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Changes in Ileum and Cecum Volatile Fatty Acids and Their Relationship with Microflora and Enteric Methane in Pigs Fed Different Fiber Levels

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Abstract: Mustard and grapeseed meals are low-cost by-products generated by the oil industry. We aimed to investigate the effects of fiber level on the concentration of volatile fatty acids (VFAs) in the ileum and cecum, as well as the microflora and enteric methane (E-CH_4) in pigs. A total of 70 Topigs hybrid pigs, 56 ± 3 days old, 20.96 ± 0.26 kg live weight, were randomly allotted to two feeding groups: (i) SM diet based on sunflower meal; (ii) MG diet based on mustard × grapeseed oil meals (MG-mixt). VFA profiles were determined by gas chromatography. E-CH_4 was calculated using our trial data along with prediction equations. The MG-mixt slightly decreased growth parameters but improved daily feed efficiency and gain cost. The MG diet increased the concentration of VFAs and the microflora level. The higher VFA level recorded in the cecum (+53.93%) was correlated with a lower pH level (Spearman correlation coefficient, rho = −0.529, $p < 0.001$). In relation to DM intake and energy retention, E-CH_4 recorded a highly significant decline in the MG group (<9.42%). A strong relationship was recorded among VFAs, microflora, predicted E-CH_4, and fiber and NDF intake. The VFAs could be predictors for the E-CH_4 level ($p < 0.001$). A significant relationship between E-CH_4 and total VFAs was noted (rho = −0.462, $p = 0.04$). We conclude that MG-mixt has the potential to replace sunflower meal, with the minor drawbacks being balanced by the advantages provided in terms of feed efficiency, E-CH_4 mitigation, and VFA levels.

Keywords: mustard meal; grapeseed meal; VFAs; microflora; E-CH_4; pigs

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1. Introduction

The swine production system entails proper nutrition management based on less expensive raw materials, which contributes significantly to profitability. Due to fluctuating prices and a trend toward the partial or total substitution of classical feedstuffs, there is a growing interest in locating and utilizing available resources. Due to their availability and low cost, many types of alternative fibrous feedstuffs (e.g., byproducts) have been used in Romania and other parts of the world for a long time. The residual meals obtained after cold-pressing oil-rich seeds are rich in nutrients and bioactive compounds, making them suitable for animal feed. Diversifying the plant source matrix by using byproducts as production residues could result in lower feed costs, improved health, and a cleaner environment.

Fibers, positively influence animal gut health, although the animal's growth performance falls short of its full potential [1,2].

The high cost of pig feed (65–70% of the total production cost) has led to increased interest in replacing or supplementing the classical soybean meal with more affordable and

accessible ingredients such as sunflower meal and rapeseed meal. The issue is related to additional costs due to processing equipment, feed wastage, and increased risk to animal health, as well as reduced performance as a result of byproduct variability and suitability for pig feed. Although the proportion of lysine in sunflower meal is lower than that in soybean meal, it is increasingly being employed as a protein source in growing-finishing pig diets. Nevertheless, as the food sector has grown, a variety of byproducts have become available and can be used to substitute sunflower meal, particularly by farmers who cannot obtain them easily.

Two lesser-known byproducts as feed sources for pigs are mustard meal and grapeseed meal, resulting from the oil extraction industry. White mustard (*Sinapis alba*) is a historical culture from the Hellenistic and Roman times commonly used in the medicinal treatment or as a spice or edible oil [3]. Mustard seed has high oil content ranging between 38% and 44% [4], along with a protein level in excess of 28% and a fiber level of ~14% [3]. Previous research has focused on using mustard byproducts as a protein source [3] as opposed to a source of bioactive substances. Grape (*Vitis vinifera*) is a widely grown fruit culture. Previous studies have focused on the metabolic profile and the anti-inflammatory properties of grape pomace biocompounds [5,6]. Antioxidant mechanisms resulting from the polyphenol concentration [7] and their role in decreasing toxicity caused by aflatoxin B1 in the mesenteric lymph nodes [8] have also been investigated. Research results indicates dietary fibrous ingredients impact the production of VFAs, also named short-chain fatty acids (SCFA). VFAs are important organic metabolites containing a maximum of five carbons, derived from the microbial anaerobic fermentation of dietary fiber, resistant starch, and amino acids. Their production depends on the degree of fermentation in the digestive tract [9–11]. The most common VFAs are acetate ($C_2H_4O_2$), propionate ($C_3H_6O_2$), and butyrate ($C_4H_8O_2$). Additionally, branched SCFAs (BCFA) are end-products of aliphatic amino acid catabolism (leucine, isoleucine, and valine) produced in lower amounts. The predominant BCFAs, isobutyrate ($C_4H_8O_2$) and isovalerate ($C_5H_{10}O_2$), have attracted less attention.

In classical studies, it has been shown that considerable amounts of VFAs are produced in the cecum and colon of pigs, whereas, in the stomach and the small intestine, the VFA concentration is lower [1,9,12], albeit slightly higher compared to rats and rabbits [13]. Recently, Li et al. [14] showed that the fermentative process of some fiber components could start in the small intestine, while Montoya et al. [12] identified, in growing pigs, soluble kiwifruit fiber levels close to 80% fermenting in the distal section of the small intestine. Furthermore, in growing pigs used as human models, 25–30% of undigested material was fermented in the ileum upon employing a high-fiber human-type diet. Bugaut [15] indicated that VFAs contribute to the basal metabolic requirement as follows: 15–28% for the total digestive tract 30% for the whole large intestine, and 1.9–2.7% in the cecum. Philippe and Nicks [16] specified that the fermentative capacity of the hindgut and fiber level could change the production of enteric methane (E-CH_4).

A number of studies have highlighted the concentration of VFAs in different sections of the gastrointestinal tract in relation to the microflora community [9,17–19]. Moreover, E-CH_4 assessment has been the subject of numerous studies, especially in ruminants [20–24]. Dämmgen et al. [25] developed a model to quantify E-CH_4 emissions from pigs, while Philippe and Nicks [16] described the processes involved in E-CH_4 emissions and reviewed the emission factors (including diets) and their effects. As far as we know, the influence of increased dietary fiber level on the concentration of SCFAs and BCFAs in the ileum and cecum, resulting from the use of a mixture of mustard meal and grapeseed meal as a replacement for sunflower meal, has not been studied.

