons of global fish production, a proportion of around 0.14 is transformed to fishmeal and fish oil (FAO, 2014), and only 0.10 of fish oil is obtained from raw material when pressed to obtain fishmeal (Arvanitoyannis and Kassaveti, 2008). Apart from other uses, fish oil is used for aquaculture feeding, increasing its price for pig feeding. Actually, specific ω-3 fatty acids such as EPA and DHA are isolated from fish oil by supercritical CO_2 for pharmaceutical, nutritional and cosmetic industries. A product derived from fish oil processing, rich in mono- and diglycerides (FOMG) may be a useful alternative as feed, even though ω-3 fatty acids content in FOMG is two- to five-fold lower than fish oil, depending on the fish species. Thus, the proportions of EPA and DHA range from 0.02 to 0.13 and 0.00 to 0.05 g/g, respectively (according to the manufacturer).

We hypothesised that the inclusion of FOMG as lipid source in diets for weaned piglets may act as a suitable energy source and at the same time as immunostimulant, with potential benefits for the animals. Thus, this work aims to study the dietary inclusion of FOMG (15 and 30 g/kg DM of total diet), to describe its potential contribution to animal health by regulating the immune system and intestinal microbiota, and its productive implications in weaned piglets. Two experiments were conducted to explore this hypothesis on farm conditions and in a controlled environment, respectively.

2. Material and methods

The FOMG to be tested was a commercial fraction of fish oil processing (MAG-MIX™, Solutex, Mallen, Spain). This product is partial extraction of EPA and DHA by distillation and supercritical CO_2 extraction, and the remaining subjected to enzymatic trans-esterification to produce mono- and diglycerides to enhance bioavailability. The FOMG was mainly composed by (as proportion of total fatty acids, according to the manufacturer): C14:0, 0.160; C16:0, 0.323; C16:1 n7, 0.161; C16:3 n4, 0.018; C16:4 n1, 0.040; C18:0, 0.020; C18:1 n9, 0.065, C18:1 n7 (vaccenic acid), 0.021; C18:2 n6, 0.011; C18:3 n3, 0.006; C18:3 n6, 0.002; C18:4 n3, 0.023; C20:0, 0.001; C20:4 n6, 0.002; C21:5 n3, 0.002; C22:5 n3, 0.001, and is specially characterized by a considerable proportion of EPA (0.031), DHA (0.006) and total ω-3 (0.069), with a ω-6 to ω-3 ratio of 0.21. Most fatty acids are in form of mono- and diglycerides.

All animal procedures were carried out under the Project License PI31/20 approved by the Ethic Committee for Animal Experimentation from the University of Zaragoza, Spain. The care and use of animals were performed according to the Spanish Policy for Animal Protection RD118/2021, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

2.1. Experiment 1: On farm evaluation of fish oil supplementation

This preliminary experiment evaluated the effects of dietary supplementation with FOMG as a fat source on a pig commercial farm. For doing so, 136 (Landrace x Large White) x Pietrain male piglets of 22–26 days of age (7.34 ± 0.22 kg initial weight) were allocated in 8 pens of 17 animals, in a completely randomised design. Two iso-lipidic dietary treatments (4 pens per treatment) were assayed: a control diet without FOMG (T0) and the same feed with FOMG at 15 g/kg DM (T15) in substitution of the same proportion of lard, that were given to four pens each. Diets were formulated to meet the nutritional requirements of piglets during the prestarter (from day

4–15 after weaning) and starter (from day 16–35 after weaning) phases along the transition period. The ingredient and chemical composition for experimental diets in experiment 1 is given in Table 1.

The experimental period started on the third day after weaning. Average weight per pen was monitored at the start and at the end of each phase (days 4, 15 and 35 after weaning). Feed refusals were weighed at the end of each phase to estimate daily feed intake per pen. Incidence of diarrhoea and their therapeutic treatment were also recorded.

2.2. Experiment 2. Dose-response effects of FOMG supplementation

Three iso-lipidic experimental diets (T0, T15 and T30) consisting of different concentrations of FOMG (0, 15 and 30 g/kg) in substitution of sunflower oil (30, 15 and 0 g/kg, respectively) were used. As described in experiment 1, two diets (prestarter and starter) were formulated for each treatment (T0, T15 and T30) to meet the nutritional requirements of the piglets from day 4–15 and from day 16–35 after weaning, respectively. The ingredient and chemical composition for diets used in experiment 2 are given in Table 2.

The experiment 2 was carried out in the facilities of the Animal Experimentation Service of the University of Zaragoza. A total of 72 (Landrace x Large White) x Pietrain male piglets (22–26 days of age, 8.09 ± 0.50 kg), were weaned and distributed in 6 groups of increasing body weight. Then, piglets were allocated by weight to 18 pens of 4 animals (6 pens per treatment, each one with a different initial weight) and assigned to the corresponding dietary treatments following a randomised complete block design. The disinfection protocol of installations from the previous feeding experiment was not followed to promote some extent of microbiological challenge to the piglets. Individual animal weight and feed refusals per pen were weekly recorded to determine average daily growth (ADG), average daily feed intake (ADFI) and gain to feed ratio (G:F) per phase, considering pen as the experimental unit. Morbidity was determined per pen as the number of piglets in the pen with no growth within a week. Blood samples were taken from the jugular vein from one randomly chosen piglet per pen at the end of the prestarter and starter phases (days 15 and 35 of experiment). Blood was collected into tubes with and without EDTA as anticoagulant to determine cell counts and serum biochemical parameters, respectively. In addition, the same animals used for blood sampling (one per pen) were slaughtered at the end of the starter phase (day 35) and their ileum and caecum were sampled to describe the microbial fermentation (concentration of volatile fatty acids, VFA) and bacterial diversity. Samples of digesta for VFA (2 mL) analysis were mixed with 0.5 mL of a solution of 0.5 M phosphoric acid with 1 mg of 4-methyl valeric acid as internal standard and immediately frozen at − 20ºC. For gut microbial analysis, ileal and caecal contents were sampled (approximately 10 mL), frozen in liquid N, stored at − 80ºC and lyophilized prior DNA extraction.

