Effects of added fermentable carbohydrates in the diet on intestinal proinflammatory cytokine-specific mRNA content in weaning piglets

S. Pié,*2 A. Awati,†2,3 S. Vida,* I. Falluel,* B. A. Williams,†5 and I. P. Oswald*

*Institut National de la Recherche Agronomique, Unité de Pharmacologie-Toxicologie, UR66, 180 chemin de Tournefeuille, 31931 Toulouse Cedex 9, France; †Animal Nutrition Group, Wageningen University and Research Centre, PO Box 338, 6700 AH, Wageningen, the Netherlands

ABSTRACT: There is increasing evidence showing that dietary supplementation with prebiotics can be effective in the treatment of intestinal inflammation. Because weaning time is characterized by rapid intestinal inflammation, this study investigated the effect of a diet supplemented with a combination of 4 fermentable carbohydrates (lactulose, inulin, sugarbeet pulp, and wheat starch) on the mRNA content of proinflammatory cytokines in newly weaned piglets. Cytokines (IL-1β, IL-6, IL-8, IL-12p40, IL-18, and tumor necrosis factor-α) were analyzed using a semiquantitative reverse-transcription PCR technique on d 1, 4, and 10 in the ileum and colon of piglets fed either a test diet (CHO) or a control diet. In addition to the diet, the effect of enforced fasting on cytokine mRNA content was also evaluated. No effect of fasting was observed on the proinflammatory cytokine mRNA content. Our results showed that the CHO diet induced an up-regulation of IL-6 mRNA content in the colon of piglets 4 d postweaning. This up-regulation was specific for the animals fed the CHO diet and was not observed in animals fed the control diet. An increase in IL-1β mRNA content was also observed on d 4 postweaning in all of the piglets. Correlations between proinflammatory cytokines and the end-products of fermentation indicated that the regulation of cytokines may be linked with some of the fermentation end-products such as branched-chain fatty acids, which are in turn end-products of protein fermentation.

Key words: pig, prebiotic, proinflammatory cytokine, weaning

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INTRODUCTION

Prebiotics were recently given a new definition as nondigestible dietary ingredients that beneficially affect the host by stimulating the activity, in terms of fermentation end-products, and stability of the diverse commensal microbiota in the gastrointestinal tract (GIT; Gibson et al., 2004; Awati and Moughan, 2006).

Prebiotics include short-chain carbohydrates, such as inulin and fructo-oligosaccharides, that are nondigestible by human digestive enzymes but that can be metabolized by colonic bacteria to produce short-chain fatty acids (SCFA), such as butyrate.

In the pig, several investigators have shown that the inclusion of dietary fermentable carbohydrates can modulate the gastrointestinal microbiota by increasing the growth of lactic acid bacteria (Houdijk et al., 2002; Smiricky-Tjardes et al., 2003; Konstantinov et al., 2004). Proliferation of endogenous bacteria such as bifidobacteria may inhibit colonization of the intestine by pathogens, and a diet supplemented with prebiotics has been shown to prevent infections in pigs (Leser et al., 2000; Letellier et al., 2000).

Apart from their effect on the intestinal microbiota, little is known concerning the possible implications of prebiotics on the host response, especially in the pig. It has been demonstrated recently that weaning is associated with increased proinflammatory cytokine mRNA content in the intestine of piglets (Pié et al., 2004).
The current study was designed to examine the effects of inclusion of fermentable carbohydrates in the diet of weaning piglets on the fermentation end-product profile and on mRNA contents of 6 proinflammatory cytokines, which are indicators of inflammatory processes in the gut, to determine whether there is any correlation among these variables. This was an attempt to elucidate how microbial activity in terms of end-products can influence the inflammatory process in the gut to design diets that could affect the gut inflammatory status by controlled stimulation of the fermentation process.

MATERIALS AND METHODS

All of the procedures involving animals were conducted in accordance with the Dutch law on experimental animals and were approved by the Wageningen University Animal Experimental Committee (Dier Experimenten Commissie).

Experimental Design

The experiment was designed as $2 \times 2$ factorial arrangement of treatments, with 2 diets and 2 feeding treatments. The experiment was conducted in 2 identical replicates. For each replicate, 9 piglets were chosen randomly from each of the 4 litters (36 piglets per replicate, and 72 piglets in total for the experiment).

On d 1 of each period, 1 piglet from each litter was killed (4 piglets), and tissue and digesta samples were collected, before the other piglets were subjected to any of the treatment combinations. On d 4 and d 10, from each litter, 1 piglet from each of the 4 treatments was killed for collection of tissue and digesta samples from the terminal ileum and the colon (Figure 1). These time points were chosen because previous studies had demonstrated that weaning is associated with a transient up-regulation of inflammatory cytokine mRNA content on d 3 to 4 after weaning and that the levels of most cytokines rapidly return to preweaning values at d 9 after weaning (Pié et al. 2004).

