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ARTICLE

Di-D-fructose Dianhydride-Enriched Caramels: Effect on Colon Microbiota, Inflammation, and Tissue Damage in Trinitrobenzenesulfonic Acid-Induced Colitic Rats

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In the present study we describe the preparation and chemical characterization of a caramel with a high (70%) content of difructose dianhydrides (DFAs) and glycosylated derivatives (DFAs). This product was obtained by thermal activation (90 °C) of highly concentrated (90% w/v) aqueous D-fructose solutions using the sulfonic acid ion-exchange resin Lewatit S2328 as caramelization catalyst. DFAs represent a unique family of cyclic fructans with prebiotic properties already present in low proportions (<15%) in commercial caramel. We report the antiinflammatory activity of the new DFA-enriched caramel in the trinitrobenzenesulfonic acid (TNBS) model of rat colitis, an experimental model that resembles human inflammatory bowel disease (IBD), and compare its effects with those obtained with a commercial sucrose caramel and with linear fructooligosaccharides (FOS). For this purpose, the effects on colon tissue damage, gut microbiota, short-chain fatty acid (SCFAs) production, and different inflammatory markers were evaluated. The administration of DFA-enriched caramel to colitic rats showed intestinal antiinflammatory effect, as evidenced macroscopically by a significant reduction in the extent of the colonic damage induced by TNBS. This effect was similar to that obtained with FOS in the same experimental settings, whereas commercial caramel was devoid of any significant antiinflammatory effect. The beneficial effect was associated with the inhibition of the colonic levels of the proinflammatory cytokines, tumor necrosis factor α (TNF α) and interleukin 1β (IL-1β), and the reduction in colonic myeloperoxidase (MPO) activity and inducible nitric oxide synthase (iNOS) expression. The DFA-enriched caramel also promoted a more favorable intestinal microbiota, increasing lactobacilli and bifidobacteria counts as well as inducing higher concentrations of SCFAs in the luminal colonic contents. These results reinforce the concept of DFAs and glycosyl-DFAs as dietary beneficial compounds with prebiotic properties and suggest that the novel DFA-enriched caramel here reported may be an interesting candidate to be explored for the dietary treatment of human IBD.

KEYWORDS: Caramel; D-fructose; di-D-fructose dianhydrides; fructooligosaccharides; inflammatory bowel disease; TNBS rat colitis; prebiotic

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disorder associated with mild to severe inflammation and ulceration in the gastrointestinal tract. The two major IBD subtypes, namely, ulcerative colitis (UC) and Crohn's disease (CD), affect millions of individuals in the Western world (*1*). Although the etiology of IBD is not well understood, it is believed that genetic, immunologic, and environmental factors may be involved (*2*). There is also increasing evidence that an altered colonic microbial environment or an elevated host immune response to the normal luminal bacteria is a possible underlying factor in the pathogenesis of IBD (*3*).

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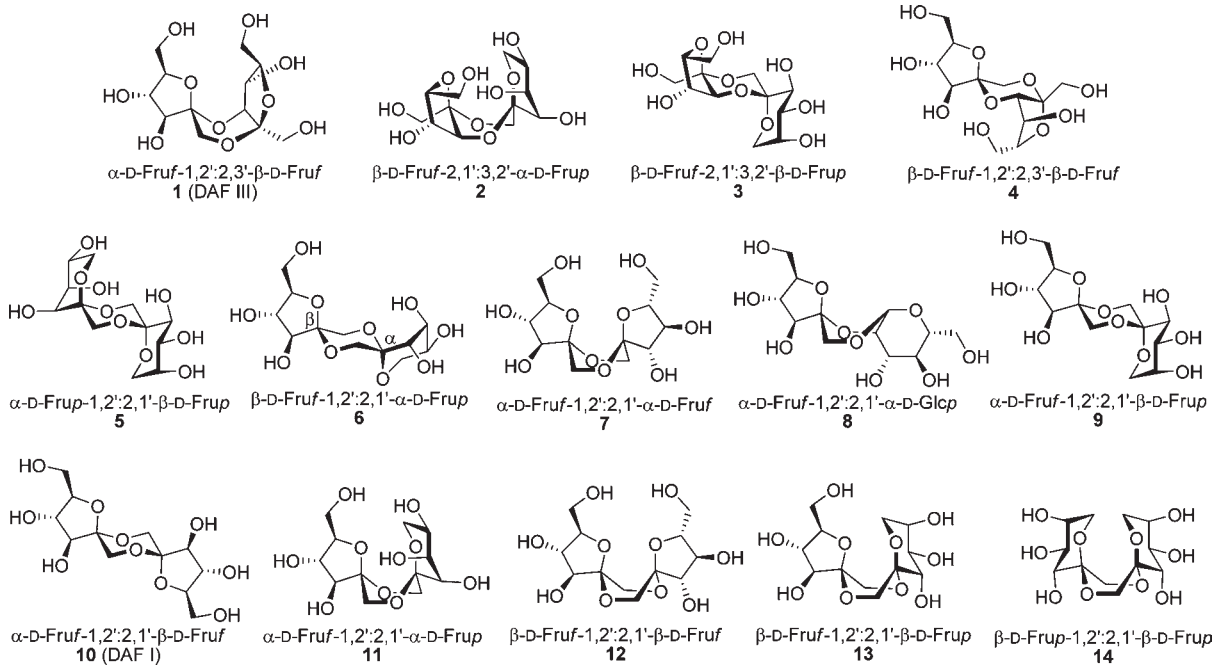

Figure 1. Chemical structures of the di-D-fructose dianhydrides (**1**−**7** and **9**−**14**) and the D-fructose−D-glucose mixed dianhydride (**8**) present in caramel.