2.3. Chemical, blood and fermentation and microbiological analyses

Dry matter (DM) of feeds was determined by oven drying at 65ºC to a constant weight. Feeds were analysed following the

Table 1
Ingredient and estimated nutrient composition of experimental feeds in experiment 1.

	Prestarter		Starter	
	T0	T15	T0	T15
Ingredients (g/kg, as fed)				
Wheat grain	182	182	250	250
Maize grain	165	165	197	197
Barley grain	162	162	240	240
Cereal flakes	123	123	–	–
Soybean meal (47% protein)	115	115	188	188
Extruded soybeans	9	9	–	–
Fishmeal	50	50	20	20
Bovine whey	80	80	25	25
Porcine plasma	15	15	–	–
Porcine hydrolysed proteins	10	10	5	5
Brewer's yeast	6.3	6.3	2	2
Wheat gluten	6.3	6.3	–	–
Lard	24	9	33	18
FOMG	–	15	–	15
Vitamin-mineral-amino acid mixture	52.4	52.4	40	40
Estimated composition (g/kg)				
Organic matter	949		952	
Crude protein	183		172	
Ether extract	63		56	
Crude fibre	26		31	
Ca	6.2		6.5	
Total P	5.5		5.4	
Lysine	13.5		12.5	
Methionine	4.7		3.9	
Threonine	9.0		8.3	

FOMG: Fish oil product rich in mono- and diglycerides.

Table 2
Ingredient and analysed nutrient composition of experimental feeds in experiment 2.

	Prestarter			Starter		
	T0	T15	T30	T0	T15	T30
Ingredients (g/kg, as fed)						
Heat processed maize	200	200	200	–	–	–
Maize grain	171	171	171	463	463	463
Barley grain	250	250	250	210	210	210
Soybean meal (47% protein)	80	80	80	140	140	140
Fishmeal (70% protein)	62	62	62	40	40	40
Potato protein	50	50	50	37	37	37
Wheat middlings	20	20	20	20	20	20
Bovine whey	120	120	120	40	40	40
Sunflower oil	30	1.5	–	30	15	–
FOMG	–	1.5	30	–	15	30
Dihydr. bicalcium phosphate	7	7	7	8	8	8
Calcium carbonate	2.5	2.5	2.5	7	7	7
Sodium chloride	3	3	3	3	3	3
L lysine HCl	2.3	2.3	2.3	0.5	0.5	0.5
DL methionine	0.3	0.3	0.3	0.3	0.3	0.3
L threonine	1.2	1.2	1.2	0.9	0.9	0.9
L tryptophan	0.7	0.7	0.7	0.4	0.4	0.4
Vitamin-mineral mixture	4	4	4	4	4	4
Nutrient composition (g/kg dry matter)						
Organic matter	948	948	947	948	946	946
Crude protein	204	207	206	208	204	202
Ether extract	56	54	52	55	53	53
Neutral detergent fibre	95	87	94	108	103	107
Starch	450	437	453	450	462	463

FOMG: Fish oil product rich in mono- and diglycerides.

procedures of AOAC (2005) for their organic matter (OM, method 942.05), crude protein (CP, method 976.05) and ether extract (EE, method 2003.05) content. Concentration of aNDFom was analysed using an Ankom 200 Fiber Analyzer (Ankom Technology, New York, NY, USA) as described by Mertens (2002) using α–amylase but not sodium sulphite, and results were expressed exclusive of residual ashes. Total starch content was determined enzymatically in samples ground to 0.5 mm by using a commercial kit (Total Starch Assay Kit K-TSTA 07/11, Megazyme, Bray, Ireland). The concentration of VFA in ileal and caecal samples was determined by gas chromatography in an Agilent 6890 apparatus (Agilent Technologies España S.L., Madrid, Spain) fitted with a capillary column (Model HP-FFAP polyethylene glycol TPA-treated, 30 m x530μm I.D. x1μm film thickness).

Blood samples for haematology analyses were taken in tubes provided with EDTA. Tubes were homogenised for 5 min at room temperature and an haemogram was performed including profiling of red blood cells (RBC), WBC and platelets. The tube without anticoagulant were centrifuged and the serum obtained was assayed for biochemical analyses. All parameters were determined spectrophotometrically in a A15 Analyzer (Biosystems SA, Barcelona, Spain), except for cortisol, that was determined by immunofluorescence with an iChroma reader (Boditech Med Inc., Korea).

The structure of the rumen prokaryotic community was explored by a meta-taxonomic approach. The DNA was extracted from lyophilized ileal and caecal samples using a commercial kit (Qiagen QIAmp DNA Stool Mini Kit, Qiagen Ltd., West Sussex, UK) following the manufacturer's recommendations. Extracted DNA was analysed for amplicon sequencing using the MiSeq V3 (600 cycles) kit (Illumina Inc., San Diego, CA, USA), as described by Palma-Hidalgo et al. (2021). The universal primers used for the amplification were Pro341F 5′-CCTACGGGAGGCAGCAG-3′ and Pro805R 5′-GACTACNVGGGTATCTAATCC-3′ targeting the V3_V4 hypervariable region of the 16 S rRNA gene (Takahashi et al., 2014). Primer-sorted and demultiplexed paired-end reads were used and downstream processing was performed using QIIME 2 (Bolyen et al., 2019). Low-quality reads and bases (PHRED quality score below 25) were trimmed at 250 bp. Chimeras were identified and removed using chimera.vsearch. Operational taxonomic units (OTUs) were identified at the 0.97 similarity level, and then representative sequences from all ASVs were aligned against Greengenes 13_8 97% (DeSantis et al., 2006). The number of sequences per sample for each microbial group was normalized across all the samples, and singletons were removed.