Animals and Housing

The 72 crossbred piglets (Hypor × Pietrain, mixed male and female group) were weaned at 4 wk of age and transported to the experimental facility. The piglets had access neither to creep feed before weaning nor any antibiotic treatment before or during the experimental period. Piglets from the same litter were kept in adjacent individual pens separated by a tough, unsplinterable, transparent acrylic thermoplastic, which is lighter than glass (Perspex, Electropa, Renskum, the Netherlands), so that they could have visual contact with their littermates without interference with the dietary or fasting treatment. This arrangement was to prevent cross-contamination between litters. The continued contact with littermates was done to reduce potential stress.

Dietary Treatments

The composition of the diets is shown in Table 1. The control diet was designed to have very low levels of fermentable carbohydrates. The test diet with added fermentable carbohydrates (CHO) was based on this same diet, but had added carbohydrates in the form of unmolassed sugarbeet pulp (pulp with most of the molasses removed), native wheat starch, lactulose, and inulin. The diets were composed in such a way that GE and CP contents were comparable. Neither diet contained antibiotics or copper beyond that of the trace mineral premix.

Fasting Treatments

The animals with enforced fasting were not offered any feed for 48 h from the moment of arrival at the experimental facility. The nonfasted animals had free access to their diet from the moment of arrival at the facility. All piglets had free access to water at all times.

Slaughtering and Sampling

The piglets were slaughtered on d 1, 4, and 10. First, ketamin (Sanaket 10%, Anisane B.V., Raamsdonksveer, the Netherlands) was used as a preanesthetic (15 mg/kg of BW), and 30 min later, the piglet was euthanized by intracardiac injection of T61 (combination of embutramide, mebenzoniumiodide, and tetraacain hydrochloride; Hoechst Roussel Vet, Frankfurt, Germany). After abdominal dissection, plastic strips were used to seal off random areas of the intestine, to reduce mixing of digesta before the entire GIT was separated from the abdominal cavity.
France), frozen in liquid nitrogen, and stored at
were placed in 1 mL of Extract-all (Eurobio, Les Ulis,
were taken to keep the
samples were mixed with chloroform
homogenizer (Labomoderne, Paris, France). Total
The tissue segments
were collected from the
At necropsy, tissue samples were collected from the
terminal ileum and the colon. Digesta particles were
removed from the terminal ileum and colon segments
by flushing with a cold saline solution. Samples were
stored at −20°C pending analysis. The tissue segments
were placed in 1 mL of Extract-all (Eurobio, Les Ulis,
France), frozen in liquid nitrogen, and stored at −80°C
before mRNA analysis. Care was taken to keep the
time between death of the piglet and tissue collection
at <10 min.

**Tissue Sample Analyses, RNA Extraction**

Intestinal samples were homogenized using a Cat
homogenizer (Labomoderne, Paris, France). Total
RNA was extracted with a monophase solution of phenol
and guanidine isothiocyanate, following the manu-
ufacturer’s recommendations. Briefly, after the homoge-
nization step the samples were mixed with chloroform
(20%, vol/vol) and shaken vigorously. The suspension
was centrifuged (12,000 × g for 15 min). The aqueous
phase was mixed with an equal volume of isopropanol,
and the RNA was then pelleted by centrifugation and
washed with 75% ethanol. The RNA was resuspended
in 50 μL of ultrapure water containing 0.02% (wt/vol)
diethyl pyrocarbonate (Sigma, St. Quentin Favallier,
France). Total RNA was quantified using a spectropho-
tometer at a wave length of 260 nm, and the purity
was assessed by determining the ratio of absorbance
at 260 and 280 (A260/A280). All samples had a ratio
ranging between 1.7 and 1.9. The quality of the RNA
was verified by electrophoresis of the samples on agar-
ose gels containing ethidium bromide and examination
of the presence of the 18S and 28S ribosomal bands.

**Reverse-Transcription PCR Detection of Cytokine mRNA and Quantification of PCR Products**

Semiquantitative determination of tumor necrosis
factor-α (TNF-α), IL-1β, IL-6, and cyclophilin was car-
rried out using reverse-transcription PCR, as pre-
viously described (Dozois et al., 1997; Fournout et al.,
2000; Marin et al., 2002), with minor modifications. As a
first step, 1.5 μg of total RNA was reverse transcribed
using random hexamers (Boehringer Mannheim, Mey-
lan, France) and murine Moloney leukemia virus re-
verse transcription (Point Mutant, Promega, Charbon-
nières, France).