Therefore, the aim of this study was to investigate the changes in VFA concentration in the ileum compared to the cecum, as well as their relationship with the microbial community and E-CH_4 level, as a function of the effect of dietary fiber level in pigs fed different types of oil industry byproducts.

2. Materials and Methods

2.1. Animal and Housing

The biological protocol was endorsed by the INCDBNA Balotesti Ethical Committee (no. 7976/12/2020). All procedures and methods applied in the experiments were carried out at the Experimental Biobase of INCDBNA Balotesti according to Romanian Law no. 199/2018, in compliance with EU Directive 2010/63/EU for animal experiments.

The experiment was conducted over 38 days on 70 growing healthy Topigs hybrid pigs (female Large White × Hybrid (Large White × Pietrain) × male Talent (mainly Duroc), in an experimental modern indoor facility (21 °C, 60% rH, cage size 2.6 × 2.3 m). The pigs, 56 ± 3 days old, 20.96 ± 0.26 kg initial body weight (BW), were ear-tagged and distributed completely randomly into four mixed-sex pens per group, 35 pigs per group, with a similar sex ratio (18 castrated male and 17 female pigs in each group). The group size was determined according to Charan and Kantharia [26].

2.2. Treatments

Two dietary treatments were formulated: (i) SM diet based on sunflower meal, with a 6.23% level of dietary fiber (Topigs guidance); (ii) MG diet based on MG-mixt, with a 7.28% level of dietary fiber. The sunflower meal was totally replaced by MG-mixt in the MG diet (Table 1).

Table 1. Diet composition for Topigs hybrid pigs fed a standard level of fiber (SM diet) or a diet based on a high level of fiber (MG diet).

Items (g as Feed Base)	SM	MG
Maize	585.8	559.1
Rice bran	150.0	150.0
Soybean meal	80.0	110.0
Sunflower meal	150.0	-
MG-mixt	-	150.0
L-Lysine-HCl	4.6	0.9
Calcium carbonate	16.0	16.4
Monocalcium phosphate	1.5	1.5
Salt	1.0	1.0
Choline premix	1.0	1.0
Phytase (500 FTU kg·feed^{-1})	0.1	0.1
Vitamin and trace mineral mixture [1]	10.0	10.0
Analyzed composition (g/kg as feed base)		
DM	876.3	880.3
CP	158.5	153.6
EE	30.3	39.2
Crude fiber	62.31	72.88
NDF	158.8	189.0
ADF	74.3	106.3
Calculated composition (g/kg as feed base)		
ME (MJ/kg) [2]	12.65	12.03
NE (MJ/kg)	9.43	9.05
ADL [3]	6.99	28.42
NSP *	207.4	207.0
Lys	10.5	10.5
Lys d [2]	8.9	8.1
Met + Cys	6.6	6.6

Table 1. *Cont.*

Items (g as Feed Base)	SM	MG
Calculated composition (g/kg as feed base)		
Met + Cys d [2]	5.8	5.5
Ca	8.1	8.1
P	6.5	6.5

Abbreviations: MG-mixt, mustard oil meal × grapeseed oil meal 7:8 *w/w*; DM, dry matter; ME, metabolizable energy; NE, net energy; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, lignin; Lys, lysine; Met + Cys, methionine + cysteine; Ca, calcium; P, phosphorus; d, digestible. * NSP = non-starch polysaccharides (OM − (starch + sugar + CP + EE)). [1] The vitamin–mineral premix provided the following per kg feed: (i) 6000 IU vitamin A, 800 IU vitamin D3, 20 IU vitamin E, 1 mg vitamin K3, 1 mg vitamin B1, 3.04 mg vitamin B2, 10 mg vitamin B3, 6.3 mg vitamin B5, 1.5 mg vitamin B6, 0.03 mg vitamin B7, 0.3 mg vitamin B9, 0.02 mg vitamin B12, 30 mg Mn, 80 mg Fe, 25 mg Cu, 100 mg Zn, 0.22 mg I, 0.22 mg Se, 0.3 mg Co, and 60 mg antioxidant. [2] ME was calculated using regression equations developed by the Oskar Kellner Institute of Animal Nutrition: ME = 5.01 DP + 8.93 EE + 3.44 GF + 4.08; digestible amino acid contents were calculated on the basis of feed composition and theoretical coefficients determined by INCDBNA Balotesti. [3] ADL = ADF–crude fiber–ash.

The mustard and grapeseed meal byproducts that resulted after oil extraction were delivered by 2E-Prod SRL, Alexandria, Romania. The price of acquisition was 24% less than that of sunflower meal. The final formula of the MG-mixt product (7:8, *w/w*) was obtained after several simulations of different proportions of the two byproducts. The MG-mixt was processed separately, dosed, and ground in a hammer mill. The resulting product was placed in a mixer for homogenization (4–6 min), and then compressed at 80 °C using a 6 mm diameter pellet press in the presence of a binder (a mixture between water + molasses in equal parts; PLT 100). Before being ground and mixed into the compound feed, the crumbly pellets were sampled for chemical analysis.

The weight of the two ingredients in the mixture was set in such a way as to cover the specific nutrient requirements as in the case of the SM diet. Whereas dietary ME and net energy (NE), as well as limiting crude amino acids were similar between groups, fiber, comprising crude fiber, neutral-detergent fiber (NDF), acid detergent fiber (ADF), and lignin (ADL), were higher in diets with MG-mixt added. Digestible amino acids were lower in the MG-mixt group. The combination MG-mixt led to a 22.1% higher crude fiber level compared to sunflower meal (g·kg DM^{-1}; Table 2). The feed intake and leftovers were recorded daily.

Table 2. Fiber composition of MG-mixt and sunflower meal.

