Effects of inulin and di-D-fructose dianhydride-enriched caramels on intestinal microbiota composition and performance of broiler chickens

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In vitro and in vivo experiments were designed to evaluate the effectiveness of laboratory-made di-D-fructose dianhydride (DFA)-enriched caramels. The DFA-enriched caramels were obtained from D-fructose (FC), D-fructose and sucrose (FSC), or D-fructose and β-cyclodextrin (FCDC). In the in vitro experiment, raftilose and all caramels increased (P < 0.05) L-lactate concentration and decreased (P < 0.05) pH. Total short-chain fatty acid concentration was higher (P < 0.05) than controls in tubes containing raftilose, FSC, FCDC and commercial sucrose caramel (CSC). Raftilose, and all caramels tested except FSC and FC (1%), increased (P < 0.01) lactobacilli log10 number of copies compared with the non-additive control. FSC, FCDC and CSC increased (P < 0.01) the bifidobacteria number of copies as compared with controls. All additives, except FCDC, decreased (P < 0.01) Clostridium coccoides/Eubacterium rectale log number of copies. Compared with controls, raftilose, FC and CSC led to lower (P < 0.01) Escherichia–Shigella and enterobacteria. For the in vivo experiment, a total of 144 male 1-day-old broiler chickens of the Cobb strain were randomly assigned to one of the three dietary treatments for 21 days. Dietary treatments were control (commercial diet with no additive), inulin (20 g inulin/kg diet) and FC (20 g FC/kg diet). Final BW of birds fed FC diet was higher (P < 0.01) than controls or inulin-fed birds, although feed: gain values were not different. Feed intake of chickens fed FC was higher (P < 0.01) than that of inulin-fed birds but not statistically different from controls. Crop pH values were lower (P < 0.01) in birds fed FC diet as compared with control diet, with inulin-fed chickens showing values not different from control- or FC-fed birds. Lower (P < 0.05) lactobacilli number of copies was determined in the crop, ileum and caeca of birds fed the inulin diet compared with the control diet. Inulin supplementation also resulted in lower (P < 0.05) C. coccoides/E. rectale, bacteroides and total bacteria in caecal contents. Addition of FC to broiler diets gave place to lower (P < 0.05) enterobacteria and Escherichia–Shigella in crop and caecal contents compared with controls. The bacteroides number of copies increased (P < 0.05) as compared with controls in the ileum, but decreased (P < 0.05) in the caeca of inulin-fed birds fed the FC diet. Energy, ADF, NDF and non-starch polysaccharides faecal digestibilities were greater (P < 0.05) than controls in chickens fed diets containing inulin or FC. Fat digestibility was higher (P < 0.05) in FC-fed birds compared with controls or inulin-fed chickens. In conclusion, DFA-enriched caramels tested here, particularly FC, may represent a type of new additives useful in poultry production.

Keywords: broiler, di-D-fructose dianhydrides, inulin, microbiota, prebiotics

Implications

Di-D-fructose dianhydride (DFA)-enriched caramels that were added to a commercial broilers’ diet resisted to some extent small intestinal digestion, gave place to lower counts of potentially pathogenic bacteria in the intestine in vivo, and increased BW, faecal energy, fat, fibre and non-starch polysaccharide faecal apparent digestibilities as compared with controls. DFA-enriched caramels may therefore represent a type of new additives useful to improve health, productivity and well-being for poultry production.

Introduction

Worldwide concern about the development of antimicrobial resistance and about transference of antibiotic resistance
genes from animal to human microbiota led to banning the use of antibiotics as growth promoters in the European Union since January 2006 (EC Regulation, 1831/2003). One of the main consequences of this ban has been a substantial increase in the use of therapeutic antibiotics (Gaggìa et al., 2010). As a consequence, there is a need to look for viable alternatives that could enhance the natural defence mechanisms of animals and reduce the massive use of antibiotics. One way is to use specific feed additives and/or dietary raw materials to favourably affect animal performance and welfare, particularly through the modulation of the gut microbiota, which plays a critical role in maintaining host health. In this context, probiotics, prebiotics and symbiotics, that is, combinations of prebiotics and probiotics, have been proposed as possible solutions (Huyghebaert et al., 2010). The main putative effects of these feed additives are the improved resistance to pathogenic bacteria colonization and enhanced host mucosa immunity, thus resulting in a reduced pathogen load, an improved health status of the animals and a reduced risk of food-borne pathogens in foods (Williams et al., 2001). However, although probiotics seem to selectively enhance lactobacilli and bifidobacteria populations and reduce colonization by pathogenic bacteria, results on performance are often contradictory and mostly affected by the microorganisms or compound chosen, the dietary supplementation level and duration of use. In many cases, the environmental and the stress status of the animals are not reported or considered, as the experimental settings are often too far from farm conditions (Gaggìa et al., 2010).