After reverse transcription, the resulting cDNA
were amplified by PCR using deoxynucleoside triphos-
phates (2 mM each; Eurobio), 0.2 mM 5'- and 3'-prim-
ers, 2 mM MgCl2, and 2.5 U of DNA Taq polymerase
(Invitrogen, Cergy Pontoise, France) in a final volume
of 50 μL. The PCR primers and the annealing tempera-
tures used for the amplification were the same as pre-
viously described (Dozois et al., 1997). The number of
PCR cycles for each transcript was as follows: 30 cycles
for TNF-α, IL-1β, and IL-6; 35 cycles for IL-6, IL-
12p40, and IL-18; and 24 cycles for cyclophilin. Two-
fold dilutions of representative samples were used to
verify that the amplification conditions were nonsatu-
rated and suitable for semiquantitative analysis.

In a second step, semiquantitative analysis of all
PCR products was done by hybridization of 32P-la-
beled, specific oligonucleotide probes to the PCR prod-
ts immobilized on nitrocellulose membranes by dot
blotting, as previously described (Darwich et al.,
2003; Techau et al., 2007). The DNA probes used for
hybridization of the different cytokines were already de-
scribed (Pié et al., 2004). The relative amounts of each
product were determined by measuring radioactivity
with a PhosphorImager (Molecular Dynamics, Sun-
nyvale, CA). For each cytokine, the relative amount of
cytokine mRNA was normalized to cyclophilin mRNA.
The data were further normalized against the control
animals (considered as 1 AU) by the following formula:

\[
\text{normalized mRNA for a given sample} = \frac{\text{mean normalized mRNA from control animals (dI)}}{1}
\]

**Digesta Sample Analyses**

Digesta were analyzed for DM, pH, and SCFA (in-
cluding lactic and formic acids in the small intestine)
as previously described. Dry matter was determined by drying to a constant weight at 103°C (ISO 6496: ISO, 1999), and ash was determined by combustion at 550°C (ISO 5984: ISO, 1978). The VFA concentrations were analyzed by gas chromatography (Fisons HRGC Mega 2, CE Instruments, Milan, Italy), using a glass column fitted with Chromosorb 101 (Supelco, Zwijndrecht, the Netherlands), N₂ saturated with methanoic acid as the carrier gas at 190°C, and iso-caproic acid as an internal standard (Awati et al., 2005). Ammonia and lactic acid concentrations were determined according to the method described by Houdijk et al. (2002) and Voragen et al. (1986), respectively. Previous studies had shown that the lactic acid concentrations were low in the colonic contents (unpublished data), so this variable was measured only in the ileal contents.

Statistical Analysis

The results were expressed as mean ± SD. Statistical analyses were performed using ANOVA, as already described (Awati et al., 2006). The following statistical model was used:

\[ Y_{ijklm} = \mu + D_i + F_j + (D \times F)_{ij} + \varepsilon_{1ijk} + G_l + (D \times G)_{ijl} + (F \times G)_{ijl} + (D \times F \times G)_{ijl} + \varepsilon_{2ijklm} \]

where \( Y \) is the dependent variable to be tested; \( \mu \) is the overall mean; \( D_i \) represents the effect of the diet; \( F_j \) is the effect of the fasting treatment; \( (D \times F)_{ij} \) is the interaction; \( \varepsilon_{1ijk} \) is error term 1, which represents the random effect of animal within diet and fasting treatment; \( G_l \) represents the effect of site of GIT; \( (D \times G)_{ijl} \), \( (F \times G)_{ijl} \), and \( (D \times F \times G)_{ijl} \) denote the respective interactions; and \( \varepsilon_{2ijklm} \) is error term 2, which represents the overall error. Fisher’s test was used to examine significant differences between treatment groups. Differences were considered significant when \( P \leq 0.05 \).

