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Dietary protein modifies effect of plant extracts in the intestinal ecosystem of the pig at weaning

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ABSTRACT: The plant extract mixture (XT) used in the present experiment, containing carvacrol, cinnamaldehyde, and capsicum oleoresin, has previously been shown to decrease diarrhea mortality and to modify the intestinal environment of pigs after weaning. However, results obtained among experiments have not been consistent. We hypothesized that dietary protein could be a main factor determining the effect of plant extracts on intestinal environment. Thus, in the present study we assessed the effects of XT in piglet diets with different protein sources and amounts. Pigs weaned at 20 ± 1 d of age (n = 240) were allocated to 1 of 6 treatments, which followed a factorial arrangement, with 2 amounts (as-fed basis) of the XT (0 and 200 mg/kg) and 3 diets with various amounts of CP and protein sources. Diet FM18 contained 10% of low-temperature fish meal (LT-FM) and a CP level of 18%; diet SBM18 contained 5% of LT-FM plus 9% of full fat extruded soy and a CP level of 18%; and SBM20 diet contained 10% of LT-FM plus 6.3% of full fat extruded soy and a CP level of 20%. Growth performance of the animals was recorded for 14 d, but no differences were detected among treatments. Eight pigs per treatment were killed to examine variables describing aspects of gastrointestinal ecology. For diets containing 18% CP, FM18 and SBM18, XT tended to decrease ileal digestibility of OM (P = 0.064 and 0.071, respectively) and decreased starch digestibility (P = 0.032 and 0.014, respectively). It also reduced villi length (P = 0.003 and 0.013, respectively) and tended to decrease intraepithelial lymphocyte number (P = 0.051 and 0.100, respectively) in the proximal jejunum. The XT inclusion also increased ileal lactobacilli:enterobacteria (P = 0.017) ratio and decreased VFA production in the cecum (P = 0.045) for all diets. A decreased CP level appeared to favor the effects of the studied plant extracts in a positive or negative way depending on the variable measured. The microbial differences produced by XT could be the reason for improved digestive health observed by the authors in stronger challenging conditions (e.g., dirtier environments or long fasting periods after weaning).

Key words: intestinal health, microbiota, pig, plant extract, protein, weaning

INTRODUCTION

Early weaning impairs growth performance of the pig, making it vulnerable to disease (Boudry et al., 2004). Both therapeutic and growth promoter antibiotics (AGP) have been effective in improving growth performance of piglets. However, bacterial resistances have motivated a large body of research on antibiotic alternatives. One of the groups of substances used as an alternative to AGP is plant extracts. Phytogenic substances, such as essential oils and oleoresins, constitute the basic ingredients of many commercial antimicrobials currently used in human health and food conservation (Burt, 2004), and lately they are used as a substitute for AGP in animal diets (Windisch et al., 2008).

In vitro, the antimicrobial effect of some plant extracts is as consistent as the effect of antibiotics (Cow-en, 1999). However, the effect of plant extracts in vivo is not consistent compared with antibiotics and for the
same product used in similar but not equal conditions, results can differ greatly (Windisch et al., 2008). Diet is one of the main factors that modify the antimicrobial effect of plant extracts in vitro (Si et al., 2006). In particular, the presence of protein in the media has been shown to directly inhibit the antimicrobial effect of plant extracts (Juven et al., 1994). The importance of diet composition, especially CP concentration, affecting the effects of AGP on pig gut (François, 1962) was described early on, so it is logical to hypothesize similar effects of comparable additives.

The plant extract mixture (XT) has shown positive effects on growth performance (Isley et al., 2003), weaning diarrhea (Manzanilla et al., 2004), lactobacilli proliferation (Castillo et al., 2006), and important morphological and immune changes in the intestinal epithelium (Nofrarias et al., 2006). The current experiment was performed to elucidate in vivo how different dietary formulations, differing in the protein source and content, modify effects of XT on the intestinal environment of pigs after weaning.

