Basic nutritional investigation

Long-term intake of resistant starch improves colonic mucosal integrity and reduces gut apoptosis and blood immune cells

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Abstract

Objective: The potential effect of a long-term intake of resistant starch on colonic fermentation and on gut morphologic and immunologic indices of interest in bowel conditions in humans was studied in pigs.

Methods: Sixteen growing pigs were meal fed for 14 wk on a diet containing a large amount of raw potato starch (RPS; resistant starch) or corn starch (CS; digestible starch). Effects were assessed in the colon from the physicochemical properties of digesta and in the intestinal morphology, including lymphocytic infiltration, apoptosis, and proliferation activities. Hematologic and blood leukocyte cell subsets analysis were performed.

Results: After 97 d, the digestive content from RPS pigs was heavier than for CS pigs, producing a hypertrophy of tunica muscularis (P < 0.05). The proportion of butyrate was two-fold higher in proximal colon digesta in RPS pigs (P < 0.05). RPS-fed pigs had reduced apoptosis in the crypts, lamina propria and lymphoid nodules in the colon, and ileal Peyer’s patches (P < 0.05). Fermentation of RPS reduced indices associated with damage to epithelial cells, such as crypt cell proliferation and magnesium excretion, whereas mucin sulfuration was increased, which promotes epithelial protection. The numbers of intraepithelial T cells and of blood leukocytes, neutrophils, and lymphocytes, mainly T-helper lymphocytes, were reduced in RPS pigs (P < 0.05).

Conclusion: Long-term intake of RPS induces pronounced changes in the colonic environment, reduces damage to colonocytes, and improves mucosal integrity, reducing colonic and systemic immune reactivity, for which health benefits in inflammatory conditions are likely to be associated. © 2007 Elsevier Inc. All rights reserved.

Keywords: Resistant starch; Butyrate; Intestinal morphology; Mucosal immunity; Colonic function

Introduction

There are well-established beneficial effects of dietary fiber on some major digestive and metabolic diseases in humans. In particular, the presence of some carbohydrates, and in particular resistant starch (RS), in the diet has been shown to prevent pathogen infections or diarrhea [1], and to be of benefit in a variety of pathologic processes, such as inflammatory bowel disease [2], colon cancer risk [3], insulin resistance and diabetes [4], and chronic renal or hepatic disease [5].

Major components of dietary fiber, i.e., non-starch polysaccharides and RS, are fermented by colonic bacteria to short-chain fatty acids (SCFAs) and may cause major changes to the physicochemical properties of digesta, such as digesta bulking, water retention capacity, and viscosity [6]. It is possible to manipulate different dietary substrates to achieve desired amounts and ratios...
of SCFAs, particularly with respect to butyrate, to influence the incidence of colonic diseases [7]. In humans, large bowel fermentation of RS appears to increase butyrate production [8,9]. Butyrate is recognized as a preferred fuel for colonocytes and modulates several functions such as proliferation, differentiation, and apoptosis of these cells. Accordingly, data support a role for butyrate in promoting a normal phenotype in colonocytes and in preventing the development of abnormal cell populations [10–12]. This could help to explain the apparent relation between increased starch consumption and the diminished risk of colorectal cancer suggested by epidemiologic studies [13].

Some studies have indicated that the nature of fiber in the diet can also affect the composition, metabolism, and function of cells of the immune system [14,15] in gut-associated lymphoid tissues and in peripheral circulation [16]. These effects of fiber may be due to changes in the SCFAs produced. Butyrate has been shown to downregulate the stimulatory function of blood-derived antigen-presenting cells [17], upregulate Kupffer cell prostaglandin E2 production [18], and inhibit B-lymphocyte function [19] and T-lymphocyte proliferation in vitro [20]. In particular, butyrate inhibits in vitro T-helper 1–type responses and this might explain the therapeutic effect of butyrate on inflammatory bowel disease, a pathologic condition characterized by a chronic inflammation of the gut mucosa [20]. Nevertheless, whether butyrate could modulate in vivo mucosal lymphoid cells and potentially peripheral leukocytes has to be elucidated.

The present experiment was designed to study the long-term effects of a chronic load of RS, offered as a large intake of raw potato starch (RPS), on gut indices of interest in human bowel conditions, in a pig model. The pig was chosen as the experimental animal because it is generally accepted to be closer to humans than other laboratory or domestic animals in terms of gastrointestinal anatomy, physiology, nutrition, and microbiota [10,21–24]. Long-term effects of RS on the hindgut were evaluated from the physicochemical properties of digesta and fermentation and intestinal morphology, including lymphocytic infiltration, apoptosis, and proliferation activities. Hematologic and immunologic parameters were also evaluated throughout the experiment.