Items (g·kg DM^{-1})	MG-Mixt	Sunflower Meal
Analyzed composition		
DM	909	897.0
Crude fiber	303.8	236.5
NDF	452	400.0
ADF	403.8	276.0
Calculated composition		
ADL (lignin)	50.83	92.0
Insoluble hemicellulose [1]	71.3	124.0

Abbreviations: NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, lignin. [1] Insoluble hemicellulose = NDF − ADF.

The dry matter and fiber chemical composition of MG-mixt and the sunflower meal are shown in Table 2. The levels of ADL and insoluble hemicellulose were calculated.

2.3. Measurements

The individual BW was determined using an electronic scale (26 and 64 days after weaning), covering a live weight range from 20 to 50 kg. The pigs fasted overnight before weighing. A total of 40 pigs randomly selected were slaughtered for biological samples

(20 pigs per group, 20 female and 20 males; 37± 3.2. kg carcass) at the end of the trial. After stunning and exsanguination, the correctly labeled pigs were transported to the necropsy laboratory. The abdominal cavity was opened, and the intestinal mass was removed without perforation of the intestines. From each pig's slaughtered, the entire cecum and approximately 15 cm of ileum distal part (~5 cm anterior to the ileocecal junction) were dissected for collecting digesta.

This study presents the value of the carcass dressing percentage. Immediately after euthanasia, the intestine content was rapidly removed and collected into sterile plastic bags, before being transported to the analytical laboratory on an ice bed in a special refrigerated container.

2.4. Chemical Analyses

2.4.1. Gross Chemical Composition

The gross chemical composition of ingredients and diets was determined in duplicate using standardized methods according to European Commission (EC) Regulation no. 152 (2009). Crude fiber (CF) extraction was performed by the intermediate filtration method, according to European Commission (EC) Regulation no. 152 (2009) and the standard SR EN ISO 6865:2002. According to Weende's method, sugar and starch were extracted first by acid hydrolysis with H_2SO_4, then proteins, some hemicellulose, and lignin were removed by alkaline hydrolysis with NaOH. The residue was filtered, dried, calcined, and weighed. For neutral and acid detergent fiber (NDF, ADF) determination, Van Soest extractions were performed, according to SR EN ISO 16472:2006 and SR EN ISO 13906:2008. The analyses were carried out using the Raw Fiber Extractor FIWE 6 (Velp Scientifica, Usmate, Italy). The ADL was calculated by the difference between ADF, crude fiber, and ash.

2.4.2. Ileum and Cecum Microflora Analyses

Tenfold serial dilutions of 1 g sample content from the distal ileum and cecum digesta were homogenized with 7 mL of Brain Heart Infusion broth (Oxoid Basingstoke, Hampshire, UK) supplemented with 2 mL of glycerol and frozen at −20 °C until the analysis. After defrosting samples, decimal dilutions in phosphate-buffered saline (Dulbecco A; Oxoid Livingstone Ltd., London, England) were conducted. The samples were assessed for lactic acid bacteria (LAB), *Escherichia coli* (*E. coli*; biotype β-hemolytic), *Salmonella* spp., *Clostridium* spp., coliform count, *Enterococcus* spp., and Enterobacteriaceae. The LAB were cultured on de Man, Rogosa, and Sharpe agar (MRS, Oxoid CM0361) incubated in anaerobic conditions at 37 °C for 48 h (Thermo Scientific jar with Anaerogen 2.5 L, Oxoid Basingstoke, Hampshire, UK). Coliforms were cultured on MacConkey agar (Oxoid CM0007) incubated aerobically at 37 °C for 24 h. *E. coli* (biotype β-hemolytic) was analyzed as previously described by [27]. *Clostridium* spp. and *Enterococcus* spp. were cultured anaerobically at 37 °C for 48 h on Reinforced Clostridia Agar (Oxoid CM0151) and Slanetz–Bartley agar (Oxoid CM0377), respectively. Enterobacteriaceae and *Salmonella* spp. were enumerated on Oxoid selective medium (Eosin Methylene Blue Agar, Levine CM 0069, Basingstoke, UK) and *Salmonella–Shigella* agar (CM0099) by incubation at 37 °C for 48 h in aerobic conditions. Each sample had three replicates. The microflora level was expressed as $\log_{10}$ CFU· g^{-1} content.

2.4.3. Volatile Fatty Acid Quantification and pH Analysis of Ileum and Cecum Content

The VFAs were quantified by gas chromatography in water extracts of distal ileum and cecum content (five replicates/sample). Briefly, the samples were mixed with distilled water in a proportion of 0.7:1 (*w/w*), and then centrifuged at 13,000 × *g* for 15 min. A sample volume of 1 µL from the centrifuged extract was injected under split mode into a gas chromatograph (Varian, 430-GC) equipped with a capillary column Elite-FFAP with a length of 30 m, an inner diameter of 320 µm, and a film thickness of 0.25 µm (Perkin Elmer, Seattle, Washington, USA). The carrier gas was hydrogen, with a flow rate of 1.5 mL/min. The injector temperature was set to 250 °C, and the split rate was 1:40. The flame ionization detector (FID) temperature was set to 200 °C, while the column oven temperature was set

to 110 °C. The oven temperature was increased to 170 °C at 12 °C/min, where it was held for 9.5 min. The analysis time was 10 min. The sample concentration was calculated using a standard commercial mixture of VFA (CRM46975, Supelco, St. Louis, MO, USA). The final results were expressed as µmol/g.

To measure the pH, 1 g of fresh sample (ileum and cecum content of each pig) was collected and transferred into 9 mL of distilled water (1:10 dilution, *w*/*v*). The pH value was measured (mean of three readings) using a portable pH meter (pH 7 + DHS, XS Instruments, Carpi, MO, Italy). After each pH measurement, the electrode was carefully washed with water and calibrated between each animal.

2.5. E-CH_4 Prediction

The E-CH_4 level equation developed by Philippe and Nicks [16], expressed as carbon dioxide equivalent (g CO_2 eq·day^{-1}), is as follows:

$$\text{E-CH}_4 = 0.012 \times \text{dRes} \times \text{DM intake kg·day}^{-1} \quad (1)$$

where dRes (g/day) refers to digestible residues calculated according to INRA-AFZ (2004), as quoted by Philippe and Nick [16]. The theoretical digestibility coefficient was obtained from the IBNA Balotesti database.