The concept of prebiotics has been recently formalized by the establishment of three scientific criteria that a food ingredient must satisfy to be considered as such: (i) resistance to gastric acidity, to hydrolysis by mammalian enzymes and to gastrointestinal absorption; (ii) be a fermentable substrate by intestinal microorganisms belonging to the human (mammalian) microbiota; and (iii) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and well-being (Roberfroid, 2007). Several complex oligosaccharides, such as inulin, galactooligosaccharides and lactulose, fulfill the three criteria and can be effectively considered as prebiotics (Candela et al., 2010). Di-β-fructose dihydrates (DFAs) and their glycosylated derivatives (glycosyl-DFAs) represent recent candidates to this list (Arribas et al., 2010; Ortiz Mellet and García Fernández, 2010). However, the number of studies on the effects of DFAs on bacterial growth is quite limited so far, and very little effort has been made specifically in poultry nutrition.

DFAs were found to be present in the non-volatile fraction of industrial soft caramel, although in a relatively modest (15–18%) proportion. Recently, caramelization technologies, based on the use of heterogeneous acid catalysts, have been developed that allow producing DFA-enriched products (up to 70–80% DFAs and glycosyl-DFAs) from fructose or fructose-containing mixtures of food-grade carbohydrates (Suárez-Pereira et al., 2010). Initial results in rats fed with a fructose-derived DFA-enriched caramel were consistent with a prebiotic behaviour associated with the preservation of a healthy microbiota equilibrium (Arribas et al. 2010). Investigating the nutritional effects of these new caramels with high DFA content in farm animals was then very appealing. Accordingly, the work here described was designed to evaluate in vitro and in vivo the effects of the use of DFA-enriched caramels, in comparison with an industrial sucrose caramel or inulin, on microbial biochemical parameters, and the eventual changes in the intestinal microbiota composition of broilers fed on diets supplemented with these products.

**Material and methods**

**Additives**

Three different DFA-enriched caramels obtained from β-fructose (FC) or 1 : 1 mixtures (w/w) of β-fructose and sucrose (FSC), and β-fructose and β-cyclodextrin (FCDC), and a commercial sucrose caramel (CSC) were tested in the in vitro trial. FC was also tested in vivo. These products were obtained by using a newly developed technology to produce caramels with high DFA and glycosyl-DFA content (>60%) on the basis of the activation of β-fructose or mixtures of different carbohydrates containing β-fructose by strongly acidic ion-exchange resins (Suárez-Pereira et al., 2010). The sucrose caramel was a commercial aromatic caramel produced by Nigay (Feurs, France, ref. Nigay 1395 SMA6) conforming to the AFNOR NF V 00-100 norm (Association Française de Normalisation, Paris, 1988) and having the following technical characteristics: dry matter 792 g/kg; pH (50% in demineralized water) 2.80; colour (absorbance at 520 nm) 6.04. Powdered raftilose® P95 (Orafti, Tienen, Belgium) used for the in vitro trial was a powder produced through enzymatic hydrolysis of chicory inulin and contains oligofructose (>932 g/kg) with degree of polymerization between 2 and 8, with an average of four residues. Inulin (92.8%) was obtained from Farmusal (Granada, Spain).

**In vitro fermentation procedure**

Fermentations were conducted in triplicate in 50 ml sterile polypropylene tubes. The composition of the semi-defined medium used for fermentations were as in Ruiz et al. (2010). Caecal contents from 10 chickens receiving a cereal-based diet (Table 1) free of any antimicrobial agent were collected in aseptic tubes, sealed, immediately frozen in liquid N2 and kept at −80°C until the inoculum was prepared. Rose et al. (2010) showed that freezing at −80°C had minimal influence on the intestinal microbiota composition or metabolism. To prepare the inoculum, caecal contents were thawed at room temperature, immediately diluted 1 : 10 in 150 mM NaHCO3 buffer adjusted to pH 7.4 (1 g of caecal content in 9 ml of buffer), and mixed in a stomacher for 2 min. Bags were previously treated with a N2 stream. Blended, diluted caecal contents were filtered through miracloth (Calbiochem, Merck Millipore, Barcelona, Spain) and sealed in serum bottles under an N2 stream. Raftilose or caramels were added to the semi-defined medium to reach a final concentration of 10 mg/ml and autoclaved. No additive (negative control),
raftilose (positive control) and sample tubes (n = 3) were
then aseptically inoculated with 4 ml of inoculum that were
added. Eluted DNA was treated with RNase and the DNA
concentration assessed spectrophotometrically by using a
NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies,
Wilmington, DE, USA). Puriﬁed DNA samples were stored
at 20°C until use (Ruiz and Rubio, 2009). Bacterial log10
copies was determined in faecal samples by using q-PCR. The 16S rRNA gene-targeted primers and PCR con-
ditions used in this study are given as Supplementary
Material S1.

q-PCR analysis for the in vivo and in vitro trials
Total DNA was isolated from freeze-dried intestinal or
fermentation samples (40 mg) using the QiAamp DNA stool
kit (Qiagen, West Sussex, UK) by following the manu-
facturer’s instructions. To increase its effectiveness, the lysis
temperature was increased to 95°C and an additional step
with lysozyme (10 mg/ml, 37°C, 30 min) incubation was
added. Eluted DNA was treated with RNase and the DNA
concentration assessed spectrophotometrically by using a
NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies,
Wilmington, DE, USA). Purified DNA samples were stored
at −20°C until use (Ruiz and Rubio, 2009). Bacterial log10
number of copies was determined in faecal samples by using q-PCR. The 16S rRNA gene-targeted primers and PCR con-
ditions used in this study are given as Supplementary
Material S1.