Materials and methods

The experiment was performed at the experimental unit of the Universitat Autònoma de Barcelona and received prior approval from the local ethical committee for animal experimentation of the institution. The treatment, housing, husbandry, and slaughtering conditions conformed to the European Union guidelines (Council of the European Communities, 1986).

Animals, housing, and diets

Sixteen Landrace × Large White growing pigs (9 wk of age, 20.69 ± 2.1 kg) were housed individually, randomly divided into two groups, and offered two experimental ground diets. Diets contained 250 g/kg of corn starch (CS diet) or 250 g/kg of RPS (RPS diet). Diets were formulated (Table 1) according to nutrient requirements of the National Research Council [25]. When animals reached an average body weight of 61.2 ± 4.5 kg, diets were adapted to the nutrient requirements of the animals (finishing diet) and the amount of purified starch increased to 350 g of CS or RPS per kilogram of feed. Purified starches (native CS: 260 g/kg of amylose, 740 g/kg of amylpectin; RPS: 200 g/kg of amylose, 800 g/kg of amylpectin) were purchased from Cerestar Iberica (Barcelona, Spain). As determined by enzyme analysis [26] CS contained 187 g/kg of type II RS and RPS 637 g/kg. Diets were offered ad libitum throughout the experimental period, which consisted of 8 wk (days 0–55) consuming the growing diet and 6 wk (days 55–96) consuming the finishing diet. The experiment was designed with the knowledge that several weeks are required for the

<table>
<thead>
<tr>
<th>Table 1 Composition and analyzed nutrient contents of experimental diets offered to pigs with 21–61 kg (growing) and 61–93 kg (finishing) body weights*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet†</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Ingredients (g/kg)</td>
</tr>
<tr>
<td>CS</td>
</tr>
<tr>
<td>RPS</td>
</tr>
<tr>
<td>Ground barley</td>
</tr>
<tr>
<td>Soybean meal</td>
</tr>
<tr>
<td>Soybean extruded</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
</tr>
<tr>
<td>Calcium carbonate</td>
</tr>
<tr>
<td>Salt</td>
</tr>
<tr>
<td>L-lysine</td>
</tr>
<tr>
<td>DL-methionine</td>
</tr>
<tr>
<td>L-threonine</td>
</tr>
<tr>
<td>Premix†‡</td>
</tr>
<tr>
<td>Nutrient analysis (g/kg DM)</td>
</tr>
<tr>
<td>Crude protein</td>
</tr>
<tr>
<td>Starch</td>
</tr>
<tr>
<td>Neutral detergent fiber</td>
</tr>
<tr>
<td>Ash</td>
</tr>
</tbody>
</table>

* Etoxiquin (Dresen, Mexico), 150 mg, as an antioxidant and 500 mg of Luctamold (Lucta, Spain) as a fungicide per kilogram of feed were added to both diets.
† Diets containing CS or high resistant starch from RPS.
‡ Premix provided the following ingredients per kilogram of diet: CaCO3, 12.3 g; CaHPO4, 9 g; NaCl, 2 g; vitamin A, 2100 μg; cholecalciferol, 45 μg; tocopherol, 10 mg; vitamin K2, 1 mg; thiamin, 1 mg; riboflavin, 4 mg; pyridoxine, 2 mg; cyanocobalamin, 20 μg; biotin, 10 μg; niacin, 18 mg; calcium-d-pantothenic acid, 10 mg; choline, 175 mg; iron, 80 mg; zinc, 110 mg; copper, 90 mg; manganese, 50 mg; cobalt, 0.1 mg; iodine, 1 mg; selenium, 0.2 mg.
intestinal microbiota to adapt to a load of fermentable substrate [3,27].

**Experimental procedures**

Individual body weight was registered at days 0, 55, and 96. For hematologic and immune cell subset studies, blood samples from the anterior vena cava were collected in tubes containing ethylene-diaminetetra-acetic acid and lithium heparin, respectively, on days 0, 7, 55, 62, and 97. Samples on days 7 and 62 allowed evaluation of early adaptation to the diets.

On day 97, pigs (90 ± 3.6 kg) were euthanized with an intravenous sodium pentobarbital overdose (200 mg/kg of body weight). Animals were bled and opened immediately, and the whole gastrointestinal tract was removed, measured, weighed, and sampled. Digesta from the proximal colon was sampled, pH was registered, and samples were stored for analysis of SCFAs. Approximately 1 g was acidified with 1 mL of solution containing 50 g/L of H₃PO₄, 10 g/L of HNO₃ and analyzed by atomic emission spectrophotometry. Calcium and magnesium were extracted from digesta with HCl and analyzed by atomic absorption spectrophotometry.