2.6. Calculations

Carcass weight was used to calculate the dressing percentage (carcass yield, %). The indicators Kleiber ratio (KR) and relative growth rate (RGR, %) were calculated using the following equations [28]:

$$\text{KR} = \text{ADG}/\text{MBW}^{0.75}, \quad (2)$$

where ADG is the average daily gain and $\text{MBW}^{0.75}$ is the metabolic weight;

$$\text{RGR} = 100 \times (\log_{10} \text{BW at the end of the test}) - (\log_{10} \text{BW at the start of the test})/(\text{age at the end of trial} - \text{the age at the start of trial}). \quad (3)$$

The cost of each diet, the feed intake, and the ADG were all taken into account when calculating economic efficiency.

For energy retention (ER) calculation (MJ·day^{-1}), the difference between *ME* intake and heat production (HP) was used [29].

The HP was calculated according to Aarnink's equation [30].

$$\text{HP} = MEm + (1 - kY)\cdot(ME - MEm); kY = 0.47 + 0.003 \times \text{BW}, \quad (4)$$

where *MEm* (MJ) is the energy for maintenance ($MEm = 0.4398 \times \text{BW}^{0.75}$) [29], and *kY* is the efficiency of protein and lipid retention [29].

The molar percentage for the main SCFAs (acetate, propionate, and butyrate) was calculated by dividing the total amount of VFAs (including valerate and BCFAs) by the concentration of each VFA.

The EvaPig tool, version 2.0.3.2 (2020), developed by the French National Institute for Agricultural Research, METEX NØØVISTAGO, and the French Association of Zootechnie, was used to calculate the energy, amino acid, and phosphorus values of pig feed.

2.7. Statistical Analyses

The descriptive statistics were calculated using IBM SPSS (2011). Data were analyzed using two-way ANOVA. In our model, the main effects were considered the diets (differing in fiber content) and the gut sections (ileum and cecum). The data distribution was verified by the Shapiro–Wilk test. Except for body weight and ADG, each pen was considered as an experimental unit irrespective of the analyses or measurements performed. The impact was considered as statistically significant at $p \leq 0.05$ and as a trend at $0.10 > p > 0.05$. The effect of replicates was omitted in the analysis due to their insignificance ($p > 0.05$).

The Pearson or nonparametric Spearman correlation was applied to evaluate the measure of bivariate association. The interpretation of correlation coefficients followed [31]. Regression analyses were used to assess the strength of the relationship among total VFAs, predicted E-CH_4, bacteria, intake of fiber and associated fractions, and amino acid intake.

3. Results

3.1. Performance and Efficiency Traits

As shown in Table 3, the intake of ADF was 26.5% higher and that of ADL was 74.86% higher in the MG-mixt group ($p < 0.0001$), whereas the NDF increased by 12.65% ($p < 0.046$). On the other hand, the Pearson correlation between fiber, nitrogen, and the limiting amino acids (lysine and methionine + cystine) was highly significant ($r = 0.75$–0.98, $p < 0.0001$).

Table 3. Descriptive statistics of growth parameters and efficiency traits of Topigs hybrid pigs fed a standard level of fiber (SM diet) or a diet based on a high level of fiber (MG diet).

Traits [1]	Diets		SEM	*p*-Value [3]
	SM	MG		
	Intake (g·day^{-1})			
ADFI	1725	1673	0.06	NS
Crude fiber	110.8	121.67	3.92	NS
ADL	12.03	47.85	2.26	***
NDF	275.59	315.49	8.50	*
ADF	130.67	177.91	5.8	***
Hemicellulose	148.6	138.41	4.80	NS
Nitrogen	43.81	41.13	1.47	NS
Lys	18.46	17.56	0.60	NS
Met + Cys	11.60	11.04	0.37	NS
ME (kJ·kg BW$^{0.75}$·day^{-1})	1225	1186	41.23	NS
Growth parameters (kg) and efficiency traits [1]				
Initial BW [2]	20.43	21.50	0.67	NS
Final BW	47.14	45.75	0.87	NS
MBW$^{0.75}$	17.98	17.58	0.10	NS
Carcass yield (%)	81.19	78.36	0.97	NS
ADG	0.703	0.638	0.01	**
Feed-to-gain ratio (g:g)	0.47	0.43	0.02	NS
RGR	0.95	0.87	0.02	**
KR	5.01	4.58	0.07	**
Economic efficiency: EUR·day^{-1}	0.43	0.32		
EUR·kg gain^{-1}	0.61	0.50		

[1] The total number of observations was 70; [2] 26 days old after weaning; the number of observations for carcass yield was 40 (20 female and 20 male). Abbreviations: ADFI, average daily feed intake; ADL, lignin; NDF, neutral detergent fiber; ADF, acid detergent fiber; ME, metabolizable energy; BW, body weight; MBW$^{0.75}$ metabolic BW; ADG, average daily gain; RGR, relative growth rate; KR, Kleiber ratio; SEM, standard error of the mean. [3] NS: nonsignificant effect; * $p < 0.05$ (significant difference between means); ** $p < 0.01$ (distinctly significant difference between means); *** $p \leq 0.001$ (highly significant difference between means).

A 9.25% decline was recorded for ADG ($p = 0.002$) in pigs fed MG-mixt. The RGR and KR recorded a highly significant decrease in the MG-fed group (<8.4% and 8.6%, respectively), whereas the intake of certain fibrous compounds (fiber, ADL, NDF, and ADF) increased. The chemical nature of nutrients influenced gastric emptiness.

In terms of economic efficiency, we found that the MG group yielded a 25% reduction in daily feed cost. In the MG fed group, the daily gain was achieved at a 16% lower cost.