Chemical and biochemical analysis and calculations
Amounts of DFAs in caramel samples were determined by GC
analysis as in Suárez-Pereira et al. (2010). Their total content
in monosaccharides, DFAs and higher oligosaccharides
(including glycosyl DFAs) were: FC, 185 g/kg D-fructose, 316
mg/kg DFAs, 480 g/kg glycosyl-DFAs; FSC, 211 g/kg ω-fructose,
77 g/kg ω-glucose, 272 g/kg DFAs, 410 g/kg glycosyl-DFAs
and glucooligosaccharides; FCDC, 100 g/kg ω-fructose, 8 g/kg
ω-glucose, 217 g/kg DFAs, 640 g/kg glycosyl-DFAs and glu-
cooligosaccharides; CSC, 167 g/kg ω-fructose, 348 g/kg ω-glucose; 136 g/kg DFAs; 311 g/kg glycosyl-DFAs and glu-
cooligosaccharides. For determination of DFAs in samples from the in vitro trial and from faecal samples, aliquots
(25–40 mg) were diluted in distilled water (60 ml) and dialysed
using a Jumbosep™ Centrifugal Devices system equipped

Table 1 Ingredient and nutrient analysis (g/kg) of the experimental diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Analysis (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>462</td>
</tr>
<tr>
<td>Soy flour</td>
<td>310</td>
</tr>
<tr>
<td>Wheat</td>
<td>150</td>
</tr>
<tr>
<td>Vit + min mixa</td>
<td>30</td>
</tr>
<tr>
<td>Animal fat</td>
<td>20</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>16</td>
</tr>
<tr>
<td>Calcium phosphate</td>
<td>3.1</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>4.5</td>
</tr>
<tr>
<td>Chromium oxide</td>
<td>2</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.2</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.2</td>
</tr>
<tr>
<td>Calculated analysis</td>
<td></td>
</tr>
<tr>
<td>Metabolizable energy (cal/g)</td>
<td>2912</td>
</tr>
<tr>
<td>CP</td>
<td>193.0</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>33.7</td>
</tr>
<tr>
<td>Fat</td>
<td>44.6</td>
</tr>
<tr>
<td>Calcium</td>
<td>7.1</td>
</tr>
<tr>
<td>Phosphorous</td>
<td>6.2</td>
</tr>
<tr>
<td>Methionine cysteine</td>
<td>8.6</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*The mineral–vitamin mix contained (per 30 kg): vitamin A, 7 500 000 IU; vitamin D3, 1 500 000 IU; vitamin E, 25 g; vitamin B12, 10 mg; vitamin B6, 67 mg; calcium pantothenate, 7.5 g; nicotinic acid, 10 g; folic acid, 25 mg; vitamin K3, 1 g; coline chloride, 250 g; Fe, 4 g; Cu, 750 mg; Co, 50 mg; Zn, 38 g; Mn, 42 g; I, 680 mg; Se, 45 mg; coccidiostate (Nistatin + Nicarbacin), 0.50 kg; BHT, 250 mg.

Birds, diets and housing
A total of 144 male 1-day-old broiler chickens of the Cobb
strain were randomly assigned to one of the three dietary
treatments. Birds were weighed on arrival and raised in wire-
floored batteries. Each treatment had eight replicates (cages)
of six birds. Cages were provided with the convenient heat-
ing and the birds received a lighting regimen of 23 h light : 1 h
darkness. Balanced commercial diets (Table 1) free of any
feed antibiotics and formulated to match the requirements
for growing birds of this age and genotype were used. Diets
were fed ad libitum for 21 days. Dietary treatments were
control (commercial diet with no additive), inulin (commercial
diet supplemented with 20 g inulin/kg diet) and FC (commercial
diet supplemented with 20 g FC/kg diet). FC was chosen
for the in vivo trial because it was at the moment better
characterized chemically than the others. The experimental
protocol was reviewed and approved by the Institutional
Animal Care and Use Committee of the Spanish Council for
Scientific Research (CSIC, Spain), and the animals were cared
for in accordance with the Spanish Ministry of Agriculture

Sacrifice and sample collection
Data on live BW and feed consumption were recorded at the
beginning and at the end of the experiment and used to
calculate feed intake (FI) and feed: gain ratio (F/G). At 21
days of age, birds (three per replicate, i.e. 18 per treatment)
were randomly selected and killed by intra-thoracic injection
of the euthanasic T61 (0.2 ml/bird) (Laboratorios Intervet SA,
Salamanca, Spain). The pH was immediately measured in the
crop content of each bird by using a Crison pH meter (Crison
Instruments SA, Alella, Spain). Immediately after killing,
contents from the crop, ileum (considered as the section
between the Meckel’s diverticulum and the ileo-cecal
junction) and caeca of each bird were collected into plastic
 tubes, stored at −20°C and freeze-dried (Ruiz and Rubio,
2009). Samples of about 1 cm taken at the mid-point of the
ileum of three randomly selected birds from each treatment
were removed for histological analysis. The samples were
flushed twice with PBS to remove luminal digesta and
immersed in formalin (10% neutral buffered formaldehyde)
for ﬁxation. After 24 h in 10% neutral buffered formaldehyde,
the tissue samples were carefully cleaned of any remaining
digesta with deionized water, and then transferred to a fresh
solution of 10% neutral buffered formaldehyde (Sigma,
Alcobendas, Spain).
with 3 kDa membranes. The system was centrifuged at 960 g for 25 min using a Hettich Rotina 35 centrifuge (Andreas Hettich GmbH, Tuttinglen, Germany). The filtrate containing the DAFs was collected and re-dialysed five times, freeze-dried and analysed as in Suárez-Pereira et al. (2010).