2.4. Calculations and statistical analyses

Microbial data was analysed following the overall approach described by Belanche et al. (2019). Alpha diversity data were analysed by one-way ANOVA. To illustrate the treatment effect on the ileal and caecal bacterial community, a permutational analysis of variance (PERMANOVA) was performed based on the Bray-Curtis distance matrix. This PERMANOVA was performed on the log10 data submitted to 999 random permutations under the reduced model and the Monte Carlo method using the PRIMER-6 software (PRIMER-E Ltd., Plymouth, UK). Pair-wise comparisons were performed along with Principal Coordinate analyses (PCoA) to show the effects of fish oil supplementation on the ileal and caecal bacterial community structure. Tripod vectors were included in the PCoA to illustrate the most discriminant OTUs based on Spearman correlations ($P > 0.75$). The abundance of the bacterial taxa were analysed using the

Kruskal-Wallis non-parametric test because they did not follow a normal distribution after performing the Shapiro-Wilk normality test.

Results were analysed statistically by one-way ANOVA using Statistix 10 (Analytical Software, 2010), considering the level of FOMG inclusion (n = 2 and 3 in experiments 1 and 2, respectively) as main effect, and in experiment 2 the initial weight (pen within treatment) was considered as random factor (block). The effect of treatment on the prestarter and starter phases, as well as on the whole experiment on productive performance and blood traits were studied. Besides, the effects of the phase and the interaction between dietary treatment and phase were tested as indicated by Littell et al. (1998). For all parameters, the pen was considered as the experimental unit. In case of detecting significant treatment effects, differences were contrasted by the least significant difference at $P < 0.05$. A trend to significance was considered when $P < 0.10$.

3. Results

3.1. Experiment 1: On farm evaluation of fish oil supplementation

No differences between treatments were recorded on the initial piglet weight (P = 0.96), which averaged 7.32 ± 0.67 kg, nor on the final weight (20.33 ± 1.38 kg). A low incidence of health problems was observed in experiment 1; particularly, during the prestarter phase eight piglets (four per treatment) presented respiratory or nervous symptoms that were associated with meningitis, but all piglets recovered after veterinary treatment. No incidences were recorded during the starter phase, and no mortality was observed during this experiment.

In terms of productive performance (Table 3), when data for the whole experiment was considered, a higher ADFI (P < 0.001) and a trend for a higher ADG (P = 0.067) and a lower G:F ratio (P = 0.084) were observed with T15. A positive response on ADFI to the additive inclusion was also manifested when the two phases were considered, either at the prestarter (P = 0.003) or starter (P = 0.017) phases, as well as a trend on ADG (P = 0.061) in starter. In this approach, the G:F ratio tended to be lower with T15 in the prestarter phase (P = 0.064).

3.2. Experiment 2: Dose-response effects of fish oil supplementation

Initial piglet weight averaged 8.09 ± 0.29 kg, with no differences among treatments. One piglet from each treatment died in the second (T30) and fourth (T0 and T15) experimental weeks. No treatment differences were recorded in accumulated morbidity, that otherwise was numerically higher with T0 (means of 2.17, 1.17 and 1.58 piglets per pen for T0, T15 and T30; sem = 0.511). Productive results of experiment 2 are given in Table 4. No treatment differences were detected on ADG or ADFI, nor on G:F ratio, during either the prestarter or starter phase, nor when the whole transition period was studied.

The effect of dietary inclusion of FOMG on blood parameters is shown in Tables 5 and 6. Because the interaction between feed and phase was not significant except for two cases (concentration of platelets, P = 0.026, and ALP, P = 0.053), indicating differences between phases within treatments and not among treatments within phases, only means for the main effect treatment are shown. No effect was detected on WBC concentration, nor on proportions of monocytes, eosinophils and basophils (Table 5); however, T15 piglets showed the highest proportion of lymphocytes and the lowest of neutrophils (P < 0.05). No dietary effects were detected on concentration of blood proteins or biochemical parameters and protein analysis (Table 6). Overall concentration of alanine aminotransferase (ALT) tended (P = 0.077) to be lower with T30 than T15. Despite it did not reached significance, probably because of its high variability (coefficient of variation 0.56), a trend for treatment differences was detected for cortisol concentration (P = 0.098), reaching numerically higher values with T0, over the reference threshold for pigs (206 nmol/L) in 8 out of 12 piglets in both prestarter and starter phases.

Total VFA concentration and molar proportions of the main VFAs at the ileum and caecum in piglets at the end of the Starting phase

Table 3
Individual body weight, average daily gain (ADG), average daily feed intake (ADFI) and gain to feed ratio (G:F) in piglets fed on control diet (T0) or supplemented with 15 g FOMG/kg (T15), in experiment 1 (n = 4).

Feeding phase	T0	T15	SEM	P-value[1]
Pre-starter (4–14 d)				
ADG (g/d)	288	293	12.7	0.789
ADFI (g/d)	378	433	8.1	0.003
G:F ratio (g/g)	0.761	0.676	0.0265	0.064
Starter (15–35 d)				
ADG (g/d)	472	528	17.1	0.061
ADFI (g/d)	667	769	21.0	0.017
G:F ratio (g/g)	0.709	0.691	0.0159	0.426
Overall (4–35 d)				
Initial body weight (kg)	7.31	7.34	0.336	0.960
Final body weight (kg)	19.75	20.90	0.673	0.272
ADG (g/d)	401	437	12.8	0.067
ADFI (g/d)	555	636	15.2	< 0.001
G:F ratio (g/g)	0.725	0.684	0.0109	0.084

SEM: standard error of means.1 The interactions between diet and feeding phase were not significant (P > 0.10) for any of the variables studied.

Table 4
Individual body weight, average daily gain (ADG), average daily feed intake (ADFI), and gain to feed ratio (G:F) in piglets fed on a control diet (T0) or supplemented with 15 (T15) or 30 g FOMG/kg (T30) in experiment 2 (n = 6).