3.2. Microflora Profile

The concentrations of the Firmicutes phylum (including *Lactobacillus* spp., *Enterococcus* spp., *Staphylococcus* spp., and *Clostridium* spp.) and Proteobacteria phylum (including

Enterobacteriaceae, *E. coli*, and coliforms) in the ileum and cecum from pigs fed both types of diet are shown in Table 4. There was no significant effect on total bacteria identified (7.03% higher in the MG group). MG-mixt positively impacted ($p < 0.05$) the levels of *Lactobacillus* spp. (>1.06-fold; 6.5–8.5 $\log_{10}$ CFU·g^{-1}) and *E. coli* (>1.41-fold; 0.05–5.69 $\log_{10}$ CFU·g^{-1}), while the level of *Enterococcus* tended ($p = 0.082$) to be lower than in the SM group (<1.11-fold; 3.0–6.17 $\log_{10}$ CFU·g^{-1}). The intensity of the fermentation processes was higher in the cecum, increasing most bacteria. A highly significant impact was noted for *Lactobacillus* spp. (+6.5%), *Staphylococcus* spp. (+21.08%), and *Clostridium* spp. (+24.48%).

Table 4. Microflora population ($\log_{10}$ CFU/g) of distal ileum and cecum gut sections from Topigs hybrid pigs (44 ± 3.30 kg) fed a standard level of fiber (SM diet) or a diet based on a high level of fiber (MG diet).

Microflora		Diets [1]		Gut Sections		Diets/Gut Sections				SEM	*p*-Value [2]	
Phylum	Genus	SM	MG	Ileum	Cecum	SM	MG	SM	MG		Diet effect	Gut Section Effect
						Ileum		Cecum				
Total bacteria identified		32.29	34.73	33.46	33.55	33.42	33.51	30.88	35.0	0.69	NS	NS
Firmicutes	*Lactobacillus* spp.	6.94	7.40	6.91	7.39	6.78	7.09	7.13	7.59	0.09	*	**
	Enterococcus spp.	4.81	4.33	4.62	4.64	5.09	4.0	4.49	4.54	0.14	T	NS
	Staphylococcus spp.	3.82	4.23	3.52	4.46	3.62	3.39	4.06	4.77	0.13	NS	***
	Clostridium spp.	5.93	6.11	5.12	6.78	5.25	4.95	6.73	6.83	0.13	NS	***
	Enterobacteriaceae	4.42	4.84	4.61	4.65	4.50	4.77	4.33	4.89	0.17	NS	NS
Proteobacteria	*E. coli*	2.80	3.96	3.80	3.02	3.27	4.53	2.25	3.61	0.29	*	NS
	Coliforms	3.54	3.84	4.83	2.70	4.88	4.77	1.96	3.25	0.25	NS	***

Data are expressed as $\log_{10}$ CFU/g and represent the means of three replicates for each animal sample. *Salmonella* (Proteobacteria) was absent; *E. coli*, *Escherichia coli*. [1] Data for each bacterium were pooled over both gut sections in order to determine the diet effect as the main factor. [2] NS: nonsignificant effect; * $p < 0.05$ (significant difference between means; ** $p < 0.01$ (distinctly significant difference between means); *** $p \leq 0.001$ (highly significant difference between means); T: trend at $0.10 > p > 0.05$. SEM, standard error of the mean. *Reference values* were sourced from [32–36]: coliforms, $\leq 10^8$–10^9; *E. coli* (biotype β-hemolytic), absent (<1 × 10^3); *Lactobacillus* spp., $\geq 10^8$–10^9; *Clostridium* spp., <10^8–10^9; *Enterococcus* spp., <10^8–10^9; *Staphylococcus* spp., <10^6–10^7; Enterobacteriaceae, 10^2–10^5.

A possible explanation consists of the greater influence of the MG diet in this gut section, with the exception of *E. coli* and coliforms, which declined in the cecum (−20.53% and −44.1%, respectively).

3.3. Volatile Fatty Acid Profile

The mean concentration of VFAs and the molar ratio of the main VFAs in both gut sections (ileum and cecum) from pigs fed differently are presented in Table 5. The total VFA concentration tended to increase in pigs fed MG-mixt (+14.38%). In our study, the BCFAs only represented approximately 1.79% of the total VFAs. A distinctly significant increase was recorded in propionic acid concentration in pigs fed a higher fiber level (+31.06%).

The isobutyric acid concentration tended to decrease (−44%), while the isovalerate acid concentration increased (+33.96%). A higher molar ratio of acetic acid was recorded in the MG group (+13.24%). The molar ratio of propionic also increased in the MG-fed group due to its greater concentration in the cecum.

We noted a highly significant impact ($p < 0.0001$) of the gut section on the mean concentration of total VFAs. Thus, the highest value of total VFAs was recorded in the cecum (>2.17-fold). The concentrations of acetic and propionic acids were greater in the cecum (>1.56-fold and >10.08-fold, respectively; $p < 0.0001$). A greater concentration of valeric acid was noted in the cecum (+97.5%).

Table 5. Concentration (μmol/g) and molar ratio of VFAs ± SEM in distal ileum and cecum gut sections from Topigs hybrid pigs (44 ± 3.30 kg) fed a standard level of fiber (SM diet) or a diet based on a high level of fiber (MG diet).

VFAs	Diets [1]		Gut Sections		Diets/Gut Sections				SEM	*p*-Value [3]	
	SM	MG	Ileum	Cecum	SM	MG	SM	MG		Diet Effect	Gut Section Effect
					Ileum		Cecum				
Total VFAs [2]	45.30	52.91	27.14	58.91	24.25	29.19	59.03	52.91	2.51	T	***
Total BCFAs (Ib and Iv)	0.79	0.76	0.41	0.92	0.52	0.26	0.98	0.76	0.08	NS	***
Acetate	29.05	30.72	21.42	33.49	19.39	22.29	34.81	30.72	1.42	NS	***
Propionate	9.39	13.62	1.59	16.03	0.62	2.95	15.52	13.62	1.04	**	***
Butyrate	3.68	5.05	3.62	4.84	3.69	3.53	3.67	5.05	0.40	NS	NS
Valerate	2.38	2.74	0.09	3.60	0.03	0.17	4.03	2.74	0.49	NS	***
Isobutyrate	0.43	0.24	0.22	0.35	0.38	0.01	0.47	0.23	0.02	T	NS
Isovalerate	0.35	0.53	0.18	0.57	0.14	0.25	0.51	0.53	0.07	T	***
Molar ratio (A:P:B)	59:17:7	58:24:9	79:8:11	57:27:8	81:5:11	74:12:12	59:26:6	60:24:9			
pH	6.08	5.63	6.58	5.62	6.44	6.29	5.18	6.08	0.10	*	***