**Analytical procedures**

Chemical analysis of the diets and digesta was performed according to the methods of the Association of Official Analytical Chemists [28] for dry matter, ash, crude protein, and fat. Neutral detergent fiber was determined by the procedures of Goering and Van Soest [29] and total fiber was measured by the method of Theander et al. [30]. Starch was determined colorimetrically as glucose released after an enzymatic incubation with thermostable α-amylase (A-4551, Sigma, St. Louis, MO, USA) for 1 h at 100°C and with amyloglucosidase (A-3514, Sigma) for 4 h at 60°C. Calcium and magnesium were extracted from digesta with HNO₃ and analyzed by atomic emission spectrophotometry (ICP-OES, Optima 4300DV, Perkin-Elmer, Shelton, CT, USA). The SCFA concentration in deproteinized digesta was determined in the colon crypts. Numbers of goblet cells with neutral, acidic, or sulfomucin granules were determined in the colon crypts.

**Morphometric analysis**

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) using a linear ocular micrometer or a grid ocular (Olympus, Microplanet) [32]. Villus height and width, crypt depth, goblet cell and intraepithelial lymphocyte (IEL) numbers, index of mitosis, and lamina propria cell density were measured on 10 well-oriented villi and crypts from each intestinal section. All colonic samples were also microscopically examined for the presence of lymphoid nodules (LNs; solitary lymphoid follicles or lymphoglandular complexes) under a light microscope [6]. Thicknesses of the whole wall and the tunica muscularis layer were also determined. Morphometric measurements were made by the same person who was blinded to the treatments.

In the colon, CD3 immunohistochemistry [33] was performed to determine the presence of T cells in the epithelium and in the lamina propria. Proliferative activity of colonocytes was studied immunohistochemically using an anti-proliferate cell nuclear antigen antibody (PCNA). Details of the staining procedure have been described elsewhere [34]. In sections from the ileum and colon, cleaved caspase-3 (CCasp3) immunohistochemistry was also performed to identify cells undergoing apoptosis [35]. Numbers of apoptotic cells were also determined in the epithelium and in the lamina propria of the ileum and colon, ileal Peyer’s patches, and colonic LNs. Compartmental distribution of the stained cells (CD3⁺, PCNA⁺, or CCasp3⁺ cells) into the basal, middle, or luminal compartment of the crypt was also evaluated.

Colonic sections were also processed for carbohydrate histochemistry using dyes. The dye methods were the periodic acid-Schiff reaction or the Alcian blue reaction at pH 2.5 or 0.5 according to routine methods of staining for neutral mucins, acidic (sialomucins), and acidic-sulfated mucins (sulfomucins), respectively [36]. Numbers of goblet cells with neutral, acidic, or sulfomucin granules were determined in the colon crypts.

**Hematologic and leukocyte cell subset analyses**

A complete hemogram and a leukocyte differential count were performed on each pig blood sample using a semiautomatic electric impedance blood cell counter (Sysmex F-800; Toa Medial Electronic Europa).

For flow cytometric analysis, immune cells were isolated from peripheral blood on Ficoll-Hypaque gradients (Histopaque, 1.077, Sigma), stained with a panel of anti-porcine monoclonal antibodies, and analyzed by flow cytometry (EPICS XL MCL, Beckman-Coulter) as previously described [32]. In particular, leukocyte cell subsets were determined by using anti-CD45 (2A5, unconjugated, INIA, Madrid, Spain) to identify all leukocytes, anti-CD21 (BB6-11C9.6, fluorescein isothiocyanate conjugated, SBA, Birmingham, England) to identify B lymphocytes, anti-CD4 (74-2-11, unconjugated, INIA) to identify T-helper lymphocytes, anti-CD8 (76-2-11, phycoerythrin conjugated, SBA) to identify cytotoxic T cells, and anti-γδTCR (PGBL22A, unconjugated, VMRD, Pullman, Washington, USA) to
identify γδ T lymphocytes. Irrelevant isotype matched monoclonal antibodies were used as negative controls.

**Statistical analysis**

Data were subjected to analysis of variance, with dietary treatment as the classification factor, using the general linear model procedure of SAS 8.1 (SAS Institute, Cary, NC, USA). Additionally, repeated measures analysis of variance with time and treatment as the within-subject factor, and animal as random effect, was used to analyze repeated blood-derived measurements over time using the model MIXED-type TOEP of SAS, and LSMEANS follow-up test was used for comparisons of means. The statistical significance for all analyses was set at $P < 0.05$, with statistical tendencies reported when $P < 0.10$.