MATERIALS AND METHODS

The experiment was performed at the Experimental Unit of the Universitat Autònoma de Barcelona and received prior approval from the Animal Protocol Review Committee of the institution. The treatment, housing, husbandry, and slaughtering conditions conformed to the European Union guidelines.

Animals, Housing, and Dietary Treatments

A total of 240 commercial crossing [(Landrace × Large White) × Pietrain] piglets excluded from receiving creep feed, 5.4 ± 0.40 kg of BW and 20 ± 1 d old, were randomly allocated to 48 pens (5 animals per pen) in an environmentally controlled room. Experimental treatments followed a 2 × 3 factorial arrangement resulting from the combination of 2 amounts (0 or 200 mg/kg, as-fed basis) of XT with 3 basal diets. The XT was standardized in 5% (wt/wt) carvacrol (Origanum spp.), 3% cinnamaldehyde (Cinnamomum spp.), and 2% capsicum oleoresin (Capsicum annum) that were included in an inert fatty carrier (85%) before inclusion in the feed. The basal diets differed in CP amounts and sources. The 3 diets (Table 1) were diet FM18 (CP = 18%), which contained 10% of low temperature fish meal (LT-FM); diet SBM18 (CP = 18%), in which 5% of LT-FM was isoproteically replaced by full fat extruded soybean meal (SBM; 5% LT-FM, 9% SBM); and diet SBM20 (CP = 20%), in which a greater protein level was obtained by supplementing SBM in excess of the 10% LT-FM (10% LT-FM, 6.3% SBM). Chronic oxide (Cr2 O3) was included as a digestibility marker.

Feeding Regimen, Controls, and Sampling

For 14 d, the animals were allowed ad libitum access to feed, and growth performance was monitored weekly to control health problems. From d 15 to 19, a controlled feed intake pattern (Manzanilla et al., 2004) was applied from 0800 to 2000 h to standardize the digestive tract conditions upon slaughter. In particular, 30-min periods of feeding (ingestion period) were alternated with 1-h fasting periods (fasting period). Pigs were fed ad libitum the remainder of the day (from 2000 to 0800 h of the next day). On d 18 and 19, after the 1200, 1330, 1500, and 1630 h ingestion period, 1 pig per treatment was weighed and killed by i.v. injection of sodium pentobarbitone (Dolethal, Vetoquinol, S.A., Madrid, Spain; 200 mg/kg of BW). Pigs to be killed were selected as the closest to the mean BW of the pen. Pigs were bled, the abdomen was opened immediately from the sternum to pubis, and the whole gastrointestinal tract was removed, weighed, and sampled. The pH in 4 segments was measured by insertion of a unipolar electrode through a small incision made in the wall of the organ (penetration pH meter Crison 507, electrode Crison 52–32, Net Interlab S.A.L., Madrid, Spain). The pH measurements were performed in the middle of the caudal portion of the stomach, 15 cm proximal to the ileocecal valve, in the lowest part of the cecum, and in the colon (20 cm distal to the cecum).

Samples for histological analysis were obtained from the proximal and distal jejunum wall, 75 cm from the stomach and 15 cm proximal to the ileum, respectively. The samples were cut open longitudinally along the mesenteric attachment and fixed by immersion in 10% (vol/vol) buffered formalin immediately after slaughter. A jejunum portion (25 cm long), 20 cm proximal to the ileum, was tied off and collected for the enterobacteria and lactobacilli counts. The jejunal portion was stored at 4°C until the culture was conducted the same day. Total contents of the ileum and rectum were collected, lyophilized, ground, and stored for subsequent analysis. A sample was taken from homogenized cecum contents, which were acidified with H3PO4 [approximately 4 g of fresh weight/mL of (wt/wt) H3PO4, 1% (wt/wt) of mercuric chloride and 50 mM 3-methyl valerate as an internal standard], and stored at –20°C for VFA analysis.