[1] Data for each VFA were pooled over both gut sections in order to determine the diet effect as the main factor. There was a standard level of fiber in the SM group and a higher level of fiber than the animal requirement in the MG group. Data are expressed as μmol/g and represent means of five replicates for each animal sample. [2] Total VFAs include acetate, propionate, butyrate, valerate, isobutyrate and isovalerate. Abbreviations: A, acetate; P, propionate; B, butyrate; Ib, isobutyrate; Iv, isovalerate; SEM, standard error of the mean. [3] NS: nonsignificant effect; * $p < 0.05$ (significant difference between means); ** $p < 0.01$ (distinctly significant difference between means); *** $p \leq 0.001$ (highly significant difference between means); T: trend at $0.10 > p > 0.05$.

Intestinal pH level. The addition of dietary fiber by replacing the sunflower meal with MG-mixt led to a significant decrease in pH level (−7.4%, $p = 0.04$; Table 5). The ileum pH was 14.59% higher. Upon applying Spearman's correlation, a strong positive relationship was recorded between pH and butyric acid (rho = 0.21, $p < 0.047$), whereas acetic acid, propionic acid, valeric acid, isovaleric acid, total VFAs, and total BCFAs were negatively correlated with pH (rho = −0.46 to −0.66, $p < 0.01$).

3.4. Predicted E-CH_4

The E-CH_4 concentration is related to the level of dietary fiber [16]. As the MG-mixt increased both the level and the intake of dietary fiber, the E-CH_4 production decreased by 12.45% ($p = 0.03$; Table 6).

Table 6. Mean E-CH_4 level (g CO_2 eq) ± SEM and regression model in pigs fed a standard level of fiber (SM diet) or a diet based on a high level of fiber (MG diet).

Item [1]	Diets		SEM	*p*-Value [2]
	SM	MG		
E-CH_4 (g CO_2 eq·day^{-1})	60.24	52.74	1.69	*
E-CH_4 (g CO_2 eq·LU^{-1}·day^{-1})	640.47	590.25	18.63	NS
E-CH_4 (g CO_2 eq·kg^{-1} ADG·day^{-1})	85.71	82.45	2.79	NS
E-CH_4 (g CO_2 eq·kg^{-1} DMI·day^{-1})	39.29	35.68	0.19	***
E-CH4 (g CO_2 eq·MJ^{-1} ER·day^{-1})	2.76	2.50	0.01	***
DMI (g)	1535	1478	44.87	NS
ER (MJ·day^{-1})	21.82	21.06	0.64	NS
OMI (g)	1415	1376	41.67	NS
dRes (g·kg DM^{-1}) [a]	131	119	0.64	

Table 6. *Cont.*

Item [1]	Diets		SEM	*p*-Value [2]
	SM	MG		
Model [b]	**β Coefficient**	***R***	***R*-Square**	***p*-Value**
1		0.664	0.440	
Constant	70.377			***
Total VFAs	2.396			**
Acetic acid	−2.628			**
Propionic acid	−3.957			***
Butyric acid	−1.723			***

Abbreviations: DMI, dry matter intake; OMI, organic matter intake; ADG, average daily gain; ER, energy retention. [1] We took into account the global warming potential of 25 for CH_4. LU, livestock unit = 500 kg LW, according to [14]. [2] NS: nonsignificant effect; * $p < 0.05$ (significant difference between means); ** $p < 0.01$ (distinctly significant difference between means); *** $p \leq 0.001$ (highly significant difference between means). [a] dRes, digestible residue. [b] Dependent variable, E-CH_4 and its predictors (total VFAs, propionic acid, acetic acid, and butyric acid).

This decline is lower than that reported by Gong et al., [18] who mentioned a 25% daily decrease when using dietary *S. cerevisiae* YST_2 supplementation. When expressed in relation to DMI or ER, the E-CH_4 recorded a significantly lower value in the MG-fed group (<9.19% and <9.42%, respectively; $p < 0.0001$). Likewise, when expressed in terms of livestock units (LU = 500 kg LW), the reduction in the E-CH_4 value was slightly lower in the fiber-rich diet (−7.84%, $p = 0.83$).

Following the application of a linear regression model, our data favored the hypothesis of a nonzero correlation between E-CH_4 and VFAs. Thus, the following factors were identified as good predictors for E-CH_4: total VFAs (β coefficient = 2.396, $p < 0.001$), propionic acid (β coefficient = −3.957, p <0.0001), acetic acid (β coefficient = −2.628, p <0.001), and butyric acid (β coefficient = −1.723, $p < 0.001$). As expected, a greater positive relationship was determined between E-CH_4 and DM, as well as fiber and NDF intake (rho = 0.92–0.97; Table 7).

In contrast to valeric acid, highly significant negative relationships were determined between E-CH_4 production and the concentrations of acetic acid, propionic acid, butyric acid, and total VFAs.

In Table 8, the Spearman correlation coefficients are presented between VFAs and microflora. In contrast to the relationship between *Enterococcus* bacteria and propionic acid (rho = −0.28, p <0.0001), no significant relationship was identified between *Lactobacillus* and *Enterococcus* bacteria and the concentrations of acetic acid, butyric acid, total VFAs, and total BCFAs. In contrast to butyric acid, total microflora recorded a highly significant Spearman correlation with acetic acid, propionic acid, and total VFAs.

Table 7. Spearman correlation coefficients among VFAs (μmol/g), fiber (g·day^{-1}), and E-CH_4 (g CO_2 eq·day^{-1}).