**Results**

Two incidents were observed during the experiment. One pig fed on the CS diet developed a rectal prolapse and was euthanized, and another pig fed on the RPS diet had a rectal prolapse and was euthanized, and another pig fed on the RPS diet had a rectal prolapse and was euthanized. Neither body weight nor average daily gain was affected by dietary treatments (91.6 kg to 96 d in CS and RPS, respectively, SEM 0.13, P versus 91 kg of final body weight in CS and RPS, respectively, SEM 0.13, P > 0.05). No clinical signs during the study and no gross findings at necropsy were recorded for any animal.

**Digesta content and composition**

Large bowel fermentation was evaluated by the composition, pH, and SCFAs in the proximal colonic digesta.

<table>
<thead>
<tr>
<th>Digestive content</th>
<th>CS</th>
<th>RPS</th>
<th>SEM</th>
<th>P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch (g/100 g DM)</td>
<td>8.54</td>
<td>16.57</td>
<td>2.199</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Neutral detergent fiber (g/100 g DM)</td>
<td>36.99</td>
<td>36.96</td>
<td>2.041</td>
<td>NS</td>
</tr>
<tr>
<td>Calcium (g/100 g DM)</td>
<td>1.23</td>
<td>0.95</td>
<td>0.132</td>
<td>NS</td>
</tr>
<tr>
<td>Magnesium (g/100 g DM)</td>
<td>0.52</td>
<td>0.34</td>
<td>0.038</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>pH</td>
<td>5.95</td>
<td>5.43</td>
<td>0.078</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>SCFA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (μmol/g fresh digesta)</td>
<td>53.27</td>
<td>81.51</td>
<td>24.651</td>
<td>NS</td>
</tr>
<tr>
<td>Acetate (mol/mol)</td>
<td>0.62</td>
<td>0.55</td>
<td>0.010</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Propionate (mol/mol)</td>
<td>0.23</td>
<td>0.19</td>
<td>0.010</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Butyrate (mol/mol)</td>
<td>0.11</td>
<td>0.20</td>
<td>0.011</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Branched SCFA (mol/mol)</td>
<td>0.02</td>
<td>0.01</td>
<td>0.003</td>
<td>NS</td>
</tr>
</tbody>
</table>

CS, corn starch; DM, dry matter; RPS, raw potato starch; SCFA, short-chain fatty acids

* Values are means, n = 7.

† NS, P > 0.10.

Table 2 Composition and physicochemical characteristics of colonic digesta in pigs fed a CS or a diet containing RPS for 96 d*

**Intestinal morphology in colon of pigs that ingested a diet containing CS or RPS for 97 d**

<table>
<thead>
<tr>
<th>Morphometry</th>
<th>CS</th>
<th>RPS</th>
<th>SEM</th>
<th>P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole wall (μm)</td>
<td>1339</td>
<td>1423</td>
<td>134</td>
<td>NS</td>
</tr>
<tr>
<td>Tunica muscularis (μm)</td>
<td>428</td>
<td>594</td>
<td>15.95</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Muscularis interna</td>
<td>296</td>
<td>407</td>
<td>10.07</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Muscularis externa</td>
<td>132</td>
<td>187</td>
<td>9.19</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Crypt depth (μm)</td>
<td>429</td>
<td>416</td>
<td>4.91</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Goblet cells (n/crypt)</td>
<td>32.0</td>
<td>31.4</td>
<td>0.606</td>
<td>NS</td>
</tr>
<tr>
<td>AB2.5+ cells</td>
<td>31.9</td>
<td>30.8</td>
<td>0.646</td>
<td>NS</td>
</tr>
<tr>
<td>AB0.5+ cells</td>
<td>28.9</td>
<td>33.8</td>
<td>0.543</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IEL/100 enterocytes</td>
<td>5.1</td>
<td>4.2</td>
<td>0.181</td>
<td>NS</td>
</tr>
<tr>
<td>Lymphocytic density</td>
<td>2.82</td>
<td>2.83</td>
<td>0.064</td>
<td>NS</td>
</tr>
<tr>
<td>Cell density</td>
<td>7.74</td>
<td>7.78</td>
<td>0.123</td>
<td>NS</td>
</tr>
<tr>
<td>Lymphoid nodules</td>
<td>0.094</td>
<td>0.037</td>
<td>0.015</td>
<td>&lt;0.10</td>
</tr>
</tbody>
</table>