RESULTS

Influence of Diet on the mRNA Content of Proinflammatory Cytokines at Weaning

The effect of the diet was first examined for nonfasted piglets (Figures 2 and 3). A significant increase in IL-1β mRNA was observed in the colon at d 4 postweaning in animals fed the control diet (\( P = 0.04 \)) and the CHO diet (\( P = 0.0002 \)), compared with the d-1 values (Figure 2). Then by d 10, for both groups of piglets, the mRNA expression levels of IL-1β returned to levels similar to those obtained on d 1. A kinetic profile similar to those observed for IL-1β mRNA was also obtained for the IL-6 cytokine in animals fed the CHO diet. Indeed, a transient up-regulation of IL-6 mRNA (\( P = 0.004 \)) was observed on d 4 postweaning in the colon of piglets fed the CHO diet (Figure 2). By contrast, no increase of IL-6 mRNA between d 1 and 4 was observed for the group of piglets fed the control diet. For TNF-α mRNA, a decrease (\( P < 0.05 \)) was observed on d 10 postweaning compared with d 1 and 4 postweaning for both groups of animals. A decrease (\( P < 0.05 \)) of IL-12p40 and IL-18 mRNA content was found on d 10 postweaning compared with d 1. Although this decrease was significant only in replicate A, the same trend was observed in replicate B (data not shown). No changes in the amounts of IL-8 mRNA were found between piglets fed the control and CHO diets (2.24 and 2.51 at d 4; 1.55 and 1.22 at d 10 for piglets fed the control and CHO diets, respectively).

Transcript levels for IL-1β, IL-6, and TNF-α were also measured in the ileum (Figure 3). An increase in IL-1β was observed on d 4 postweaning in this part of the gut in animals fed the control diet (\( P = 0.04 \)) and the CHO diet (\( P = 0.04 \)). No change in IL-6 mRNA level was observed for either diet treatment. No change was observed for the level of TNF-α mRNA (Figure 2). An analysis of IL-8, IL-12p40, and IL-18 mRNA content were also performed because we had already demonstrated that weaning could modulate the level of these cytokines (Pié et al., 2004). No significant alteration of the level of these cytokines in piglets fed the control diet and the CHO diet were observed (data not shown).

Influence of Fasting on the mRNA Content of Proinflammatory Cytokines at Weaning

Low voluntary feed intake, a characteristic often occurring in piglets just after weaning, can also modulate gut inflammation. For this reason, a comparison was also made of the profiles for proinflammatory cytokine mRNA expression on d 4 postweaning in 2 groups of piglets: 1 group fasted for 2 d postweaning and the other group given free access to feed. Not surprisingly, total feed intake during the first 4 d postweaning was lower (\( P < 0.05 \)) in the group of fasted piglets than in the group of nonfasted piglets (Table 2). However, a comparison of cytokine levels on d 4 postweaning did not show any differences between the fasted and nonfasted piglets fed the control diet or the CHO diet (Table 2). Similar results were obtained for the ileum (data not shown). Fasting also had no influence on the cytokine mRNA content measured at d 10 after weaning in the ileum and the colon of piglets fed the control or the CHO diet (data not shown).

Influence of Diet and Fasting on Digesta Measurements in the Ileum and Colon

Slaughter day affected all variables (\( P < 0.05 \)) except pH and branched-chain proportion (BCP; Table 3). Total VFA; acetic, propionic and butyric acids; and ammonia concentrations all increased with time,
Figure 2. Content of IL-1β, IL-6, and tumor necrosis factor-α mRNA in the colon of piglets weaned (d 1) and then placed on either the test diet with added fermentable carbohydrates (CHO, dark bars) or the control diet (hatched bars). Results are expressed in arbitrary units (AU) as the ratio between the cytokine-specific and the cyclophilin reverse-transcription PCR values. The data were further normalized to the values obtained in samples from control, unweaned (d 1) animals (considered as 1 arbitrary unit, AU). Values are the means ± SEM of 2 independent replicates (replicates A and B). a–cWithin a panel, means with different superscript letters are different (P < 0.05).

 whereas DM, lactic acid, and formic acid concentrations decreased. Differences between the GIT sites were highly significant (P < 0.001) for all variables except lactic acid and formic acid. Volatile fatty acid concentrations, ammonia concentrations, DM, and BCP were greater in the colon. However, surprisingly, pH was lower in the ileum compared with the colon (Table 3). Diet also influenced these variables; VFA (P < 0.01) (except butyric acid) and lactic acid concentrations (P < 0.1) were greater in the CHO diet, whereas the BCP and ammonia concentration were less (P < 0.001) in this diet compared with the control diet. Fasting had an effect on ammonia and the propionic, acetic, and lactic acid concentrations (P < 0.05). There were lower ammonia and lactic acid concentrations, coupled with greater propionic and acetic acid concentrations for the nonfasting compared with fasting animals.

 There were interactions (P < 0.01) between GIT site and slaughter day for most of the fermentation end-products (see Table 3). There was also an interaction (P < 0.01) between diet and GIT site for propionic acid, DM, pH, and ammonia concentrations.