Feeding phase	T0	T15	T30	SEM	P-value[1]
Pre-starter (1–14 d)					
ADG (g/d)	196	271	195	48.1	0.462
ADFI (g/d)	305	384	293	46.1	0.337
G:F ratio (g/g)	0.678	0.700	0.646	0.0440	0.699
Starter (15–35 d)					
ADG (g/d)	333	429	365	38.2	0.226
ADFI (g/d)	599	758	686	66.9	0.277
G:F ratio (g/g)	0.563	0.567	0.534	0.0305	0.716
Overall (1–35 d)					
Initial body weight (kg)	8.10	8.17	8.01	0.499	0.976
Final body weight (kg)	17.8	21.0	18.0	1.58	0.315
ADG (g/d)	278	366	322	42.5	0.382
ADFI (g/d)	482	609	528	58.9	0.344
G:F ratio (g/g)	0.557	0.602	0.606	0.0272	0.404

SEM: standard error of means.
[1] The interactions between diet and feeding phase were not significant (P > 0.10) for any of the variables studied.

Table 5
Blood cell concentration and proportions in piglets fed on a control diet (T0) or supplemented with 15 (T15) or 30 g FOMG/kg (T30) during the post-weaning period in experiment 2.

	T0	T15	T30	SEM	P-value[1]
Total white blood cells (10^6/mL)	29.3	22.6	31.8	3.67	0.205
Lymphocytes (%)	38.8[ab]	47.9[a]	32.5[b]	3.30	0.015
Monocytes (%)	8.55	9.88	9.36	0.785	0.488
Neutrophils (%)	50.1[a]	39.6[b]	56.7[a]	3.29	0.006
Eosinophils (%)	1.31	2.29	1.09	0.487	0.195
Basophils (%)	0.29	0.26	0.33	0.062	0.684
Red blood cells (10^{12}/mL)	5.66	5.84	5.57	0.144	0.414
Haemoglobin (mg/mL)	105	104	102	3.2	0.753
Haematocrit (%)	32.1	31.9	30.5	0.98	0.449
MCH (mg/mL)	324	328	331	2.5	0.370
RDW (%)	19.0	18.0	18.5	0.58	0.178
Platelets (10^6/mL)	559	572	498	44.9	0.477

SEM: standard error of means; MCH: mean corpuscular haemoglobin; RDW: red blood cells distribution width. Means indicate the average treatment values from samples taken at day 15 and 35 after weaning. Within a row, letters indicate significant differences (P < 0.05).
[1] The interactions between diet and feeding phase was only significant for platelet concentration (P = 0.026).

Table 6
Average concentration of blood proteins and metabolites in piglets fed on a control diet (T0) or supplemented with 15 (T15) or 30 g FOMG/kg (T30) during the post-weaning period in experiment 2.

	T0	T15	T30	SEM	P-value[1]
Blood proteins					
Serum proteins (mg/mL)	47.3	47.3	44.4	1.42	0.280
Plasma proteins (mg/mL)	52.4	52.8	50.1	1.55	0.413
Albumin (mg/mL)	21.7	21.0	20.7	1.06	0.795
Globulins (mg/mL)	25.6	26.4	23.8	1.11	0.236
Blood metabolites					
AST (IU/L)	72.2	42.5	49.3	14.66	0.336
ALT (IU/L)	43.6	48.2	36.7	3.48	0.077
ALP (IU/L)	505	585	579	42.8	0.348
Urea (mg/L)	172	186	174	16.2	0.813
Creatinine (mg/L)	14.6	15.7	14.3	0.84	0.462
Cortisol (nmol/L)	254	177	162	31.7	0.098

SEM: standard error of means; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase. Means indicate the average treatment values from samples taken at day 15 and 35 after weaning.
[1] The interactions between diet and feeding phase only tended to be significant for ALP concentration (P = 0.053).

is shown in Table 7. A major variability in total VFA concentration was observed (coefficient of variation 0.31), and therefore results must be taken with care. A trend (P = 0.064) for a higher ileal VFA concentration was observed with T15 than T30. A trend (P = 0.076) for differences was also detected on butyrate proportion, being higher for T15 respect to the other treatments. However, it has to be considered that butyrate proportion only accounted for 0.04–0.07 of total ileal VFA because acetate proportion averaged more than 0.90 of total VFA. Piglets given T0 recorded a higher caecal VFA concentration (P < 0.05) compared with T30, with intermediate values with T15, but no differences in molar proportions were detected at the caecum level.

Next generation sequencing generated a total of 0.89 million of prokaryotic high-quality sequences after quality filtering. The proportion of archaeal sequences was small (0.068% in the ileum and 0.75% in the caecum) and was unaffected by the treatments (supplementary Tables S1 and S2), therefore this community was not further analysed. Bacterial sequencing depth was normalized to 15.186 sequences per sample which provided sufficient coverage at the ileum (70%) and caecum (80%) levels.

Analysis of the ileum bacterial community by PCoA showed that increasing supplementation with FOMG promoted a progressive modification of the overall structure of the bacterial community along PCO1 (Fig. 1A). These significant differences across the three treatments were confirmed by the pair-wise PERMANOVA analysis, with the greatest differences noted between T0 and T30 (P < 0.05). Tripod vectors showed that several taxa belonging to Gammaproteobacteria and Leuconostocaceae were positively correlated with the ileal bacterial community of T0 piglets. The inclusion of FOMG in diet reduced the small intestine bacterial diversity as it was measured by both Richness and Shannon indexes (Fig. 2). The structure of ileal microbiota at phylum level (Fig. 2 and supplementary Table S1) showed that Firmicutes was the most abundant phylum, reaching higher abundances in T30 and T15 than in T0 (proportions of 93.0%, 88.4% and 78.1%, respectively; P = 0.015). The T0 piglets tended to show the higher proportion of unknown bacterial sequences (P = 0.057). The most abundant families at the ileum were Clostridiaceae (on average 42.7%), Peptostreptococcaceae (17.0%) and Lactobacillaceae (4.6%), not observing treatment differences in any of them. Among other minor families (all belonging to the phylum Firmicutes) piglets fed T0 tended to have numerically higher abundances of Lachnospiraceae (P = 0.050) and Ruminococcaceae (P = 0.084), which includes the genus *Gemmiger* (P = 0.050). Similarly, pigs fed T0 the highest abundance of Leuconostocaceae (P = 0.030) and its genus *Weissella* (P = 0.012), across treatments. A trend to differences (P = 0.087) was observed in abundances of family Coriobacteriaceae, which includes the genus *Olsenella* (P = 0.060), and in *Pediococcus* (P = 0.086), whereas treatment T30 led to high abundances of the *Lactobacillus* genus (P < 0.05) whereas it was mostly absent in the other treatments (Fig. 2).