The pH of the in vitro fermentation solutions was measured immediately after the 24 h fermentation period. l-lactate and raftilose concentrations were determined by using Megazyme kits (K-LATE 10/04 and K-FRUC 12/04, respectively) (Megazyme International, Wicklow, Ireland).

Short-chain fatty acids (SCFA) (acetate, propionate, butyrate, isobutyrate, valerate and isovalerate) concentrations were determined by GLC (Playne, 1985). Briefly, 2.0 ml of the fermentation product was centrifuged (4°C, 15 000 g, 15 min) and the supernatant (0.5 ml) mixed with 0.5 ml of a mix of crotonic acid (4 g/l) + metaphosphoric acid (20 g/l) + HCl (0.5 N), and left overnight at −20°C. After centrifugation, 1 µl of the supernatant was injected in a Perkin Elmer model AutoSystem (PerkinElmer, Madrid, Spain) gas chromatograph fitted with a Supelco SP 2380 (Sigma-Alrich Quimica SL, Madrid, Spain) capillary column (30 m × 0.25 mm × 0.2 µm). Samples were analysed in triplicate. Appropriate SCFA standards were produced with crotonic acid (2 g/l) as internal standard.

The N contents in feed, ileal contents and faeces were determined according to the Dumas procedure using a LECO Truspec CN analyser (LECO Corporation, St. Joseph, MI, USA). Deduc and ADF were carried out according to van Soest et al. (1991) by using an Ankon® fiber analyzer unit (Ankom Technology Corp., Macedon, NY, USA). NSP in diets and faecal samples was determined as in Englyst et al. (1991) by using an Ankom 220 ber analyzer unit (Ankom Technology Corp., Macedon, NY, USA).

The pH of the in vitro fermentation solutions was measured immediately after the 24 h fermentation period. l-lactate and raftilose concentrations were determined by using Megazyme kits (K-LATE 10/04 and K-FRUC 12/04, respectively) (Megazyme International, Wicklow, Ireland).

Chromatography was performed at 20°C. After centrifugation, 1 ml of the supernatant was injected in a Perkin Elmer model AutoSystem (PerkinElmer, Madrid, Spain) gas chromatograph fitted with a Supelco SP 2380 (Sigma-Alrich Quimica SL, Madrid, Spain) capillary column (30 m × 0.25 mm × 0.2 µm). Samples were analysed in triplicate. Appropriate SCFA standards were produced with crotonic acid (2 g/l) as internal standard.

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Carcinogenic and mutagenic substances were determined following a colorimetric micromethod (Fenton and Fenton, 1979). AMEn was calculated as in Hill and Anderson (1958), and gross energy in feed and faeces was determined in a bomb calorimeter (Parr Instruments Co., IL, USA).

Statistical analysis
Data were analysed as a one-way ANOVA using the GLM procedure of SAS (SAS Institute, 2003, Cary, NC, USA), with the pen serving as the experimental unit for performance parameters, and the individual chicken or the fermentation tube as the experimental unit for biochemical or microbiological parameters. All microbiological counts were subject to base-10 logarithm transformation before analysis. Treatment means were separated using Bonferroni’s multiple comparison tests. Statistical significance was declared at a probability of $P < 0.05$.

Results
Effects of caramels on biochemical parameters and microbial populations in vitro
The pH of the solutions (Table 2) decreased in vitro fermentation with all additives compared with the non-additive control, the lowest values being reached with raftilose, FC (2%) and CSC. l-lactate concentration in vitro (Table 2) was higher ($P < 0.05$) for raftilose than for the other additives, of which the highest values were found for FC (2%) and CSC. Regarding SCFA concentrations (Table 2), acetate concentration was highest ($P < 0.05$) for FSC and FCDC followed by raftilose. Propionate values were increased ($P < 0.05$) compared with controls for all additives tested, with the highest values for FSC and raftilose. FCDC was the only additive that increased ($P < 0.05$) butyrate and valerate concentrations. No effect was detected with respect to control values on isobutyrate or isovalerate production. The total SCFA concentration ($P < 0.05$) was higher than the concentration of control in tubes containing raftilose, FSC, FCDC and CSC, with FC (1%) showing a tendency for increased values as compared with control diets.

Incubation for 24 h within the defined media, regardless of the additive, resulted in significant ($P < 0.01$) differences in all groups of bacteria examined in this study (Table 3). Raftilose and all caramels tested, except FSC and FC (1%), increased ($P < 0.01$) lactobacilli log10 number of copies compared with the non-additive control. No differences in lactobacilli number of copies were found between raftilose, FC (2%) and CSC. FSC, FCDC and CSC induced increased ($P < 0.01$) bifidobacteria log10 number of copies as compared with control tubes. Only FCDC induced an increase ($P < 0.01$) in the bacteroides log10 number of copies respect to raftilose. All additives, except FCDC, decreased ($P < 0.01$) Clostridium cocoides/Eubacterium rectale number of copies. Raftilose decreased ($P < 0.01$) and FCDC increased ($P < 0.01$) C. leptum log number of copies. Compared with controls, raftilose, FC and CSC gave place to lower ($P < 0.01$) Escherichia–Shigella and enterobacteria log number of copies.