 **Correlation Between Proinflammatory Cytokine mRNA Content and Digesta Measurements**

 Tables 4 and 5 show the correlations obtained using step-wise regression for the 6 cytokines correlated
Figure 3. Content of IL-1β, IL-6, and tumor necrosis factor-α mRNA in the terminal ileum of piglets weaned (d 1) and then placed on either the test diet with added fermentable carbohydrates (CHO, dark bars) or the control diet (hatched bars). Results are expressed in arbitrary units (AU) as the ratio between the cytokine-specific and the cyclophilin reverse-transcription PCR values. The data were further normalized over the values obtained in samples from control, unweaned (d 1) animals (considered as 1 AU). Values are the means ± SEM of 2 independent replicates (replicate A and B). Within a panel, means with different superscript letters are different ($P < 0.05$).

Table 2. Effect of feed intake on the expression of proinflammatory cytokines on d 4 postweaning in the colon of piglets fed the CHO diet or the control diet.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Fasting</th>
<th>Feed intake, g</th>
<th>Feed intake, 1 g</th>
<th>Day postweaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>Yes</td>
<td>220 ± 40</td>
<td>1.46 ± 0.35</td>
<td>0.46 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>294 ± 76</td>
<td>2.10 ± 0.95</td>
<td>1.70 ± 0.41</td>
</tr>
<tr>
<td>Control</td>
<td>Yes</td>
<td>182 ± 53</td>
<td>2.63 ± 1.04</td>
<td>1.22 ± 0.42</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>325 ± 78</td>
<td>1.97 ± 0.70</td>
<td>1.26 ± 0.19</td>
</tr>
</tbody>
</table>

Table 2. Effect of feed intake on the expression of proinflammatory cytokines on d 4 postweaning in the colon of piglets fed the CHO diet or the control diet.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Fasting</th>
<th>Feed intake, 1 g</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-8</th>
<th>IL-12p40</th>
<th>IL-18</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>Yes</td>
<td>1.46 ± 0.35</td>
<td>1.84 ± 1.27</td>
<td>0.61 ± 0.41</td>
<td>0.74 ± 0.31</td>
<td>0.58 ± 0.22</td>
<td>0.93 ± 0.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>2.10 ± 0.95</td>
<td>2.46 ± 1.76</td>
<td>1.70 ± 1.03</td>
<td>0.69 ± 0.26</td>
<td>0.89 ± 0.27</td>
<td>1.70 ± 0.41</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Yes</td>
<td>2.63 ± 1.04</td>
<td>1.34 ± 1.13</td>
<td>1.87 ± 1.25</td>
<td>1.13 ± 0.62</td>
<td>0.80 ± 0.10</td>
<td>1.22 ± 0.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>1.97 ± 0.70</td>
<td>1.09 ± 0.76</td>
<td>0.89 ± 0.20</td>
<td>0.67 ± 0.04</td>
<td>0.86 ± 0.29</td>
<td>1.26 ± 0.19</td>
<td></td>
</tr>
</tbody>
</table>

*a,b*Within a column, means with different letters differ ($P < 0.05$).

1Feed intake was calculated as the total feed intake during the 4 d after weaning. Values are means ± SEM (n = 8).

2Results are expressed in arbitrary units (AU) as the ratio between the cytokine-specific and the cyclophilin reverse-transcription PCR values. The data were further normalized over the values obtained in samples from control, unweaned (d 1) animals (considered as 1 AU). Values are means ± SD (n = 8). TNF-α = Tumor necrosis factor-α.

3The effect of fasting was tested within the animals fed the same diet.
Table 3. Effect of diet, fasting, slaughtering day, and gastrointestinal tract region on intestinal DM, pH, and end-product profile of digesta of the weanling piglets