PCNA immunohistochemistry

<table>
<thead>
<tr>
<th>Crypt</th>
<th>CS</th>
<th>RPS</th>
<th>SEM</th>
<th>P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminal compartment</td>
<td>9.6</td>
<td>9.5</td>
<td>0.313</td>
<td>NS</td>
</tr>
<tr>
<td>Middle compartment</td>
<td>12.8</td>
<td>12.7</td>
<td>0.382</td>
<td>NS</td>
</tr>
<tr>
<td>Basal compartment</td>
<td>12.5</td>
<td>10.0</td>
<td>0.243</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Whole crypt</td>
<td>35.7</td>
<td>32.5</td>
<td>0.833</td>
<td>&lt;0.10</td>
</tr>
</tbody>
</table>

CD3 immunohistochemistry

<table>
<thead>
<tr>
<th>Crypt</th>
<th>CS</th>
<th>RPS</th>
<th>SEM</th>
<th>P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminal compartment</td>
<td>4.1</td>
<td>3.5</td>
<td>0.148</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Middle compartment</td>
<td>1.2</td>
<td>1.4</td>
<td>0.065</td>
<td>NS</td>
</tr>
<tr>
<td>Basal compartment</td>
<td>0.9</td>
<td>0.7</td>
<td>0.127</td>
<td>NS</td>
</tr>
<tr>
<td>Whole crypt</td>
<td>6.1</td>
<td>5.6</td>
<td>0.233</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Lamina propria</td>
<td>1.57</td>
<td>1.52</td>
<td>0.057</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 3 Intestinal morphology in colon of pigs that ingested a diet containing CS or RPS for 97 d*

AB0.5+, Alcian blue at pH 0.5; AB2.5+, Alcian blue at pH 2.5; CS, corn starch; RPS, raw potato starch; PAS, periodic acid-Schiff; IEL, intraepithelial lymphocytes; PCNA, proliferate cell nuclear antigen antibody

* Values are means, n = 7.
† NS, P > 0.10.
‡ Goblet cells stained with PAS.†, AB2.5+, and AB0.5+.
§ Number of lymphocyte-like cells or total cells per 1000-μm² area in the lamina propria.
¶ Number of positive cells per compartment or per whole crypt.
†‡ Number of CD3+ cells (T cells) per 1000-μm² area.
Digesta) did not differ between treatments. The proportion of acetate (moles per mole) in the large bowel digesta was larger in the proximal colon for CS pigs than for RPS pigs \((P < 0.05)\), whereas the inverse was observed for the proportion of butyrate. Therefore, the concentration of butyrate (micromoles per gram) was three-fold higher for RPS than for CS pigs in the proximal colon (5.86 versus 16.30, SEM 2.55, \(P = 0.02\)).

Intestinal morphology and immunohistochemistry

In the small intestine, RPS pigs tended to have increased goblet cells in the crypt of the jejunum (21.9 versus 26.1 goblet cells/100 enterocytes, SEM 1.6, \(P = 0.09\)) and reduced villus height (416 versus 378 \(\mu\)m, SEM 10.9, \(P = 0.030\)) and increased cell density (7.8 versus 8.9 cells/1000-\(\mu\)m\(^2\) area, SEM 0.3, \(P = 0.014\)) in the ileum. Crypt depth,
Table 4
Hematologic parameters and leukocyte cell flow cytometric analysis in pigs fed experimental diets (CS and RPS) for 97 d*

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 55</th>
<th>Day 62</th>
<th>Day 97</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CS</td>
<td>RPS</td>
<td>CS</td>
<td>RPS</td>
<td></td>
</tr>
<tr>
<td>Red blood cell count (10^6/µL)</td>
<td>6.1</td>
<td>6.6</td>
<td>6.7</td>
<td>7.3</td>
<td>7.2</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>11.2</td>
<td>12.0</td>
<td>12.5</td>
<td>13.0</td>
<td>13.5</td>
</tr>
<tr>
<td>Hematocrit value (%)</td>
<td>36.3</td>
<td>37.5</td>
<td>38.0</td>
<td>39.8</td>
<td>40.2</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>59.1</td>
<td>56.6</td>
<td>56.9</td>
<td>54.7</td>
<td>55.9</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>30.9</td>
<td>31.9</td>
<td>32.9</td>
<td>32.5</td>
<td>33.5</td>
</tr>
<tr>
<td>Platelet count (10^3/µL)</td>
<td>613</td>
<td>579</td>
<td>542</td>
<td>436</td>
<td>375</td>
</tr>
<tr>
<td>Leukocyte count (10^3/µL)</td>
<td>16.0</td>
<td>16.6</td>
<td>14.3</td>
<td>17.6</td>
<td>15.2</td>
</tr>
</tbody>
</table>