Analytical Procedures

Chemical analysis of the diet was conducted according to the Association of Official Analytical Chemists (AOAC, 1995) standard procedures. The GE was determined by an adiabatic calorimeter, and Cr concentration in the diet, ileum, and feces was analyzed following the procedure described by Williams et al. (1962) of atomic absorption spectrophotometry. Total starch of feed and digesta samples was measured by the method of Theander (1991). Briefly, total starch was determined as glucose liberated after enzymatic incubation with thermostable α-amylase (Sigma, Ref. A-4551, Sigma, Madrid, Spain) for 1 h at 100°C, and amyloglucosidase (Sigma, Ref. A-3514) for 4 h at 60°C.
Tissue samples for the morphometric study were dehydrated and embedded in paraffin wax, sectioned at 4 µm, and stained with hematoxylin and eosin. Morphometric measurements were performed with a light microscope (BHS, Olympus, Barcelona, Spain). Villus height (VH), crypt depth (CD), intraepithelial lymphocyte (IEL) number in the villi, the number of mitotic cells in the crypt, and intravillus lamina propria cell density were measured. Measurements were taken in 10 well-oriented villi and crypts from each intestinal section of each animal. On the basis of cellular morphology, differences between the nuclei of enterocytes, mitotic figures, and lymphocytes were clearly distinguishable at 400× magnification.

The VH and CD were measured using a linear ocular micrometer (Olympus, Ref. 209-35040, Microplanet, Barcelona, Spain). Villus:crypt ratio was calculated by dividing villus height by crypt depth. The same villus and crypt columns were used to determine the number of IEL, and mitoses (meta- and anaphases); these variables were expressed per 100 enterocytes. Intravillus lamina propria cell density was determined by counting total visibly stained nuclei in 10 fields (total area of 4,000 µm²) from each section using a grid ocular (Olympus, Microplanet, Barcelona, Spain). Cell density was expressed as number of total stained cells per 1,000 µm². All the morphometric analyses were conducted by the same person, who was blinded to the treatments.

For bacterial counts, 1 g of sample was weighed, serially diluted, and 100-µL aliquots were plated in MacConkey agar (Oxoid, Ref. CM 115, Oxoid S.A, Madrid, Spain) for enterobacteria counts (dilutions 10⁻³ to 10⁻⁷) and in rogosa agar (Oxoid, Ref. CM 627) for lactobacilli counts (dilutions 10⁻⁵ to 10⁻⁹). Enterobacteria were counted after 24-h incubation (37°C), and lactobacilli were counted after a 48-h incubation (37°C, 5% CO₂). Concentration of VFA (µmol/g of fresh matter) in deproteinized cecal digesta was determined by GLC, following the procedures of Jouany (1982).

**Calculations and Statistical Analysis**

Ileal and rectal apparent digestibility of each nutrient fraction was calculated by standard marker ration methodology using the equation digestibility = [1 − (nutrient digesta/nutrient diet) × (Cr-digesta/Cr-diet)], where nutrient diet and nutrient digesta represent the nutrient concentrations (g/kg) in digesta and diet DM, respectively.
respectively, and Cr-diet and Cr-digesta represent the marker concentrations (g/kg) in diet and digesta DM, respectively.

All results were analyzed by ANOVA with the GLM procedure (SAS Inst. Inc., Cary, NC). Diet and XT were included in the model as classification factors. The XT × diet interaction was also included in the model. In growth analysis, initial BW was used as a covariate. Pen was the experimental unit. Mean separation was done using Tukey-Kramer’s correction. As suggested by Lowry (1992) for this factorial arrangement, the interaction was studied when its P-value was significant or when it was <0.15 without any significant main effect. In these cases, the P-values of the effect of the XT inclusion within each diet are presented in the table, and the effect of the diet within XT at the 0 mg/kg rate is indicated by superscript. The α level used for determination of significance for all analyses and contrasts was 0.05, and all trends (P < 0.10) are reported. Additionally, the REG procedure of SAS was used for regression determinations presented in the Discussion section.

RESULTS

Growth Performance and Digestibility

Growth performance did not differ among experimental groups. All pigs consumed the diet provided with an ADFI of 240 ± 6.2 g; ADG was 177 ± 5.6 g and G:F was 0.73 ± 0.014 g/g (data not shown).