Items	pH	Acetate	Propionate	Butyrate	Valerate	Isobutyrate	Isovalerate	Total VFA	Total BFA	DM	NDF	Fiber	E-CH_4
pH	1	−0.460 **	−0.523 **	0.218 *	−0.565 **	−0.217 *	−0.669 **	−0.529 **	−0.626 **	0.305 **	0.327 **	0.330 **	0.227 **
Acetate		1	0.792 **	0.257 *	0.475 **	0.085	0.604 **	0.930 **	0.518 **	−0.39 **	−0.362 **	−0.378 **	−0.371 **
Propionate			1	0.332 **	0.547 **	0.012	0.558 **	0.925 **	0.406 **	−0.511 **	−0.427 **	−0.486 **	−0.532 **
Butyrate				1	−0.397 **	−0.160	−0.360 **	0.387 **	−0.337 **	−0.26 *	−0.282 **	−0.243 *	−0.326 **
Valerate					1	0.184	0.785 **	0.522 **	0.669 **	−0.15	−0.048	−0.132	−0.149
Isobutyrate						1	0.170	0.076	0.649 **	0.49 **	0.419 **	0.475 **	0.527 **
Isovalerate							1	0.598 **	0.821 **	−0.23 *	−0.165	−0.214	−0.200
Total VFA								1	0.496 **	−0.451	−0.405 **	−0.430 **	−0.462 *
Total BFA									1	0.06	0.048	0.060	0.118
DM										1	0.98 **	0.99	0.97 **
NDF											1	0.984 **	0.921 **
Fiber												1	0.956 **

** Distinctly significant correlation at the 0.01 level; * significant correlation at the 0.05 level.

Table 8. Spearman correlation coefficients between main VFAs (μmol/g) and microflora (log_{10} CFU/g).

Items	Acetate	Propionate	Butyrate	Total VFA	Coliforms	*E. coli*	*Lactobacillus*	*Clostridium*	*Enterococcus*	*Staphylococcus*	Enterobacteriaceae	Total Microflora
Acetate	1	0.792 **	0.257 *	0.930 **	−0.616 **	−0.586 **	0.128	0.558 **	−0.043	0.247	−0.224	−0.396 **
Propionate		1	0.332 **	0.925 **	−0.784 **	−0.432 **	0.002	0.584 **	−0.285 *	0.356 **	−0.500 **	−0.512 **
Butyrate			1	0.387 **	0.516 **	0.336 *	−0.002	−0.478 **	0.096	−0.394 **	0.398 **	0.201
Total VFA				1	−0.691 **	−0.593 **	0.215	0.651 **	−0.107	0.283 *	−0.342 *	−0.408 **
Total BFA					−0.641 **	−0.491 **	0.227	0.715 **	−0.076	0.425 **	−0.363 **	−0.275 *
Coliforms					1	0.767 **	0.191	−0.501 **	0.632 **	−0.170	0.799 **	0.839 **
E. coli						1	0.184	−0.519 **	0.356 **	−0.223	0.665 **	0.723 **
Lactobacillus							1	0.390 **	0.320 *	0.202	0.190	0.495 **
Clostridium								1	0.020	0.576 **	−0.276 *	−0.224
Enterococcus									1	0.295 *	0.815 **	0.803 **
Staphylococcus										1	0.082	0.113
Enterobacteriaceae											1	0.813 **

** Distinctly significant correlation at the 0.01 level; * significant correlation at the 0.05 level.

4. Discussion

Fiber can be considered an important component of the pig diets that attracted attention due to their health advantages. Fiber fermentation serves as the source of energy for bacterial communities [37], influencing several physiological processes by filling the intestinal tract and producing gases and VFAs known as physiologically active compounds [38]. Many studies have previously investigated VFAs levels in different segments of human and animal gastrointestinal tracts [9,39], but there is no information on the concentration of VFAs in the ileum and cecum of adult pigs fed mustard and grapeseed oil meals, which increase the level of dietary fiber.

Our study confirmed the theory of fiber-producing satiety through decreasing feed intake by delaying gastric emptying. As a result, despite the fact that pigs have increased their abilities to use dietary fiber across their life cycle, our data have shown a decrease in feed intake in groups fed fiber-rich diets, resulting in a slight decline in growth performance. In fact, a high fiber level has often adverse effects on the digestibility of other nutrients presumably linked to the rate of passage of digesta through the gastrointestinal tract [14]. However, this lower performance in the MG group was accompanied by a decrease in the feeding prices and cost per kilogram of daily body gain. This result is consistent with previous studies [14,38] mentioning that fibrous byproducts are frequently used in many countries, although fiber-rich diets do not maximize and sometimes inhibit performance. Evidence of the nutritional potential of black and yellow mustard seed meal was also provided by Sarker et al., [3].

Pigs' growth performance and their intestinal health are linked to the intestinal microbial community. The intestine hosts a complex bacterial community (Firmicutes, Proteobacteria, etc.), releasing metabolites important for health. However, the small intestine hosts fewer bacteria than the large intestine; consequently, the fermentation process occurs at a lower rate in this gastrointestinal segment. In this study, a diverse community of microflora, within the reference limits [32–36], was identified in the ileum and cecum. Wang et al. [40] specified that Firmicutes are the most dominant bacteria, followed by Bacteroidetes across each age stage. Our results support this theory. Thus, as dominant phyla in both gut sections, we identified Firmicutes (*Lactobacillus* spp., *Enterococcus* spp., *Clostridium*, and *Staphylococcus* spp.), as well as Proteobacteria (*E. coli* and coliforms related to Enterobacteriaceae) to a lower extent. We have observed that data pooled across all Firmicutes bacteria in each gut section indicated that their level was higher in the cecum than in the ileum. This is consistent with the explanation given by Jaworski and Stein [37] who consider that the cecum plays a significant role in fiber fermentation. This should also explain the stimulating effect recorded for pigs fed the MG diet; however, in contrast, the data pooled across Proteobacteria showed a lower concentration in the cecum vs. the ileum. Hence, consumption of MG-mixt influenced the level of *E. coli* bacteria. According to the previous study of Umu et al. [41], *Lactobacillus* bacteria almost disappear after the growing period, being negatively correlated with age. At the genus level, we found *Lactobacillus* spp. as the major bacteria in both the ileum and the cecum digesta, followed by *Clostridium* spp. in the growing pigs category considered in this work. Furthermore, we noticed that the fiber-rich diet in the MG group had a significantly positive effect on *Lactobacillus* spp. Hence, the higher level in the ileum confirms the theory that the level of *Lactobacillus* spp. is increased at a lower pH [42]; however, this theory was found to be invalid in the cecum where both the pH and the *Lactobacillus* level were higher. This may have been influenced by the type of fiber, their physicochemical characteristics, and the lignification grade, which all support diverse microflora. As mentioned previously by Gao et al. [43], a high pH level increases intestinal osmotic pressure, whereas the digestion function decreases with changes in microflora composition. In contrast, *E. coli* exhibited the lowest density, regardless of gut section or type of pig feed. However, the increase in ileal *Lactobacillus* concentration in fiber-rich diets was accompanied by an increase in *E. coli* levels. This could have been due to the low pH, but this correlation was not confirmed in the cecum. In contrast with the results obtained by Franklin et al. [42], our data revealed