Lymphocytes
- Absolute count (10^3/µL) | 8.8 | 9.7 | 8.5 | 10.4* | 8.0* | 11.4* | 9.0* | 10.5 | 8.8 | 0.7 |
- Relative count (%) | 56.3 | 59.2 | 59.9 | 58.9 | 53.0 | 57.9 | 60.6 | 52.6 | 52.0 | 2.8 |

Monocytes
- Absolute count (10^3/µL) | 1.0 | 0.62 | 0.64 | 0.61 | 0.69 | 0.72 | 0.73 | 1.02 | 1.05 | 0.1 |
- Relative count (%) | 6.3 | 3.9 | 4.7 | 3.5* | 4.5* | 3.7* | 4.6* | 5.2 | 6.2 | 0.5 |

Neutrophils
- Absolute count (10^3/µL) | 6.7 | 5.5 | 4.6 | 6.0 | 5.8 | 6.8* | 4.4* | 7.7* | 6.1* | 0.7 |
- Relative count (%) | 35.9 | 33.3 | 32.0 | 33.6 | 38.0 | 34.6 | 30.4 | 37.8 | 35.6 | 2.8 |

CD21+(%) | 15.0 | 16.0 | 16.7 | 14.2 | 17.4 | 17.6 | 19.8 | 16.5 | 18.2 | 1.8 |

γβTCCR+(%) | 13.3 | 18.5 | 17.1 | 20.1 | 18.9 | 20.8 | 20.3 | 14.6 | 11.3 | 1.6 |

CD4+(%) | 11.8 | 12.9 | 11.9 | 17.5* | 12.0b | 17.2 | 15.0 | 16.0* | 12.1* | 1.6 |

CD8+(%) | 38.5 | 43.1 | 42.6 | 35.0 | 29.8 | 32.5 | 25.9 | 35.8 | 38.0 | 3.3 |

CD8low+(%) | 17.0 | 26.6 | 25.7 | 19.4 | 15.6 | 14.0 | 13.4 | 13.6 | 16.0 | 2.1 |

CD8high+(%) | 21.5 | 16.6 | 16.8 | 15.6 | 14.2 | 17.7* | 12.5* | 22.2 | 22.0 | 1.9 |

CD4+CD8+(%) | 3.1 | 3.5 | 2.6 | 5.7* | 4.1* | 6.7 | 8.2 | 9.6* | 6.0* | 0.9 |

CD4+CD8low+(%) | 12.6 | 9.6 | 9.0 | 10.0* | 7.1b | 9.8* | 6.7b | 10.2 | 10.0 | 1.3 |

CD4+CD8high+(%) | 23.9 | 29.8 | 30.3 | 26.5 | 21.5 | 26.2 | 23.2 | 26.5 | 31.9 | 2.9 |

CD4+CD8ratio | 0.3 | 0.3 | 0.3 | 0.5 | 0.4 | 0.5 | 0.6 | 0.4* | 0.3b | 0.05 |

CS, corn starch; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; RPS, raw potato starch
* Values are means, n = 7. *b Means differ within sampling days (P < 0.05).

numbers of IEL, and the index of mitosis in the small intestine were not affected by dietary treatments.

Morphologic characteristics of the colon are presented in Table 3. Thickness of the tunica muscularis was higher for RPS pigs due to the muscularis interna and the muscularis externa (P < 0.05). Conversely, CS pigs had deeper colonic crypts (P = 0.045). Further, RS pigs increased (P = 0.033) cells with sulfomucin granules (Alcian blue–cells at pH 0.5). In contrast, even when the numbers of IEL did not differ between diets, numbers of CD3+ IELs (T cells) in the crypts were reduced in RPS pigs (P = 0.027), especially in the luminal compartment (P = 0.044). In the colonic lamina propria, no differences were observed in cell density or in the numbers of T cells. Nevertheless, a reduction of colonic LNs per millimeter of mucosa and its diameter was observed in RPS pigs (P < 0.05). In both groups, the luminal compartment was the main apoptotic compartment and represented 69% and 65% of all apoptotic cells in the CS and RPS pigs, respectively. In the lamina propria, a decrease (P = 0.030) in the absolute numbers of apoptotic cells was observed in RPS pigs (Fig. 2). In this compartment, the percentage of CCasp3+ cells ranged from 12.4% to 2.5% and was lower in RPS pigs (P < 0.05). In addition, cells belonging to the colonic LNs reduced their apoptotic activity (P = 0.052). In the ileum, apoptosis did not differ between groups in the epithelium and the lamina propria. Apoptosis in the villus epithelia was mainly found in the luminal and middle compartments (69% and 23% of all apoptotic cells, respectively). However, a significant reduction in apoptotic activity was observed in the follicular zones of ileal Peyer’s patches in RPS pigs (P = 0.004).