Table 2 shows ileal and whole-tract digestibility in the piglets. An interaction was observed for ileal digestibility of OM (P = 0.032) and starch (P = 0.016). Thus, inclusion of XT tended to decrease OM ileal digestibility of the FM18 (P = 0.064) and SBM18 (P = 0.071) diets, but not of the SBM20. These differences are partially explained by the decrease in the ileal digestibility of the starch with the XT inclusion in the FM18 (P = 0.032) and SBM18 (P = 0.014) diets. No differences were detected among dietary treatments for the ileal digestibility of the protein (data not shown) or whole-tract digestibility of OM.

Morphology of the Small Intestine

Figure 1 and Table 3 show the structural characteristics of the mucous membrane in the proximal and distal jejunum. Among dietary treatments, various interactions were observed. Diets FM18 and SBM18 resulted in a greater VH in the proximal jejunum compared with SBM20 when XT was not included in the diet (483.7 and 485.6 vs. 373.3, respectively). Proximal jejunum villi were shorter with XT inclusion in diets FM18 (P < 0.001) and SBM18 (P = 0.013), but were not affected in the SBM20 diet (P = 0.714). A similar response was observed in the distal jejunum, but this effect was only significant in diet SBM18 (P = 0.015). Crypt depth was only affected in the proximal jejunum where XT diminished CD in diet FM18 (P < 0.001) (Figure 1).
From these results, the calculated VH:CD ratio did not show differences as affected by the XT inclusion, but in the proximal jejunum, this ratio was greater \( (P = 0.003) \) for diet SBM18 \( (2.26 \pm 0.069) \) compared with diets FM18 \( (1.95 \pm 0.069) \) and SBM20 \( (1.93 \pm 0.069; \) Table 3). There was a different pattern between the proximal and the distal jejunum in IEL, mitoses, and lamina propria cell density. In proximal jejunum, diet SBM20 increased lamina propria cell density \( (P = 0.004) \) and mitoses \( (P = 0.037) \). The number of mitoses expressed as total number in a crypt tended to be decreased by XT in diets FM18 \( (P = 0.070) \) and SBM18 \( (P = 0.095) \) (data not shown). However, no differences were shown for the number of mitoses when expressed relative to 100 cells. The inclusion of XT decreased total IEL in villi when included in diets FM18 \( (P = 0.002) \) and SBM18 \( (P = 0.040; \) data not shown). In the distal jejunum, IEL decreased in total number with XT inclusion \( (P = 0.032) \). Mitoses were different for each diet when XT was not included, were increased by XT when included in diet FM18 \( (P = 0.003) \), and tended to be greater when XT was included in diet SBM18 \( (P = 0.088) \). Lamina propria cell number was least for diet FM18 and was increased by XT when included in diet FM18 \( (P = 0.002) \).

**Microbial Counts and Hindgut Fermentation**

Table 4 shows data relative to microbiota in the digestive tract characterized as the lactobacilli and enterobacteria counts in the distal jejunum. The inclusion of XT increased lactobacilli counts \( (7.6 \pm 0.16 \text{ vs. } 8.2 \pm 0.16; \) \( P = 0.005) \). As a result, lactobacilli:enterobacteria ratio \( (\text{Lact:Ent}) \) was greater for XT-treated pigs \( (0.84 \pm 0.300 \text{ vs. } 1.94 \pm 0.321; \) \( P = 0.017) \).

Table 5 shows total VFA concentrations and profile in the cecum. Total VFA concentrations decreased in XT-treated pigs \( (208.6 \pm 8.62 \text{ vs. } 184.2 \pm 8.01; \) \( P = 0.045) \). These changes were simultaneous with a decrease in acetic acid percentage \( (P = 0.033) \) and an increase in butyric \( (P = 0.050) \) and valeric acid percentages \( (P = 0.027) \). Among diets, acetate percentage was greater for the SBM20 \( (P = 0.008) \) diet, and valeric acid percentage was greater for the SBM18 diet \( (P = 0.009) \). The pH measurements did not show differences among treatments (data not shown). Mean pH obtained were 3.9 \( \pm \) 0.28 in stomach, 6.6 \( \pm \) 0.12 in ileum, 5.7 \( \pm \) 0.12 in cecum, and 6.1 \( \pm \) 0.13 in colon.