<table>
<thead>
<tr>
<th>Item</th>
<th>pH</th>
<th>DM, g·kg⁻¹</th>
<th>Ammonia, mmol·L⁻¹</th>
<th>Propionic acid, mmol·L⁻¹</th>
<th>Acetic acid, mmol·L⁻¹</th>
<th>Butyric acid, mmol·L⁻¹</th>
<th>Branched chain,¹ proportion</th>
<th>Lactic acid, mmol·L⁻¹</th>
<th>Formic acid, mmol·L⁻¹</th>
</tr>
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<tbody>
<tr>
<td>Diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.51</td>
<td>173.8</td>
<td>24.39</td>
<td>10.0</td>
<td>36.9</td>
<td>4.7</td>
<td>4.7</td>
<td>2.11</td>
<td>46.8</td>
</tr>
<tr>
<td>CHO</td>
<td>6.45</td>
<td>161.1</td>
<td>16.89</td>
<td>11.8</td>
<td>42.3</td>
<td>5.3</td>
<td>1.53</td>
<td>4.7</td>
<td>54.1</td>
</tr>
<tr>
<td>¹MSE²</td>
<td>0.02</td>
<td>3.62</td>
<td>0.77</td>
<td>0.37</td>
<td>1.10</td>
<td>0.25</td>
<td>0.1</td>
<td>3.03</td>
<td>2.04</td>
</tr>
<tr>
<td>P-value</td>
<td>0.246</td>
<td>0.102</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>0.007</td>
<td>0.129</td>
<td>&lt;0.001</td>
<td>0.092</td>
<td>0.119</td>
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<td>Fasting</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>6.45</td>
<td>173.2</td>
<td>22.01</td>
<td>10.2</td>
<td>41.8</td>
<td>5.3</td>
<td>1.75</td>
<td>55.4</td>
<td>17.2</td>
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<tr>
<td>No</td>
<td>6.52</td>
<td>161.7</td>
<td>19.26</td>
<td>11.5</td>
<td>37.4</td>
<td>4.7</td>
<td>1.89</td>
<td>45.5</td>
<td>16.9</td>
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<tr>
<td>¹MSE²</td>
<td>0.02</td>
<td>3.62</td>
<td>0.77</td>
<td>0.37</td>
<td>1.10</td>
<td>0.25</td>
<td>0.1</td>
<td>3.03</td>
<td>2.01</td>
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<tr>
<td>P-value</td>
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<td>0.135</td>
<td>0.015</td>
<td>0.016</td>
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<td>0.351</td>
<td>0.024</td>
<td>0.911</td>
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<tr>
<td>Slday³</td>
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<td>4</td>
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<td>23.51</td>
<td>12.9</td>
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<td>5.8</td>
<td>1.76</td>
<td>41.5</td>
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<tr>
<td>¹MSE²</td>
<td>0.02</td>
<td>3.62</td>
<td>0.77</td>
<td>0.37</td>
<td>1.10</td>
<td>0.25</td>
<td>0.09</td>
<td>0.03</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.475</td>
<td>&lt;0.001</td>
<td>0.005</td>
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<tr>
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<td>6.55</td>
<td>112.1</td>
<td>4.91</td>
<td>0.2</td>
<td>8.8</td>
<td>0.5</td>
<td>1.21</td>
<td>50.5</td>
<td>17.08</td>
</tr>
<tr>
<td>Colon</td>
<td>6.41</td>
<td>222.9</td>
<td>36.37</td>
<td>21.6</td>
<td>70.5</td>
<td>9.6</td>
<td>2.43</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>¹MSE²</td>
<td>0.02</td>
<td>3.62</td>
<td>0.77</td>
<td>0.37</td>
<td>1.10</td>
<td>0.25</td>
<td>0.09</td>
<td>2.14</td>
<td>1.46</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Diet × fasting</td>
<td>0.704</td>
<td>0.313</td>
<td>0.84</td>
<td>0.382</td>
<td>0.553</td>
<td>0.541</td>
<td>0.579</td>
<td>0.141</td>
<td>0.006</td>
</tr>
<tr>
<td>Diet × Slday</td>
<td>0.081</td>
<td>0.971</td>
<td>0.507</td>
<td>0.966</td>
<td>0.444</td>
<td>0.734</td>
<td>0.827</td>
<td>0.510</td>
<td>0.122</td>
</tr>
<tr>
<td>Diet × GIT</td>
<td>0.005</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.232</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>GIT × Slday</td>
<td>0.150</td>
<td>0.079</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.018</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

¹Proportional branched-chain fatty acid production in weaning piglets, expressed as % of the total VFA.
²MSE = Mean SE.
³Slday = Slaughter day.

with DM, pH, and fermentation end-products in the piglet digesta. Table 4 shows the correlations obtained from the colon samples, and Table 5, those for the terminal ileum.

For the colon, one can see that there was a positive correlation between DM (P < 0.05) and IL-1β, IL-12p40, IL-18, and TNF-α. In addition, IL-8 correlated negatively (P = 0.043) with butyrate, whereas IL-12p40 (P = 0.004) and IL-18 (P = 0.026) had a negative correlation with BCP and acetic acid, respectively. No cytokines in colonic tissue were correlated with ammonia or propionic acid.