Immune cell subsets

Hematologic results are summarized in Table 4. The effect of age was observed for nearly all the studied blood parameters. An increase with time was observed in red blood cell count, hemoglobin concentration, hematocrit value, blood leukocytes, monocytes, neutrophils, and eosinophils (P < 0.05), whereas platelet counts decreased (P < 0.05). RPS pigs had a greater hemoglobin concentration than CS pigs, especially in the long term. Leukocyte, lymphocyte, and neutrophil total counts were reduced in RPS
pigs ($P < 0.05$). Although the counts of monocytes did not differ between treatments, its percentage in RPS pigs increased ($P = 0.012$) as a consequence of the increase in absolute counts of neutrophils and lymphocytes. No statistical differences between groups were observed in eosinophil and basophil counts.

Flow cytometric analyses also demonstrated significant differences associated with age and dietary treatments (Table 4). Percentages of blood CD4$^+$ T cells diminished in RPS pigs compared with CS pigs ($P = 0.03$) due to a decrease in CD4$^+$CD8$^+$ cells on days 55 and 62 and CD4$^+$CD8$^-$ cells on days 55 and 97 ($P < 0.05$). The mean percentage of CD8$^+$ cells was not affected. Nonetheless, CS pigs showed higher values ($P = 0.051$) of cytotoxic T cells (CD8$^{\text{high}}$) on day 62 (7 d after introducing the finishing diet), whereas no differences were observed on other days. The percentage of B lymphocytes and γδ T cells did not differ between dietary treatments.

Discussion

Starch is a substantial component of the human and swine diets. It may represent greater than 50% of the daily energy intake in pigs and in traditional agrarian cultures, and as little as 25% in Westernized diets [37]. It is generally accepted that most of the starch is digested in the small intestine. However, a fraction of starch and starch degradation products resists small intestine digestion (RS) and enters the large bowel, where it may be fermented [38]. We chose RPS to generate a supply of RS to the large bowel of growing pigs. The addition of RPS to the diet of pigs is known to increase the amount of starch entering the large bowel daily, which becomes an available source for the colonic microflora [27]. Our results demonstrate that the RPS diet modified the luminal environment and especially increased the butyrate concentration in colon digesta.

In general, major changes are observed in the weight of the gastrointestinal tract after long-term consumption of large amounts of RS. According to the starch content of the finishing diets (572.4 and 589.7 g/kg for the CS and RPS diets, respectively) and ileal starch digestibility (93.69 and 74.82% for CS and RPS; unpublished results), the estimated flow of starch to the hindgut was 36.1 and 148.5 g/kg dry matter for the CS- and RPS-fed pigs. Unfortunately, feed intake was not determined, and differences due to different levels of feed intake could not be excluded. Besides, the RPS diet increased the weight of the hindgut wall probably due to hypertrophy of the tunica muscularis. These adaptive trophic effects could be mediated by the weight of the hindgut content or their physicochemical characteristics, such as a higher butyrate concentration.

Our results also suggest that feeding RPS could improve hindgut epithelium integrity as indicated by the raised mucin sulfatation, lower luminal magnesium concentration, and lower epithelial proliferative activity. Sulfatation of mucin is considered to be an indicator of mucin maturity and is associated with increased protection of the intestinal epithelium against bacteria and proteases [36,39]. Conversely, reduced mucin sulfatation is closely correlated to colitis in humans [40]. Thus, the composition of mucins modified by RS fermentation could have raised the resistance of the mucosa to bacterial infection and could have an influence on gut bacterial colonization [41]. Conversely, the lower luminal magnesium concentration observed in RPS pigs could be a marker of a reduced epithelial cell damage because epithelial cells contain relatively large amounts of magnesium. Previous studies by Govers et al. [42] demonstrated a relation between cell damage and colonocyte hyperproliferation, in addition to excretion of endogenous magnesium. Moreover, animals fed on the RPS diet showed a reduction in cytotoxicity as a result of starch fermentation explained by the increasing bulking of digesta and probably by the suppression of secondary bile acid formation [8] or protein fermentation, which produces toxic endproducts [43]. Increases in stool weight have a diluting effect on potential carcinogens and irritant compounds, and epidemiologic studies have shown a reduced risk of colon cancer under these circumstances [44]. In addition, lower colonic pH in the RPS group may reduce procarcinogenic enzyme activity [7] and enhance the conversion of ammonia (NH3) into ammonium (NH4$^+$). Ammonium is less well absorbed by the colon and is excreted in the feces, reducing blood urea [44].