Effects of inulin and caramels on performance and crop pH
Final BW of birds fed the FC diet was higher ($P < 0.01$) than controls or inulin-fed birds (Figure 1), although feed:
gain values were not different. Feed intake of chickens fed the FC diet was higher \( P < 0.01 \) than inulin but not different from controls. Crop pH values were lower \( P < 0.01 \) than controls in birds fed the FC diet, with inulin-fed chickens showing values not different from controls or FC-fed birds.

**Effects of inulin and caramels on intestinal microbiota composition in vivo**

Decreased \( P < 0.05 \) lactobacilli \( \log_{10} \) number of copies was determined in crop, ileum and caecal contents of birds fed the inulin diet (Table 4). Inulin supplementation also resulted in lower \( P < 0.05 \) \( C. \) coccoides/E. rectale, bacteroides and total bacteria in caecal contents. Addition of \( \alpha \)-fructose caramel (FC, 2%) to broilers’ diets resulted in higher \( P < 0.05 \) \( C. \) coccoides/E. rectale number of copies in the crop, and in lower \( P < 0.05 \) enterobacteria and \( E. \) coli – \( S. \) shigella \( \log_{10} \) number of copies in crop and caecal contents compared with controls. Compared with controls, bacteroides number of copies increased \( P < 0.05 \) in the ileum, but decreased \( P < 0.05 \) in the caeca, of chickens fed the FC diet.

**In vivo intestinal digestibility of substances**

In vivo ileal apparent digestibility of DAFs in birds fed FC diet was 0.81 (Table 5). No DAFs were detected in the control diet or in the ileal contents of birds fed the control diet. Inulin digestibility was 14% and 85% at the ileal and fecal levels, respectively. Ileal N digestibility was not affected \( P < 0.05 \) by the supplementation with either inulin or FC. On the
contrary, faecal energy, ADF and NDF apparent digestibilities were greater ($P < 0.05$) than controls in chickens fed diets containing either inulin or FC. Fat faecal apparent digestibility was higher ($P < 0.05$) in FC-fed birds compared with controls or inulin-fed chickens. NSP digestibility was higher ($P < 0.05$) than controls in inulin-fed birds, but not in those fed the FC diet.

**Morphology of the ileal mucosa**
Inclusion of 20 g of FC/kg in diets for broiler chickens between 1 and 21 days had no significant effect on the histological parameters measured (Table 6). Nevertheless, inulin supplementation with 20 g/kg diet increased ($P < 0.01$) villus height and the ratio of villus height/crypt depth as compared with both control- and FC-fed birds.

### Table 4 Bacterial log$_{10}$ number of copies/mg of freeze-dried contents in the crop, ileum and caeca of broiler chickens fed on control, inulin or DFA-enriched caramel supplemented diets from 1 to 21 days