Table 4. Stepwise multiple regression analysis of factors ¹ responsible for variation in the proinflammatory cytokine mRNA expression in the colon

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Intercept</th>
<th>pH</th>
<th>DM, g·kg⁻¹</th>
<th>Ammonia, mmol·L⁻¹</th>
<th>Propionic acid, mmol·L⁻¹</th>
<th>Acetic acid, mmol·L⁻¹</th>
<th>Butyric acid, mmol·L⁻¹</th>
<th>Branched-chain,² proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>0.054</td>
<td>—</td>
<td>0.007</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>1.428</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-12p40</td>
<td>—9.223</td>
<td>0.644</td>
<td>0.003</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-18</td>
<td>0.558</td>
<td>—</td>
<td>0.0016</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.537</td>
<td>—</td>
<td>0.0025</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Where significant, the value is the slope of the regression curve. TNF-α = Tumor necrosis factor-α.
²Proportional branched-chain fatty acid production in weaning piglets, expressed as % of the total VFA.
For the terminal ileum, the number of digesta measurements that were correlated with cytokines was greater than for the colon. In this case, ammonia had a negative correlation with IL-1β (P < 0.1), IL-6 (P < 0.05), and also with IL-8, although not a significant one. Dry matter, butyrate, BCP, and lactic acid, when correlated with most cytokines, had a positive influence. The pH and acetic acid were not correlated with any of the cytokine values.

### DISCUSSION

During the experimental periods, none of the piglets showed any signs of diarrhea or any other illness. This was not unexpected, given that this was not an infection challenge study, and was conducted in an experimental facility where husbandry may be more fastidious than may be possible on a commercial farm.

In a recent study, Pié et al. (2004) demonstrated that weaning is associated with an early inflammatory cytokine response in the intestine of newly weaned piglets. Because diet can modulate the inflammatory response in the gut (Kudsk, 2003), the current study investigated the effect of a diet supplemented with indigestible but fermentable carbohydrates on the intestinal level of a set of proinflammatory cytokines, and then correlated these values with variables obtained for digesta chemistry. These results showed that the diet enriched with CHO induced an up-regulation of IL-6 mRNA level in the colon of piglets at 4 d postweaning. An increase in IL-1β mRNA was similarly observed at d 4 postweaning in piglets fed the CHO diet, but also in pigs fed the control diet.

Many studies have shown that diet can influence the immune response (Cunningham-Rundles, 2001), so this factor may represent one important influence on the regulation of GIT inflammation in newly weaned animals. In the current study, a significant up-regulation of IL-6 mRNA was observed in the colon of weaned piglets fed the CHO diet, but not in piglets fed the control diet, indicating that the regulation of IL-6 mRNA in the colon depends on dietary factors, or at least on microbiological factors that have been influenced by diet. Short-chain fatty acids, which are predominant end-products of dietary carbohydrate fermentation in the colon, and which can increase IL-6 abundance in the intestine of piglets (Milo et al., 2002), represent a good candidate that could modulate IL-6 mRNA content in the colon of piglets. However, comparisons of IL-6 mRNA contents with SCFA (acetic, propionic, and butyric acids) concentrations in the ileum and colon of weaned piglets showed no correlation between the levels of IL-6 mRNA and SCFA, indicating that the increased level of this cytokine was not directly dependent on the presence of SCFA. On the other hand, these results also demonstrated that lactic acid, in the ileum, was positively correlated with IL-6 mRNA. This may indicate that the IL-6 mRNA content is linked in some way to bacterial fermentation, most probably by lactobacilli or other lactic acid-producing species, at least in the ileum. In agreement with this finding, other studies have shown that non-pathogenic bacteria such as lactic acid bacteria can induce the production of cytokines such as IL-6 (Miettinen et al., 1996; Morita et al., 2002a,b). Because carbohydrates favor the growth of these bacteria in the colon, it is possible that the increase of IL-6 mRNA observed in piglets fed the CHO diet resulted from stimulation of specific bacterial species. Further studies are needed to elucidate the pathway by which carbohydrates could modulate IL-6 content.

The increase in IL-6 mRNA level in animals fed the CHO diet was surprising. Indeed, several reports have indicated that prebiotics tend to suppress inflammatory responses rather than the reverse (Kanauchi et al., 1998; Videla et al., 2001; Cherbut et al., 2003).
However, in the case of IL-6, it is noteworthy that this cytokine, first regarded as proinflammatory, also has other properties. In particular, this cytokine plays an important role in the terminal differentiation of B cells to antibody-producing plasma cells, and in the driving of naive CD4+ T cells into effector Th2 cells (Van Snick, 1990). Furthermore, IL-6 is known to have anti-inflammatory activities, such as inhibition of macrophage TNF and IL-1 release (Akira et al., 1990; Van Snick, 1990).