**DISCUSSION**

The 3 diets used in this experiment were initially considered to promote a range of dietary insults for piglets after weaning. The FM18 diet was initially considered the decreased risk diet for intestinal problems. The isoproteic replacement of fish meal in the SBM18 diet or the supplementation in the SBM20 diet with extruded soybean were considered risk factors because of the inclusion of soy in the diet, even when it was extruded (Makkink et al., 1994), and the increase in the CP concentration of the diet (Pluske et al., 1997), respectively. However, no significant effects were observed on growth performance, and no symptoms of diarrhea were observed.

Results from our study showed that XT promoted changes in nutrient digestion (decreased ileal digestibilities for OM and starch) and mucosal histology (shorter villi, less CD and decreased IEL number in the proximal jejunum) when included in 18% CP level diets, and modified microbial fermentation (increased jejunal lactobacilli counts and decreased cecal VFA production) for all diets, but especially in 18% CP diets. Despite the variation due to the treatments in the current study, VH and CD measurements were in a range comparable with data obtained for healthy animals by Cera et al. (1988), Zijlstra et al. (1996), and Pluske et al. (1996). Villus height reflects a balance between mitotic activity of the crypt cells and the desquamation produced principally by external aggressors (Cera et al., 1988; Nabuurs, 1995). Inclusion of XT reduced...
**Table 3. Intestinal histology of pigs fed the experimental diets**

<table>
<thead>
<tr>
<th>Item</th>
<th>Proximal jejunum</th>
<th>Distal jejunum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Villi/crypt ratio, µm</td>
<td>1.867 ± 0.247</td>
<td>1.600 ± 0.156</td>
</tr>
<tr>
<td>Mitoses, n/100 cells</td>
<td>1.947 ± 0.348</td>
<td>4.317 ± 0.487</td>
</tr>
<tr>
<td>IEL, n/100 cells</td>
<td>3.17 ± 0.347</td>
<td>32.5 ± 6.58</td>
</tr>
<tr>
<td>Lamina propria cells, n/1,000 µm²</td>
<td>9.257 ± 1.65</td>
<td>8.607 ± 1.56</td>
</tr>
</tbody>
</table>

**Contrast**

| FM18 vs. SBM18               |
| FM18 vs. 0                   |
| FM18 vs. SBM20               |
| SBM18 vs. 0                  |
| SBM18 vs. SBM20              |

**P-values**

<table>
<thead>
<tr>
<th>Diet</th>
<th>0 vs. 200</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEM</td>
<td></td>
</tr>
<tr>
<td>Proximal jejunum</td>
<td></td>
</tr>
<tr>
<td>Villi/crypt ratio, µm</td>
<td>0.004</td>
</tr>
<tr>
<td>Mitoses, n/100 cells</td>
<td>0.071</td>
</tr>
<tr>
<td>IEL, n/100 cells</td>
<td>0.084</td>
</tr>
<tr>
<td>Lamina propria cells, n/1,000 µm²</td>
<td>0.091</td>
</tr>
<tr>
<td>Distal jejunum</td>
<td></td>
</tr>
<tr>
<td>Villi/crypt ratio, µm</td>
<td>0.004</td>
</tr>
<tr>
<td>Mitoses, n/100 cells</td>
<td>0.041</td>
</tr>
<tr>
<td>IEL, n/100 cells</td>
<td>0.003</td>
</tr>
<tr>
<td>Lamina propria cells, n/1,000 µm²</td>
<td>0.051</td>
</tr>
</tbody>
</table>

**Notes:**

1. Values are least squares means (n = 8). Histology measurements were done in proximal jejunum, 75 cm from the stomach, and distal jejunum, 15 cm proximal to the ileum. IEL = intraepithelial lymphocyte.