At the caecal level, PCoA showed that FOMG supplementation tended to modify the structure of the bacterial community in comparison to T0 treatment (pair-wise differences P < 0.1, Fig. 1B) whereas no differences were noted between T15 and T30. Tripod vectors showed the presence of a large number of taxa (e.g., Lachnospiraceae and Ruminococcaceae) correlated with the sample distribution. Bacterial diversity indexes in the caecum were unaffected by the treatments (Fig. 3). The caecal bacterial community was dominated by the phyla Firmicutes (average 65.1%) which showed no differences across treatments (supplementary Table S2), whereas Bacteroidetes was less abundant in T0 than in T15 and T30 (13.2%, 18.7% and 16.3%, respectively; P = 0.030). Thus, the Firmicutes to Bacteroidetes ratio was reduced with FOMG addition (6.39, 3.38 and 3.39 in T0, T15 and T30; P = 0.042). The FOMG supplementation promoted a decrease in the abundances of the families Leuconostocaceae (P = 0.007) and its genus *Weisella* (P = 0.016), and Ruminococcaceae (P = 0.050) and the absence of sequences from the families Paraprevotellaceae (P = 0.049) and Enterobacteriaceae (P = 0.078) in comparison to T0 (Fig. 3).

4. Discussion

The FOMG was included in diet at the expense of the other fat sources, which were lard in experiment 1 and sunflower oil in experiment 2. Because the estimated net energy content of the FOMG was 6830 kcal/kg (NRC, 2012), similar to that reported for the substituted fat sources (lard and sunflower oil), minimal deviations in the dietary energy content were estimated (4 and 19 kcal/kg feed in experiments 1 and 2), so diets could be assumed as isoenergetic. Despite of this, in experiment 1 the T15 piglets tended to have a 0.12 higher growth rate during the starter phase that the T0 piglets, being proportional to the 0.14 increase in feed intake. Feed intake during the post-waning period is key to maximize growth rates and to prevent weight variability and health problems, because lighter piglets do not catch up during the fattening period (López-Vergé et al., 2018). In experiment 2 this increment in performance was more

Table 7
Concentration of volatile fatty acids (VFA) and molar proportions (mol/mol) of the main VFA at the ileum and caecum in piglets fed on a control diet (T0) or supplemented with 15 or 30 g FOMG/kg (T15 and T30, respectively) at the end of Starting phase (35 days after weaning) in experiment 2 (n = 6).

	Ileum					Caecum				
	T0	T15	T30	SEM	P-value	T0	T15	T30	SEM	P-value
VFA (mM)	12.4	16.1	10.2	1.64	0.064	146.9^{a}	123.8^{ab}	100.8^{b}	6.68	< 0.001
Acetate	0.944	0.901	0.931	0.0153	0.148	0.497	0.478	0.455	0.0280	0.583
Propionate	0.007	0.013	0.015	0.0048	0.509	0.309	0.298	0.343	0.0149	0.119
Butyrate	0.045	0.070	0.039	0.0096	0.076	0.154	0.174	0.161	0.0184	0.748
Valerate	0.0003	0.0041	0.0053	0.0021	0.241	0.029	0.038	0.027	0.0076	0.576
BCFA	0.0034	0.011	0.011	0.0031	0.173	0.011	0.013	0.014	0.0021	0.682

BCFA: branched-chain fatty acids (sum of isobutyrate and isovalerate).
SEM: standard error of means.
Within a row, letters within each organ indicate significant differences (P < 0.05).

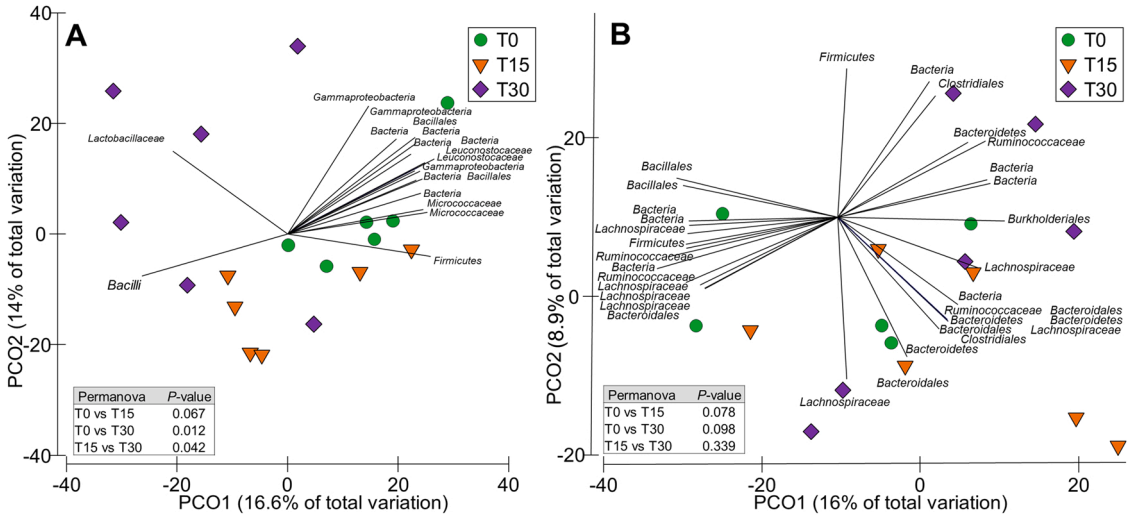

Fig. 1. : Principal coordinate analysis (PCoA) of the ileal (A) and caecal bacterial community (B) in piglets a control diet (T0) or supplemented with 15 (T15) or 30 g FOMG/kg (T30) at the end of the starter phase in experiment 2. Tripod vectors describe the direction and intensity of the most discriminant taxa (Spearman's correlation > 0.75). Pair-wise PERMANOVA values are shown based on the Bray-Curtis dissimilarity distance.

obvious since piglets fed T15 showed higher growth rates than observed for T0 during the prestarter (0.38 higher) and starter phase (0.29 higher), that can also be partially explained by the higher feed intake recorded (0.26 higher across both phases). However, other factors such as a higher immune status or an improved gut microbial development during the post-weaning period should not be ruled out (Blavi et al., 2021). Besides, considering that most fatty acids from the fish oil fraction are in form of mono- and diglycerides, this could favor fatty acid absorption and bioavailability (Chevalier et al., 2021).