In addition to IL-6, analysis of IL-1β mRNA content in weaned piglets also demonstrated that this cytokine was up-regulated by 3 d postweaning. However, this up-regulation was similar in piglets fed the CHO diet and the control diet, suggesting that the level of this cytokine was not influenced by the diets used. In support of this hypothesis, analysis of IL-1β mRNA in relation to dietary factors did not show that the level of this cytokine, both in the colon and the ileum, was correlated with any of the end-products of fermentation (except for ammonia in the ileum, but the probability of correlation was low). In agreement with these results, an increase in IL-1β concentration was also observed in the blood of weaned piglets independent of the diet (McCracken et al., 1999). It is noteworthy that in a previous study (Pié et al., 2004), in which different experimental conditions were used, there was also a rapid mRNA increase in IL-1β after weaning. This may indicate that an increase in IL-1β mRNA is a common gut phenomenon at the time of weaning, and may be considered to be a general and early immunological change associated with weaning (or other stress) in piglets.

In the ileum, a positive correlation was observed between BCP and IL-8, IL-12p40, and IL-18 mRNA, indicating that the products of protein fermentation may favor inflammatory responses at weaning. In contrast, no correlation between these inflammatory cytokines and BCP was found in the colon. The lymphoid structures, Peyer’s patches, present in the ileum but not in the colon (Pabst and Rothkotter, 1999), may explain the variation in the responses observed between these 2 areas of the GIT.

Despite a strong correlation between IL-8, IL-12p40, and IL-18 mRNA and BCP in the ileum, no postweaning changes in the mRNA content of these cytokines were observed in piglets fed the CHO or the control diet. This may indicate that the proportion of branched-chain fatty acids, increased as a result of protein fermentation, were not sufficient to induce significant changes of the cytokine level or that other factors may be implicated in the regulation of these cytokines. In the colon, a positive correlation between DM and the cytokines IL-12p40, IL-18, and TNF-α was also obtained. Dry matter, which reflects movement of the colonic water, is affected by dietary factors such as fiber (Low et al., 1978) and SCFA (Ruppin et al., 1980) and by microbial factors including enteric bacterial toxins (Sears and Kaper, 1996). Because these factors are also potential inducers of proinflammatory cytokines, the DM content may represent a coincidental indicator of some inflammatory responses in the colon of weaned piglets.

In addition to the diet, the low feed intake commonly observed immediately after weaning in piglets may represent an important factor that can influence the cytokine response. Indeed, recently it has been reported that lack of intestinal nutrient provision (i.e., parenteral feeding) favors the development of inflammation in the intestine of piglets (Ganessunker et al., 1999; Zijlstra et al., 1999). McCracken et al. (1999), who examined numbers of inflammatory T cells during the weaning period, suggested that impaired feed intake immediately after weaning may contribute to inflammation in the intestine of piglets. In the current study, however, there was no correlation between the quantity of feed intake and the mRNA content of the proinflammatory cytokines IL-1β, TNF-α, and IL-6 by d 4 postweaning. Indeed, up-regulation of IL-1β and IL-6 mRNA was observed on d 4 postweaning at the same levels in animals fasted for 2 d after weaning as for the control, nonfasted animals. For IL-6, this is in agreement with other results, which may indicate that this cytokine is regulated by dietary factors. For IL-1β, for which the level seems to be independent of the diet and feed intake, it was demonstrated in a previous study that IL-1β mRNA was increased in nearly all parts of the intestine (Pié et al., 2004). Together, these results strongly support our previous hypothesis that the up-regulation of IL-1β mRNA is probably related to the general stress associated with weaning. In support of this hypothesis, Kanitz et al. (2004) recently demonstrated that social stress (i.e., maternal deprivation and social isolation) during the neonatal period resulted in an increase in IL-1β in the brain of piglets. Alternatively, the up-regulation of IL-1β or TNF-α mRNA or both could be the result of an increased resorption of lipopolysaccharide from the intestine, because of an alteration of the intestinal barrier at weaning (Boudry et al., 2004). Indeed, lipopolysaccharide is a well-known inducer of inflammatory cytokines in all animal species, including pigs (Touche et al., 2002). Local inflammation caused by weaning anorexia (McCracken et al., 1999) may also contribute to the observed changes.

In conclusion, our results demonstrate that a diet enriched in fermentable carbohydrates can modulate the IL-6 mRNA content during the weaning period in piglets. Correlations between several proinflammatory cytokines and the end-products of fermentation demonstrated that the regulation of cytokines is strongly linked to fermentation end-product measurements such as BCP and ammonia, particularly in the ileum. Cytokines play a major role in the maintenance of gastrointestinal homeostasis, and their regulation by nutritional factors may be an important consideration in the future for the development of new diet formulas for newly weaned piglets.
LITERATURE CITED


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