2. Diet FM18 (18% CP) contained fish meal but not soybean meal. Diets SBM18 (18% CP) and SBM20 (20% CP) contained fish meal and extruded soybean meal. Diets contained 0 or 200 mg/kg of plant extract mixture (XT).

3. INT = interaction (XT × diet).

4. Interaction was studied when the **P**-value was significant or when it was less than 0.15 without any significant principal effect. Tukey-Kramer correction was used for mean separation. **P**-values for the XT effect within each diet are presented.

5. Differences due to diet (**P** < 0.05), when XT is not included, are indicated by different superscripts in the same row.
we use the Lact:Ent ratio as an index of intestinal equilibrium (Hillman et al., 1995). Increased number of lactobacilli in the gut microbiota have also been reported using different antibiotics (Collier et al., 2003), which could be related to a decreased diarrhea incidence in piglets (Manzanilla et al., 2004) and to the decreased number of IEL in piglets fed diets with 18% CP supplemented with XT.

The mechanism of action of these plant extracts on microbiota is not clear. A direct antimicrobial effect of XT against determined microbial groups could allow the proliferation of lactobacilli as was observed by Collier et al. (2003) when using antibiotics. However, the dose of carvacrol and cinnamaldehyde used in this experiment was approximately 10-fold less than the antimicrobial dose (Burt, 2004). In this respect it is surprising that the concentration of VFA, which are the major end products of bacterial metabolism in the large intestine of swine (Bergman, 1990), were also decreased by XT supplementation. In the present experiment, we used VFA concentration and profile as an index of the changes in the microbial population and of the quantity and source of substrates fermented in the hindgut. The most important factors affecting VFA production are the quantity and source of substrate arriving in the hindgut (Bergman, 1990; Macfarlane and Macfarlane, 2003). Surprisingly, the decreased ileum digestibility promoted by XT supplementation was simultaneous to a decrease in cecal VFA concentrations, especially in diets containing 18% CP. This could be due to a direct effect of XT on cecal microbiota. However, it could also be due to a carryover effect of the increased lactobacilli in the proximal segments (i.e., through an increase in the production of lactic acid), which was not measured in the current study. It is also relevant that XT included in 18% CP diets promoted a decrease in acetic acid and an increase in butyric acid percentage. It is accepted that starch and brans from wheat or oats stimulate the formation of butyrate (Bach-Knudsen et al., 1993; Anguita et al., 2007). Lactate has been recently suggested as a major precursor for butyrate synthesis (Bourriaud et al., 2005). Thus an increase in the production of lactate by lactic acid-producing bacteria may increase the conversion to butyrate (Tsukahara et al., 2002).

François (1962) attributed the improved effects of antibiotics in a decreased protein diet to limited nutrient availability for the microbiota and to physical-chemical effects of the ingredients. Specifically, François (1962) hypothesized that nutrient limitation induced competition among the different groups of microorganisms and the host, thereby making bacteria more sensitive to environmental effects. This competition could potentially explain why the XT effect was more marked with the low protein level diets in the present experiment. Decreased pH values in the stomach reduce the gastric emptying rate (François, 1962). Protein is 1 of

### Table 4. Microbial counts in distal jejunum of the pigs fed the experimental diets

<table>
<thead>
<tr>
<th>Item</th>
<th>FM18&lt;sup&gt;2&lt;/sup&gt;</th>
<th>SBM18&lt;sup&gt;2&lt;/sup&gt;</th>
<th>SBM20&lt;sup&gt;2&lt;/sup&gt;</th>
<th>SEM</th>
<th>XT</th>
<th>Diet</th>
<th>INT&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterobacteria</td>
<td>6.5</td>
<td>6.1</td>
<td>6.8</td>
<td>5.8</td>
<td>6.6</td>
<td>6.8</td>
<td>0.39</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>7.8</td>
<td>8.4</td>
<td>7.1</td>
<td>8.3</td>
<td>7.8</td>
<td>8.0</td>
<td>0.25</td>
</tr>
<tr>
<td>Lactobacilli:enterobacteria</td>
<td>1.34</td>
<td>2.19</td>
<td>0.24</td>
<td>2.50</td>
<td>0.94</td>
<td>1.13</td>
<td>0.482</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values are least squares means (n = 8).
<sup>2</sup>Diet FM18 (18% CP) contained fish meal but not soybean meal. Diets SBM18 (18% CP) and SBM20 (20% CP) contained fish meal and extruded soybean meal. Diets contained 0 or 200 mg/kg of plant extract mixture (XT).
<sup>3</sup>INT = interaction (XT × diet).