From the observed results we have no conclusive arguments to associate the positive effects in growth rate during experiment 1 to a better health status since no differences in mortality were apparent in any experiment; however, it is noticeable that morbidity was almost two-fold higher in T0 piglets. Shin et al. (2017) reported that reducing the ω-6 to ω-3 polyunsaturated fatty acid proportion by including 12–17 g/kg fish oil in diet increased piglet growth and reduced the incidence of diarrhoea. It has been reported that ω-3 polyunsaturated fatty acids (PUFA) are precursors of lipid mediators and, through the production of eicosanoids, play an important role in attenuating the intestinal inflammation that is normally observed at weaning (Wall et al., 2010). Although gut inflammation was not evaluated in our study, our results support the hypothesis that supplementation with a fish oil fraction as a source of ω-3 could alleviate and prevent the development of inflammatory process during the piglet post-weaning resulting on superior intestinal barrier function and higher weight gain (McAfee et al., 2019). Lower plasma concentrations of tumour necrosis factor-α (TNF-α), the principle mediator of inflammation, have been found in piglets supplemented with ω-3 fatty acids than in their un-supplemented counterparts (Gaines et al., 2003; Li et al., 2014).

Blood parameters recorded in experiment 2 did not give any clear reference for an incidence of pathology for any treatment. Haematological parameters related with red blood cells, haemoglobin and platelets were within the standards considered as normal for healthy piglets around weaning (Egeli et al., 1998; Perri et al., 2017; Ventrella et al., 2017). However, despite no treatment differences were recorded on lymphocyte concentration, a high concentration respect to the reference values given by those authors was observed in most piglets at the prestarter and starter phases, which otherwise might be associated with the stress of sampling (Li et al., 2019). The higher levels of neutrophils detected in T0 and T30, in comparison to T15 may indicate the presence of an infective or inflammatory process, since these cells might be related to digestive disorders (Ventrella et al., 2017; McAfee et al., 2019). In any case, a potential beneficial effect of the FOMG on digestive health that might be inferred from results of T15 is not apparent considering results from T30, possibly as a result of an excessive dosage of fish oil (Liu et al., 2003), an insufficient vegetable oils, or both. In this sense, it has been suggested that vegetable oil have a superior ability, compared to tallow or fish oil, to maintain intestinal villus height in piglets (Jung et al., 2003).

High concentrations of AST were recorded for all treatments, being particularly evident in the T0 group in which one-third of the piglets were well over the physiological range (Egeli et al., 1998), and it was also observed that T30 decreased the plasmatic levels of ALT. Since Increased levels of AST and other transaminases such as ALT and ALP are generally associated to hepatic damage, both results suggest that supplementation with fish oil, in our study the FOMG, might have a role in preventing hepatic damage or inflammation (Huber et al., 2018). Moreover, the trend for significance ($P = 0.098$) in blood cortisol concentration highlights that T0 piglets had 57% higher values than FOMG supplemented to piglets at 30 g/kg, which may indicate a higher level of stress (Colson et al., 2012). However, this parameter should be considered with care since it may rapidly increase with animal handling and sampling (Stilwell et al., 2008).

In pig production, the high frequency of digestive processes occurring immediately after weaning is a clear indication that digestive microbiota is a major factor on determining animal adaptation to solid diets (Blavi et al., 2021). The small intestine microbiota is low