a higher level of *E. coli*. Many *E. coli* species are harmless due to the presence of bacteriocins that prevent pathogenic bacteria from colonizing the gut. However, according to Schierack et al. [44], *E. coli* are among the most pathogenic bacteria dominating most samples from the gastrointestinal tract but not from feces [45]. Enterobacteriaceae, including *E. coli*, coliforms, and *Salmonella*, are known as pathogenic bacteria [46].

The higher level of the total bacteria identified in this study in pigs fed a fiber-rich diet, influenced the total VFAs. This is consistent with previous findings [47], where alfalfa fiber was administered to 28–48-days-old pigs. The intensity of the fermentative process at the cecum converted dietary fiber into VFAs in a higher concentration than the ileum (more than double). Likewise, we found a total concentration of VFAs higher in the distal ileum than in the cecum of the fiber-rich fed group. The insignificant increase in total VFAs in the ileum of MG-fed pigs was especially due to the concentrations of acetic acid and propionic acid, as the concentration of both these VFAs decreased in the cecum. In contrast, the cecum's elevated butyrate proportions were noted, maybe due to the cecum microflora's metabolism and acidic fermentation.

Beneficial bacteria regulate the pH of the intestine. In the current investigation, a lower pH was linked to a higher generation of VFAs measured in the cecum. Acetic acid was the predominant VFA found both in the ileum and cecum. While the molar proportion of acetic acid decreased in the cecum, the concentration of propionate increased significantly. Results pointed toward a significant increase in propionic acid in pigs fed a fiber-rich diet probably due also to the pH level and associated bacteria. On the other hand, we observed a nonsignificant increase in butyrate concentration in the cecum as an effect of the fiber-rich diet. These data support the results obtained by Heinritz et al. [17], despite a reduced dietary fat level in a group fed a fiber-rich diet. The concentration of total BCFAs was only significantly impacted in the ileum.

The lower pH in the cecum inhibited the concentration of *E. coli* but did not affect that of *Clostridium* bacteria, which are known to be acid-sensitive bacteria. In the cecum, the pH level was acidic, whereas it was near neutral in the ileum.

The Spearman correlations were strong between *Clostridium* spp. and total VFAs (rho = 0.65) and total BCFAs (rho = 0.71) due to their association with acetic acid and propionic acid (rho > 0.6). Although VFAs are known as possible inhibitors of certain pathogen bacteria, unexpected increases in total VFAs and *E. coli* were noticed in the MG-mixt group.

No support can be found in the literature regarding the low correlation between VFAs and *Lactobacillus*. A possible explanation, given with caution, could be the lower intensity of the microbial fermentation process in the ileum and the difference in fermentability rate of the three fiber types. Many studies are contradictory; the amount and type of substrate, the source of fiber fractions, and their digestibility are essential factors. However, substantial fermentation of soluble dietary fiber was found in the pig small intestine by Jha and Leterme [1].

Using regression analyses, we found: *Clostridium* bacteria, pH, and fiber intake to be potential good predictors for the concentration of total VFAs, with an *R*-square value of 0.65 ($p < 0.0001$), indicating that 65% of the variability of total VFAs can be explained by our predictors. Lysine intake was also identified as a good predictor (β coefficient = −15.12).

The production of E-CH_4 was altered but not significantly. To our knowledge, a higher efficacy in lowering the E-CH_4 level, using nutritional tools, has not been specified in the literature. In this study, by developing a model to predict E-CH_4 using our experimental data integrated into equations from the literature, we identified a significant decline in the MG-fed group. The values obtained were close to those mentioned by Guingand et al. [48], higher than those obtained by Dong et al. [49], and lower than those reported in other studies [50,51], as quoted in Philippe and Nicks [16], which took into consideration the total CH_4 (enteric and from manure). Our values ranged between 0.26 and 1 kg CO_2 eq·LU^{-1}. DMI and ER significantly influenced the E-CH_4 level.

Upon applying Spearman correlation, we found a significant negative relationship of E-CH_4 with total VFAs (rho = −0.462, $p = 0.04$), but a positive one with pH (rho = 0.227,

$p = 0.03$), in line with the theory of Yadah and Jha [36] that the negative correlation between pH and VFAs can have a negative effect on E-CH_4 due to the decrease in methanogens and protozoa. The regression analysis revealed total VFAs and their major components, as well as ADF and lysine intake, as potential predictors for the E-CH_4 level.

5. Conclusions

The results of this study indicated that VFA concentrations can be altered through dietary intervention by certain components such as fiber and its associated fractions. Due to the close link between VFAs and the gut microflora, increase bacterial density leads to an increase in VFA concentration. However, the pH may be a contributing factor. A cost-effective alternative method to E-CH_4 determination is to develop new prediction models by identifying the strongest predictors and by reducing estimation errors. This study contributes to a better understanding of the associated mechanisms, allowing us to speculate that dietary strategies and models could be developed to assess E-CH_4. The regression analysis revealed total VFAs and their major components as potential predictors for the E-CH_4 level.

On the basis of these results, we conclude that MG-mixt has the potential to replace sunflower meal, which is particularly important for farmers who are compelled to identify substitutes due to a lack of regularly used ingredients in their area, despite the slight decrease in growth recorded. However, the benefits provided by the feed in terms of gain efficiency, the level of VFAs, and E-CH_4 mitigation outweighed the minor negative effects.

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