### Table 5. Volatile fatty acid concentration and profile in cecum and colon of pigs fed the experimental diets

<table>
<thead>
<tr>
<th>Item</th>
<th>FM18&lt;sup&gt;2&lt;/sup&gt;</th>
<th>SBM18&lt;sup&gt;2&lt;/sup&gt;</th>
<th>SBM20&lt;sup&gt;2&lt;/sup&gt;</th>
<th>SEM</th>
<th>XT</th>
<th>Diet</th>
<th>INT&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total VFA, µmol/g of fresh matter</td>
<td>233.1</td>
<td>187.1</td>
<td>204.2</td>
<td>181.3</td>
<td>188.4</td>
<td>184.2</td>
<td>13.87</td>
</tr>
<tr>
<td>Acetic acid, %</td>
<td>53.1&lt;sup&gt;y&lt;/sup&gt;</td>
<td>51.7</td>
<td>53.1&lt;sup&gt;x&lt;/sup&gt;</td>
<td>46.6</td>
<td>55.3&lt;sup&gt;x&lt;/sup&gt;</td>
<td>54.7</td>
<td>1.54</td>
</tr>
<tr>
<td>Propionic acid, %</td>
<td>31.0</td>
<td>30.0</td>
<td>29.5</td>
<td>30.4</td>
<td>28.2</td>
<td>29.8</td>
<td>1.26</td>
</tr>
<tr>
<td>Butyric acid, %</td>
<td>13.0</td>
<td>16.2</td>
<td>13.9</td>
<td>16.6</td>
<td>13.6</td>
<td>12.8</td>
<td>1.03</td>
</tr>
<tr>
<td>Branched VFA, %</td>
<td>0.76</td>
<td>0.69</td>
<td>0.51</td>
<td>0.45</td>
<td>0.74</td>
<td>0.66</td>
<td>0.116</td>
</tr>
</tbody>
</table>

<sup>x,y</sup>Differences due to diet (P < 0.05) are indicated by different letters in the same row.
<sup>1</sup>Values are least squares means (n = 8).
<sup>2</sup>Diet FM18 (18% CP) contained fish meal but not soybean meal. Diets SBM18 (18% CP) and SBM20 (20% CP) contained fish meal and extruded soybean meal. Diets contained 0 or 200 mg/kg of plant extract mixture (XT).
<sup>3</sup>INT = interaction (XT × diet).
the main factors regulating pH and gastric emptying due to the buffering effect that proteins have. It is well known from studies investigating the effects of acidifiers that increasing the relative protein level of the diet increases the buffering capacity of the diet, facilitating gastric emptying (Partanen and Mroz, 1999). Makkink et al. (1994) demonstrated that soy products promoted decreased pH in the stomach compared with fish meal. In the current experiment, no differences were detected in gastric pH among diets, mostly due to the variability of the gastric pH (gastric emptying was not measured in the present study). However, previous studies by Manzanilla et al. (2004) demonstrated an association between supplementation of XT and delayed gastric emptying. Capsaicin in XT could stimulate receptors in the pylorus, thereby retarding gastric emptying independently of pH. Therefore, protein and XT could have interactive effects on gastric emptying.

The incorporation of carvacrol, cinnamaldehyde, and capsicum oleoresin promotes positive and negative changes in the digestive function, intestinal epithelium, microbial ecology, and fermentation in weaned pigs depending on the amount of protein included in the diet. In the current study, the interaction among different variables concerning epithelium structure, microbial populations, and digestive function were shown. These results should encourage the scientific community to continue screening and studying plant extracts as effective alternatives to antibiotics and consider possible interactions with diet.

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