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Inulin</th>
<th>FC</th>
<th>Pooled SD</th>
<th>Significance level</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Crop</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>6.2$^a$</td>
<td>5.3$^b$</td>
<td>5.8$^{ab}$</td>
<td>0.8</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>2.9</td>
<td>3.0</td>
<td>2.9</td>
<td>0.2</td>
<td>0.092</td>
</tr>
<tr>
<td>Clostridium cocoides/Eubacterium rectale</td>
<td>3.8$^a$</td>
<td>3.9$^{ab}$</td>
<td>4.2$^b$</td>
<td>0.3</td>
<td>0.023</td>
</tr>
<tr>
<td>C. leptum</td>
<td>4.0</td>
<td>3.9</td>
<td>4.0</td>
<td>0.4</td>
<td>0.648</td>
</tr>
<tr>
<td>Enterobacteria</td>
<td>5.5$^a$</td>
<td>5.3$^{ab}$</td>
<td>4.7$^b$</td>
<td>0.7</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Escherichia–Shigella</td>
<td>5.5$^a$</td>
<td>5.2$^{ab}$</td>
<td>4.8$^b$</td>
<td>0.8</td>
<td>0.014</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>4.5</td>
<td>4.7</td>
<td>4.5</td>
<td>0.3</td>
<td>0.027</td>
</tr>
<tr>
<td>Total bacteria</td>
<td>6.8</td>
<td>6.9</td>
<td>7.0</td>
<td>0.3</td>
<td>0.317</td>
</tr>
<tr>
<td><strong>Ileum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>5.2$^a$</td>
<td>4.5$^b$</td>
<td>5.0$^{ab}$</td>
<td>0.7</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>2.7</td>
<td>2.8</td>
<td>2.9</td>
<td>0.4</td>
<td>0.545</td>
</tr>
<tr>
<td>C. cocoides/E. rectale</td>
<td>4.7</td>
<td>4.4</td>
<td>4.3</td>
<td>0.5</td>
<td>0.205</td>
</tr>
<tr>
<td>C. leptum</td>
<td>4.2</td>
<td>4.1</td>
<td>3.8</td>
<td>0.5</td>
<td>0.100</td>
</tr>
<tr>
<td>Enterobacteria</td>
<td>4.4</td>
<td>4.4</td>
<td>4.2</td>
<td>0.6</td>
<td>0.521</td>
</tr>
<tr>
<td>Escherichia–Shigella</td>
<td>3.2</td>
<td>3.0</td>
<td>3.0</td>
<td>0.8</td>
<td>0.666</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>3.9$^a$</td>
<td>3.9$^a$</td>
<td>4.5$^b$</td>
<td>0.6</td>
<td>0.013</td>
</tr>
<tr>
<td>Total bacteria</td>
<td>6.0</td>
<td>5.8</td>
<td>5.9</td>
<td>0.5</td>
<td>0.289</td>
</tr>
<tr>
<td><strong>Caecum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>7.2$^a$</td>
<td>6.7$^b$</td>
<td>6.9$^{ab}$</td>
<td>0.6</td>
<td>0.004</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>4.3</td>
<td>4.0</td>
<td>3.9</td>
<td>0.4</td>
<td>0.062</td>
</tr>
<tr>
<td>C. cocoides/E. rectale</td>
<td>8.3$^a$</td>
<td>7.8$^b$</td>
<td>7.8$^b$</td>
<td>0.2</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>C. leptum</td>
<td>8.1$^a$</td>
<td>8.0$^{ab}$</td>
<td>7.8$^b$</td>
<td>0.3</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Enterobacteria</td>
<td>5.9$^a$</td>
<td>5.6$^a$</td>
<td>5.1$^b$</td>
<td>0.6</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Escherichia–Shigella</td>
<td>5.9$^a$</td>
<td>5.7$^{ab}$</td>
<td>5.2$^b$</td>
<td>0.6</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>5.7$^a$</td>
<td>5.3$^b$</td>
<td>5.0$^c$</td>
<td>0.3</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Total bacteria</td>
<td>8.7$^a$</td>
<td>8.3$^b$</td>
<td>8.3$^b$</td>
<td>0.2</td>
<td>$&lt;0.001$</td>
</tr>
</tbody>
</table>

DFA = D-fructose dianhydride; FC = D-fructose caramel.
Means in the same row with different superscript letters differ ($P < 0.05$).

---

### Table 5 AMEn (cal/g) and ileal and faecal apparent digestibility (%) in growing broiler chickens fed control, inulin or DFA-enriched caramel supplemented diets from 1 to 21 days of age

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Inulin</th>
<th>FC</th>
<th>Pooled SD</th>
<th>Significance level</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AMEn</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>83</td>
<td>85</td>
<td>85</td>
<td>2.2</td>
<td>0.059</td>
</tr>
<tr>
<td>DFAs</td>
<td>nd</td>
<td>nd</td>
<td>81 (SD 4.1)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Inulin</td>
<td>nd</td>
<td>14 (SD 9.2)</td>
<td>nd</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Ileal digestibility</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy</td>
<td>84$^b$</td>
<td>85$^b$</td>
<td>85$^b$</td>
<td>0.8</td>
<td>0.036</td>
</tr>
<tr>
<td>Fat</td>
<td>94$^b$</td>
<td>94$^b$</td>
<td>95$^b$</td>
<td>0.7</td>
<td>0.004</td>
</tr>
<tr>
<td>ADF</td>
<td>39$^b$</td>
<td>45$^b$</td>
<td>46$^b$</td>
<td>2.9</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>NDF</td>
<td>66$^b$</td>
<td>68$^b$</td>
<td>69$^b$</td>
<td>1.3</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>NSP</td>
<td>59$^b$</td>
<td>66$^b$</td>
<td>64$^{ab}$</td>
<td>4.3</td>
<td>0.038</td>
</tr>
<tr>
<td>Inulin</td>
<td>nd</td>
<td>85 (SD 3.1)</td>
<td>nd</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

DFAs = di-D-fructose-dianhydrides; nd = not detected.
Means in each row with different superscript letters differ ($P < 0.05$).
Table 6 Morphology of the ileal sections of broiler chickens fed control, inulin or FC diets from 1 to 21 days of age

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Inulin</th>
<th>FC</th>
<th>Pooled SD</th>
<th>Significance level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Villus height (μm)</td>
<td>784a</td>
<td>982b</td>
<td>763a</td>
<td>89</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Crypt depth (μm)</td>
<td>96</td>
<td>86</td>
<td>87</td>
<td>15</td>
<td>0.222</td>
</tr>
<tr>
<td>Villus height/crypt depth</td>
<td>9a</td>
<td>12b</td>
<td>9a</td>
<td>2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Villus width (μm)</td>
<td>131</td>
<td>118</td>
<td>116</td>
<td>62</td>
<td>0.536</td>
</tr>
<tr>
<td>Villus surface area (μm²)</td>
<td>325 940</td>
<td>368 549</td>
<td>281 272</td>
<td>183 053</td>
<td>0.175</td>
</tr>
<tr>
<td>Mucosal thickness (μm)</td>
<td>47</td>
<td>36</td>
<td>39</td>
<td>16</td>
<td>0.043</td>
</tr>
<tr>
<td>Muscular layer thickness (μm)</td>
<td>173</td>
<td>152</td>
<td>177</td>
<td>39</td>
<td>0.144</td>
</tr>
</tbody>
</table>

Means (n=3, with five measurements per sample) with different superscripts in each row were significantly different (P < 0.01).