In contrast, pigs fed on the RPS diet showed a higher concentration of butyrate in the colonic digesta, which is in accordance with previous reports of RS fermentation [45,46]. Butyrate, which is the preferred fuel for colonocytes [12], has a paradoxical effect on normal and neoplastic colonocytes [10]. The presence of butyrate stimulates proliferation and inhibits apoptosis of normal colonocytes in vivo [46–49]. In our experiment, a reduction in the degree of epithelial apoptosis was observed in the RPS pigs; therefore, this could be explained by a higher concentration of butyrate in the colonic digesta. However, the present results of RPS pigs appear to disagree with the proliferative effects of butyrate on colonic epithelium. Thus, cell proliferation was enhanced in the CS pigs compared with the RPS pigs, and it explains the increased crypt depth observed in the CS pigs. Increases in epithelial proliferation are known to be an unspecific response to damage to epithelial cells [42,50].

Dietary changes also promoted changes in immune cell subsets in local and peripheral compartments. We observed that RS fermentation reduced in vivo infiltration of T cells in the epithelium. These effects may be due to the SCFAs produced. In particular, butyrate, but not acetate or propionate, may inhibit lymphocyte proliferation and interleukin-2 and interferon-γ production in vitro [20]. However, in vivo, results are not conclusive and SCFAs, and especially butyrate from fermentable dietary fiber, may potentiate the localization of cytotoxic IELs and natural killer cells in the large intestinal crypts [51]. The proliferation of IEL has also
been associated with exposure to bacterial antigens [52], evidenced by changes in bacterial biodiversity and major strains of colonic microflora [53,54]. Further, increased numbers of LNs in the mucosa of CS pigs compared with RPS pigs could indicate a higher immune reactivity to bacteria or other harmful substances, potentially caused by impairment of the mucosa barrier function. Interestingly, lamina propria T cells were not affected, indicating that not all gut-associated lymphoid tissues respond equally. In vitro studies in humans and pigs have suggested that lamina propria T cells enter the S phase after activation less efficiently than systemic T cells [55], providing an explanation because systemic T cells were substantially affected, more than the lamina propria.

This is the first report of decreasing apoptosis in the ileal and colonic mucosal lymphoid tissues after ingestion of RS. Apoptosis in the lymphoid tissues is directly related to cell proliferation and differentiation as a consequence of antigen stimulation [56]. Therefore, reduced numbers of apoptotic lymphocytes could indicate lower numbers of antigen-specific activated cells in these primary inductive sites that sample antigens. Butyrate released by fermentation of dietary RS could be involved in this process because it downregulates activation of T cells [17]. Consequently, dietary RS may diminish inflammatory bowel diseases because fermentation of RS cannot induce aberrant T-lymphocyte responses, which are implicated in their pathogenesis [57].

In peripheral blood, leukocytes and neutrophils were reduced in RPS pigs. In a rat model of enteritis, high blood leukocyte values and hypomotility coincided with bacterial invasion of the intestinal wall [58]. Interestingly, 1 wk after introducing the second diet, cytotoxic T cells were raised in the CS group, suggesting a proinflammatory response that might be due to bacterial changes or a disturbed intestinal environment. Therefore, the present results further support the improved mucosal integrity and function in RPS pigs. However, flow cytometry demonstrated that changes in lymphocytes were due to reduction in T-helper lymphocyte subsets. Bohmig et al. [17] reported that butyrate downregulates in vitro the stimulatory function of peripheral blood–derived antigen-presenting cells, modulating T-cell responses. T-lymphocyte decreases in peripheral blood could have resulted from differential effects of SCFAs on lower proliferation and production of anti-inflammatory cytokines in RPS pigs. Moreover, butyrate may induce a hemoglobin concentration increase on peripheral circulation by effects on gene expression [59], although the clinical importance is not known. It could be that the enhanced gut production of butyrate also raised its arterial level, suggesting that butyrate might have effects on cells that are not in direct proximity to the gut [60]. Conversely, other studies found that pigs fed with high amylose starch (RS) had lower concentrations of butyrate in portal venous plasma than in those fed CS [61]. Unfortunately, we could not determine the amount of butyrate in peripheral blood. Nevertheless, changes in blood parameters were observed on day 7, being evident from day 55 onward, suggesting that it may be important to consider the time required for the intestinal microbiota to adapt to a load of fermentable RS [3,27].

Conclusion

Long-term intake of RPS, as a source of type II RS, modifies the luminal environment and especially increases the butyrate concentration in colon digesta, which might improve mucosal integrity. Fermentation of RPS lowers peripheral T-cell populations and immune reactivity and apoptosis in the colon. These gut indices could indicate the possible health benefits of RS intake with regard to bowel conditions in humans, such as in inflammatory bowel disease.

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