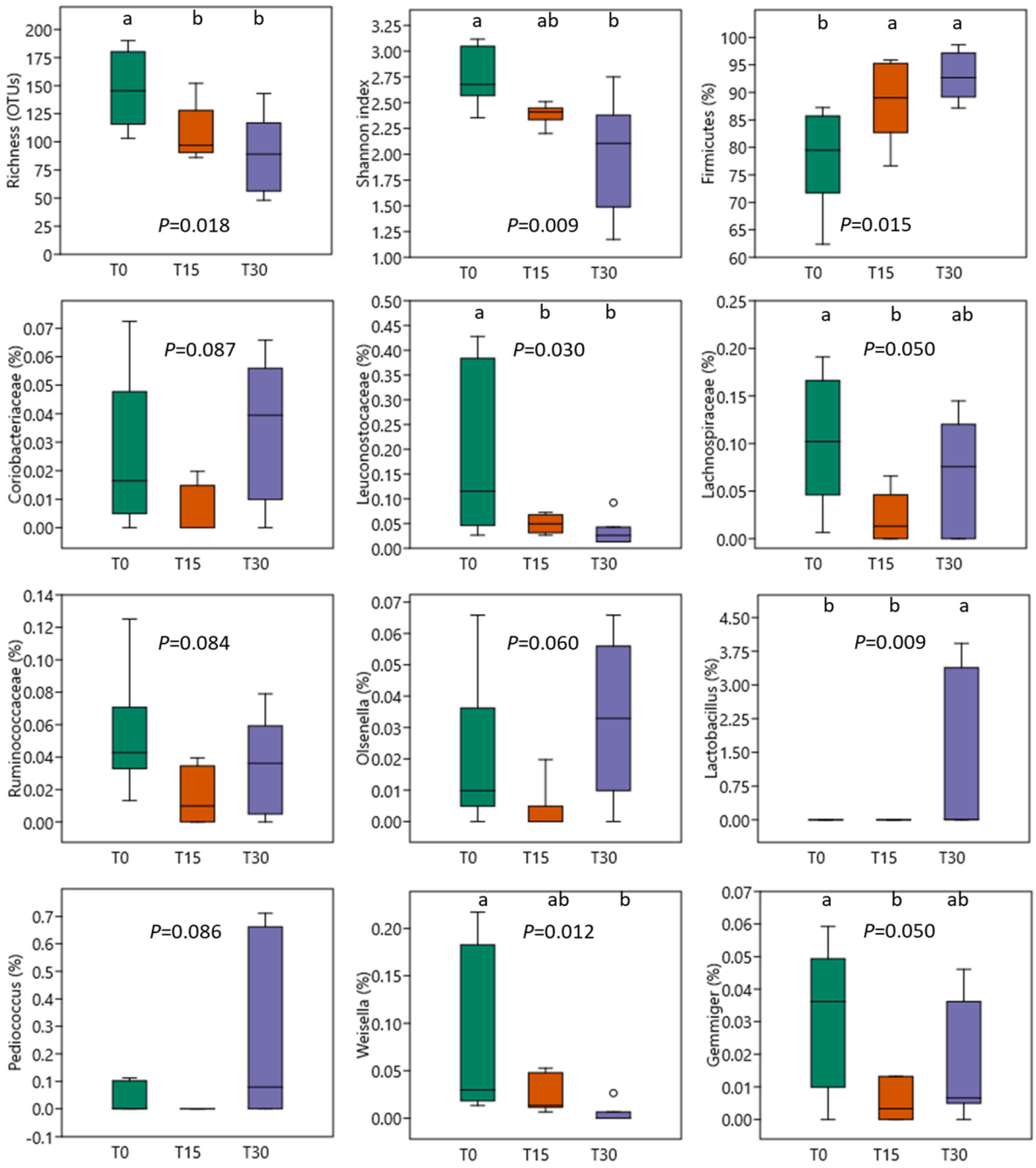

Fig. 2. : Boxplots showing the ileal bacterial diversity and taxa abundances in piglets given feed on a control diet (T0) or supplemented with 15 (T15) or 30 g FOMG/kg (T30) at the end of the starter phase in experiment 2. Only taxa which tended to differ ($P < 0.10$) among treatments are shown. Letters indicate treatment differences ($P < 0.05$) from a Kruskal-Wallis non-parametric test.

diverse and highly variable in piglets, and can led to digestive disorders and failure in nutrient absorption (Adhikari et al., 2019). Presence gut VFA as products of the microbial fermentation are considered indicators of gut health, the butyrate being of particular interest because it represents main energy source for the enterocytes (Leonel and Alvarez-Leite, 2012). In the present experiment, piglets fed T15 diets tended ($P < 0.10$) to have a higher VFA concentration (0.30 and 0.58 higher, respectively) and butyrate molar proportion (0.56 and 0.79 higher) at the ileum than those fed T0 and T30 diets, suggesting more active microbial fermentation and gut health. These findings are in line with the evidence indicating that dietary supplementation with ω-3 fatty acids exert a positive action by reverting the microbiota composition in the disease, and increase the production of anti-inflammatory compounds such as VFA, and