Discussion

The nutritional effects of sucrose-derived DFA-enriched products and biotechnologically produced individual DFAs have been previously investigated to some extent in laboratory animals (rat), but studies on production animals (pig, broiler chicken) are very scarce. Arribas et al. (2010) recently showed that the administration of a DFA-enriched caramel obtained from fructose (containing 70% of an isomeric mixture of 13 DFAs and glycosyl-DFAs, identical to FC in this work) to colitic rats promoted a more favourable intestinal microbiota, increasing lactobacilli and bifidobacteria \( \log_{10} \) number of copies, as well as inducing increased concentrations of SCFA in the luminal colonic contents. Previous results on the prebiotic potential of a caramel obtained by pyrolysis of sucrose in the presence of citric acid (Manley-Harris and Richards, 1997) containing \( \approx \)34% of DFAs and 43% of monosaccharides indicated that DFAs and their glycosylated derivatives might represent promising candidates as prebiotic agents (Orban et al., 1997). It is important to mention here that in the present study fructose-derived caramels were used. These were obtained through a different procedure that involves thermal activation at a much lower temperature (90°C) in the presence of an acid resin approved for its use in the food industry that is then removed from the final product by filtration. The content of DFAs and glycosyl-DFAs, assumed to be the active prebiotic components, in the caramels assayed in vitro amounts to 60–80%, being of \( \approx \)80% in the fructose caramel used for the in vivo evaluation.

The DFA-enriched caramels and the commercial sucrose caramel used here increased the numbers of potentially beneficial bacteria in vitro. Although numerous studies indicate that inulin and oligofructose selectively stimulate the health-promoting groups of the human intestinal microbiota (Candela et al., 2010), in vitro studies on oligosaccharides involving animal microbiota are scarce, and as far as we know there are no references using chicken microbiota. Caramels used here mostly resembled the effects obtained with raftilose. Thus, raftilose and most caramels tested increased lactobacilli, bifidobacteria, bacteroides and clostridia log number of copies as compared with the non-additive control diet, whereas coliforms and enterobacteria number of colonies were decreased by two of the caramels tested (i.e. FC and FSC) but not by raftilose. This effect is similar to those found with a number of substances with potential (isomalto-oligosaccharides, lactosucrose, xylo-oligosaccharides) or confirmed (inulin, transgalacto-oligosaccharides, lactulose) prebiotic effects in humans (Candela et al., 2010). Regarding the products of microbial metabolism, it is well established that the principal end products of bacterial fermentation processes are SCFA, with inulin and fructooligosaccharides (FOS) giving rise mainly to acetate (Candela et al., 2010). The results found with raftilose and caramels in our in vitro trial (lower pH values, and higher lactate and SCFA, mainly acetate, production) are similar to those reported by Tzortzis et al. (2005) using galacto-oligosaccharides and inulin.

Although in vitro results provide a preliminary indication, the final demonstration of a prebiotic effect of any food ingredient must be carried out in vivo (Roberfroid, 2007). In the current work, the addition to the diet of \( \alpha \)-fructose-derived caramel (FC, 2%) did not significantly affect lactobacilli or bifidobacteria number of copies, but led to lower enterobacteria and Escherichia-Shigella numbers in crop and caecal contents compared with controls. Compared with controls bacteroides number of copies increased controls in ileal contents but decreased in the caeca of chickens fed the FC diet. As for the inulin-fed birds, lower lactobacilli \( \log_{10} \) number of copies was determined in the crop, ileum and caeca compared with controls. Inulin supplementation also resulted in lower bacteroides and total bacteria in caecal contents. These differences in the effects found with inulin and DFAs are likely to be linked to their different intestinal digestibility. Surprisingly, although most of the research interest on prebiotics has focused on their role as modulators of the intestinal microbiota, little effort has been made on their intestinal digestibility, a major criterion to determine their prebiotic potential and mechanism of action (Roberfroid, 2007). However, given that DFAs are highly stable in acidic media and that they are inert to the action of mammalian intestinal glycosidases, it is likely that substantial amounts reach the final sections of the intestinal tract essentially unaltered (Manley-Harris and Richards, 1997). In the current work, ileal apparent digestibility of DFAs in birds fed FC diet was 81%, which means that about 19% of the amounts present in the diet would reach the caeca. The ileal digestibility of inulin was 14% at the ileal level and 85% at the faecal level, which indicates that higher amounts (about 86% of the inulin in the diet) reach the chickens’ caeca. These data suggest that the effectiveness of inulin and
caramels is mainly at the caecal and ileal levels, respectively. In fact, this higher digestibility of DFAs in the upper parts of the intestinal tract is likely to explain the lower pH in the crop of birds fed diets containing caramel with respect to control or inulin-fed chickens. In addition, lactobacilli number of copies was not different from controls in the upper gut of caramel-fed birds, but was affected in inulin-fed chickens.