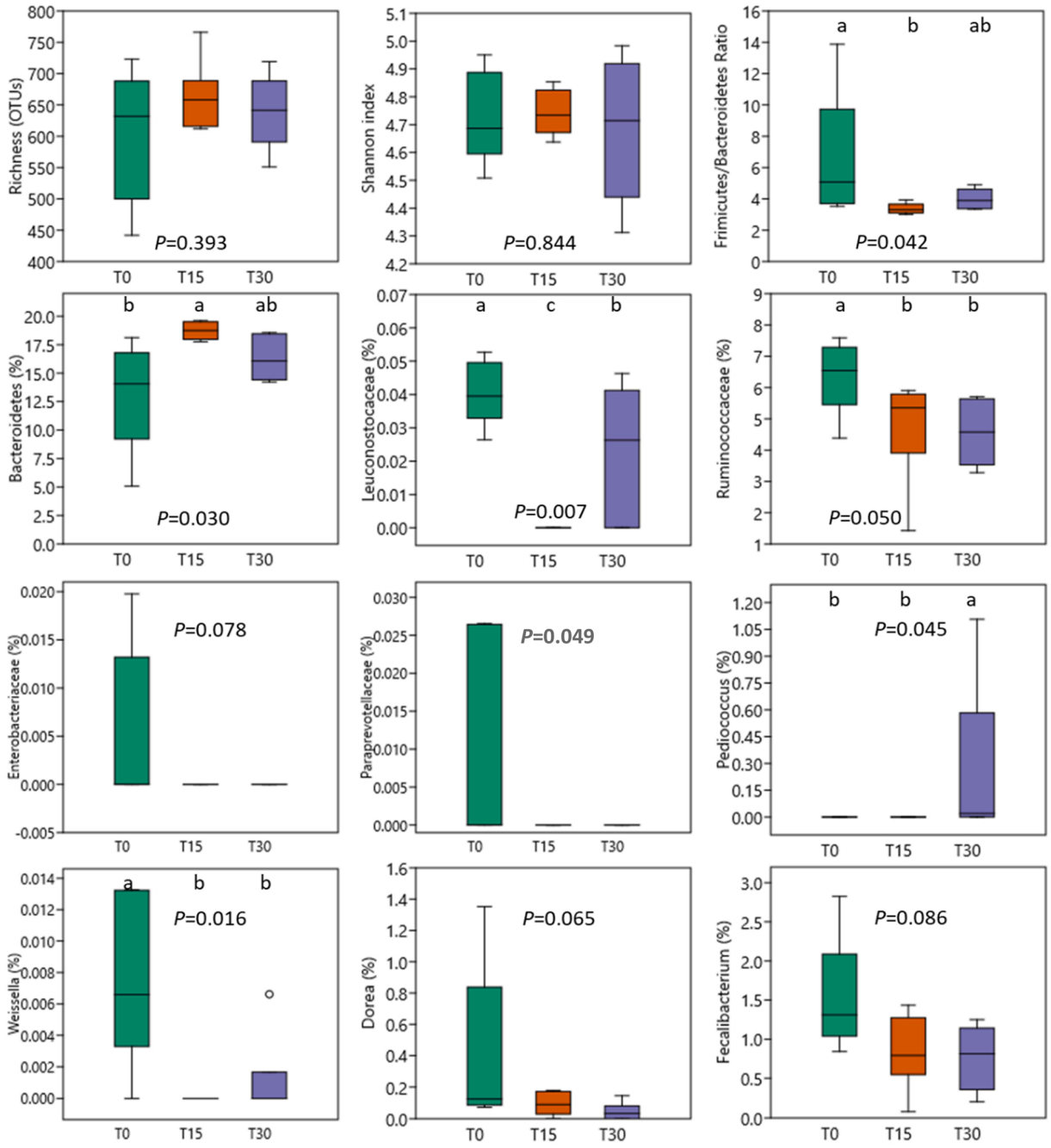

Fig. 3. : Boxplots showing the caecal bacterial diversity and taxa abundances in piglets given feed on a control diet (T0) or supplemented with 15 (T15) or 30 g FOMG/kg (T30) at the end of the starter phase in experiment 2. Only taxa which tended to differ ($P < 0.10$) among treatments are shown. Letters indicate treatment differences ($P < 0.05$) after a Kruskal-Wallis non-parametric test.

more specifically butyrate through the proliferation of butyrate-producing bacteria (Fu et al., 2021). In our study, supplementation with FOMG modified the ileal bacterial community structure and progressively decreased the bacterial diversity in relation to un-supplemented piglets (40 OTUs and 58 OTUs less for T15 and T30, respectively). Previous studies have shown that the decreased bacterial diversity observed in the ileum (Tao et al., 2015) is often accompanied by an increase in the colon bacterial diversity (Konstantinov et al., 2004), being both elements an indication of a microbial adaptation from milk diets (fermented in the small intestine) to solid diets which are mostly fermented in the hindgut.

A meta-analysis compiling 15 metagenomic studies reported that the most prominent butyrate-producing species in the human colon (based on the butyrate transferase and butyrate kinase activity) are the families Lachnospiraceae and Ruminococcaceae (Vital

et al., 2017). Despite the observed decrease in the two aforementioned families in piglets supplemented with FOMG, the increase in Firmicutes suggests that other taxa from this Phylum may play a more relevant role in butyrate production at the ileum than in the colon due to physiological (e.g. higher passage rate) and microbiological differences (Yu et al., 2017). In this sense, treatment T30 promoted the presence of two beneficial Firmicutes genera (*Lactobacillus* and *Pediococcus*) at both ileal and caecal levels, which were mostly undetectable in the rest of the piglets. These two genera have been shown to prevent fatty liver disease by modulating the hindgut microbiome, when used as probiotics in mice (Lee et al., 2020).

The environmental conditions are more stable in the caecum than in the ileum as noted by the higher bacterial diversity and fermentative activity. This, together by the fact that most fatty acids are expected to be absorbed in the small intestine, could explain the smaller treatment differences at a microbiological level observed at the caecum respect to the ileum. Instead, a higher extent of fermentation in this organ might indicates a lower digestion and absorption of nutrients at the small intestine, that leads to a higher arrival of fermentable substrate to the hindgut. As a result, the decrease in the VFA concentration with the level of FOMG supplementation (0.16 and 0.31 lower in T15 and T30 respect to T0) could be associated to a higher nutrient utilisation and absorption in former sites of the digestive tract. Despite molar VFA proportions and bacterial diversity indexes at the caecum did not differ across treatments, this does not necessarily indicate a similar population since the same metabolic role can be performed by different bacterial species (Weimer, 2015). Piglets fed T15 and T30 diets exhibit a similar bacterial community structure and taxa distribution, that tended to differ from that observed in control piglets. In humans, a high Firmicutes-to-Bacteroidetes ratio at the hindgut has been described as microbial dysbiosis associated with obesity and gut inflammation (Fu et al., 2021). Our study noted that FOMG supplementation decreased the caecal Firmicutes-to-Bacteroidetes ratio, as observed with ω-3 supplementation in mice fed high-fat diets (Onishi et al., 2017). This shift in the ratio may be explained by the decrease in the abundance of two Firmicutes families (Ruminococcaceae and Faecalibacterium) and the increase in Bacteroidetes. These findings are in agreement with the lower faecal levels of bacterial diversity and presence of *Faecalibacterium prausnitzii* in 45 days old piglets supplemented with 600 mg of ω-3 per kg (Noriega et al., 2016), despite our study did not detected a drop in diversity at the ileum level.

5. Conclusions

Supplementation of diets for weaned piglets with a fish oil processing fraction rich in ω-3 fatty acids at a level of 15 g FOMG/kg DM increased weight gain during the transition period. This increase in growth was partially explained by an enhanced feed intake, but it is also related to an improvement in animal health, manifested in lower blood levels of neutrophils and cortisol, and a higher nutrient utilization in the small intestine, suggested by a lower VFA concentration at the caecum. Moreover, the FOMG supplementation exerted positive modulatory effects of the gut microbiota as noted by the lower bacterial diversity and higher butyrate production at the ileum, and a lower and most favourable Fibrobacter-to-Bacteroidetes ratio in the caecum. These findings shed light on the underlying mechanisms explaining beneficial effects of fish oil supplementation and the importance of providing appropriate levels of ω-3 mono- and diglycerides supplementation in weaned piglets.

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CRediT authorship contribution statement

A. Belanche: Methodology, Data curation, Writing – original draft, Writing – review & editing. **S. Diago:** Methodology, Investigation. **M. Fondevila:** Conceptualization, Investigation, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of interest

None.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2022.115517.

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