The effectiveness of a given additive in broiler feeding depends on a variety of factors. Variables such as concentration, diet type, animal characteristics, hygiene and husbandry conditions, and environmental stress can influence the response to inulin or FOS in broiler feeding (Patterson and Burkholder, 2003). Dietary supplementation with chito-oligosaccharide (COS) improved the growth rate of broilers, which was likely mediated through the effects of COS on FI and nutrient digestibility. COS may also serve as a growth promoter in broiler production by modulating the concentrations of intestinal microbial flora, as this additive increased the concentrations of cecal lactobacilli and reduced the caecal concentrations of Escherichia coli (Li et al., 2007). In the current work, BW was greater than controls in animals fed the FC diet, and faecal energy, ADF, NDF and NSP apparent digestibilities were greater than controls in chickens fed diets containing inulin or FC. Fat faecal apparent digestibility was higher in FC-fed birds compared with controls or inulin-fed chickens. The mechanism by which NSP exert their anti-nutritive effects in poultry is usually linked to the increased bulk and viscosity of the intestinal contents, which decrease the rate of diffusion of substrates and digestive enzymes, thus hindering their effective interaction at the mucosal surface (Chot et al., 1996). The concentrations of soluble NSP in wheat were inversely correlated with their metabolizable energy (MEn)-values in broiler chickens (Annison, 1991). The viscous conditions in the small intestine may interfere with crude fat digestibility owing to draining of bile acids from enterohepatic circulation (Malayo et al., 2010). It has been suggested that an increased digesta viscosity may reduce the amounts of conjugated bile acids, affect fat emulsification negatively and thus decrease fat digestibility (Langhout et al., 1997). Therefore, a higher NSP digestibility as reported here would decrease NSP amounts within the intestine and consequently increase nutrients digestibility.

Although it has been reported that a high level of bird performance may be supported by a range of microbial compositions (Geier et al., 2009), and despite its scientific and practical relevance, the study on the relationship between variations of the microbiota composition and productive/physiological parameters is still at the beginning in poultry production. In a pioneering work, by using T-RFLP analysis together with multivariate statistical methods, Torok et al. (2011a) identified and characterized changes in gut microbiota development in chickens in response to enzyme or antimicrobial agents in feed that had performance implications. More specifically, an overgrowth of some microorganisms including enterobacteria in the intestine has been reported (Bourlinoux et al., 2003; Pelicano et al., 2005) to result in mucosal impairment, villus erosion and damage to the intestinal cells, thus reducing their nutrients’ absorptive potential. Fonseca et al. (2010) linked a decrease in the quantity of caecal enterobacteria to improved performance in broilers, and Kim et al. (2011) proved that lower numbers of certain gut pathogens such as E. coli may improve broiler performance. Peinado et al. (2012) recently showed that a garlic derivative lowered the intestinal numbers of enteropathogens and improved the ileal histological structure and productive parameters of broilers. On the other hand, in the current work, lactobacilli numbers were lower in the crop, ileum and caeca of chickens fed on the inulin diet and tended to be lower in the FC-fed chickens, which was accompanied by an increase in the bacteroides number of copies in the ileum. Although generally regarded as a beneficial group, higher numbers of lactobacilli have been implicated in broiler growth depression owing to competition for nutrient uptake or impaired fat absorption linked to deconjugation of bile acids (Torok et al., 2011b). Meanwhile, species from the Bacteroidetes family are involved in many important metabolic activities including fermentation of carbohydrates, induction of critical glycolytic enzymes in the enterocytes, utilization of nitrogenous substances, biotransformation of bile acids and prevention of pathogen colonization (Bry et al., 1996; Phillips, 2009). In summary, according to the literature and the current results, it could be concluded that a decrease in the numbers of some bacterial groups such as enterobacteria, and probably lactobacilli, accompanied by an increase in others such as bacteroides in the intestine might be related to improved performance in poultry. However, with the information at present available, it is not possible to rule out other mechanisms such as modifications in the microbiota metabolism, effects on bacterial groups other than those studied here, effects on the immune system, absorption of substances and so on.

Inulin-type fructans are regarded as prebiotics in various intestinal environments of diverse animal genera (mammal, fish, bird, etc.). The specific intestinal fermentation results in a bacterial ecosystem that is less prone to pathogen invasion and in an increased production of SCFA characterized by a higher proportion of butyrate. As a result, the increased absorptive capacity, reflected in a longer intestinal length and increased villus height or crypt depth of the intestine, results in improved feed conversion and better growth in the young animal. The only effect observed in the present work on the histological structure of the chickens’ mucosa was that inulin supplementation increased villus height and the ratio between villus height and crypt depth as compared with both control- and FC-fed birds, which is in keeping with previous reports (Rebolé et al., 2010).

In summary, the results presented in this study show that DFAs in DFA-enriched caramels resisted to some extent chickens’ small intestinal digestion in vivo, were fermented in vitro by birds’ caecal microbiota and selectively stimulated the growth of bacteria associated with health and well-being in vitro. FC supplementation also gave place to lower counts of potentially pathogenic bacteria in the broiler’s intestine.
in vivo. Faecal energy, fat, ADF, NDF and NSP apparent digestibilities were greater than controls in chickens fed diets containing DFA-enriched caramels.

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Supplementary material
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References

Di-o-fructose dianhydrides enriched caramels for broilers