At present, the drugs available for the treatment of IBD exhibit severe side effects and long-term toxicity, stressing the need for novel safe and effective therapeutic agents for this pathology. It has been suggested that dietary management, including the administration of prebiotics (*4*), particularly oligosaccharides, may be an attractive alternative to drug therapy of IBD (*5*). Prebiotic oligosaccharides are not sensitive to gastric acid, escape degradation and adsorption in the upper gastrointestinal tract, and reach the colon intact (*6*), where they promote the proliferation of beneficial bacteria such as *Bifidobacteria* and *Lactobacilli* (*7*) and have a positive effect in maintaining the intestine homeostasis (*8*, *9*). In addition, the fermentation of these carbohydrates produces short chain fatty acids (SCFA), mainly, acetate, propionate, and butyrate, which clearly contribute to their beneficial properties in a multifactorial manner (*10*).

Among carbohydrate prebiotics, fructose-based oligo- and polysaccharides, such as inulin, fructooligosaccharides (FOS), and lactulose, have been reported as promising candidates for IBD management (*11*−*13*). Difructose dianhydrides (DFAs) and their glycosylated derivatives (glycosyl-DFAs) (*14*) represent promising candidates to be incorporated to this list. From the structural point of view, DFAs can be considered as fructans with a cyclofructodisaccharide framework. They have been isolated from microorganisms and higher plants and can be also produced enzymatically or chemically from fructose or fructose-containing raw materials (*15*). The identification of DFAs and glycosyl-DFAs as the major components of the saccharide fraction in caramel (13 DFAs and a mixed D-fructose−D-glucose dianhydride have been identified in sucrose caramel; see **Figure 1**) has further raised an intense research on their nutritional behavior (*16*). Interestingly, some individual DFA representatives, such as α-D-fructofuranose β-D-fructofuranose 1,2′:2,3′-dianhydride (**1**, DAF III) have been shown to behave as prebiotics, promoting *Bifidobacteria* growth and mineral absorption in the small and large intestine of rats (*17*−*19*) and humans (*20*). Likewise, products containing DFAs and glycosylated (glucosylated or fructosylated) DFAs (up to 40% altogether), obtained by thermolysis of sucrose (*21*), inulin (*22*, *23*), or isomaltulose (6-*O*-α-D-glucopyranosyl-D-fructofuranose, palatinose) in the presence of citric acid (*24*) (**Figure 2**), were claimed to promote a beneficial microbiota.

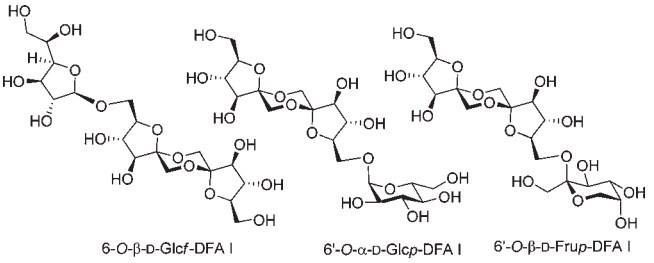

Figure 2. Chemical structures of some glycosyl-DFAs formed by pyrolysis of sucrose, inulin, or isomaltulose in the presence of citric acid. Glycosyl-DFAs with a higher degree of polymerization are formed by addition of successive units of D-glucose or D-fructose.

Recently, we developed a new technology to produce caramels with high DFA and glycosyl-DFA content (> 60%) based in the activation of D-fructose by strongly acidic ion-exchange resins (*25*). The aim of the present study was to demonstrate the suitability of the heterogeneous caramelization procedure for scaling up schemes and to ascertain if a DFA-enriched caramel prepared in this manner is able to exert intestinal antiinflammatory properties. For this purpose, the effect of a DFA-enriched caramel was evaluated, both histologically and biochemically, in the trinitrobenzenesulfonic acid (TNBS) model of rat colitis, and its effects were compared with those obtained after administration to colitic rats of either linear fructooligosaccharides (FOS) or a commercial sucrose aromatic caramel.

EXPERIMENTAL PROCEDURES

Materials. Pure standards of α-D-fructofuranose β-D-fructofuranose 1,2′:2,1′-dianhydride (*26*), di-α-D-fructofuranose 1,2′:2,1′-dianhydride (*27*), di-β-D-fructofuranose 1,2′:2,1′-dianhydride (*28*), α-D-fructopyranose β-D-fructopyranose 1,2′:2,1′-dianhydride (*37*), di-β-D-fructopyranose 1,2′:2,1′-dianhydride (*29*), α-D-fructofuranose β-D-fructopyranose 1,2′:2,1′-dianhydride (*37*), and β-D-fructofuranose β-D-fructopyranose 1,2′:2,1′-dianhydride (*30*) were prepared by boron trifluoride or trifluoromethanesulfonic acid (triflic acid) promoted spirocyclization of suitably protected D-fructose

precursors, column chromatography of the protected derivatives, and final deprotection of the individual DFAs after the reference indicated in each case. Their identity and purity (>98%) were confirmed by mycroanalysis, ^{1}H and ^{13}C NMR, gas chromatography (FID−GC; see Supporting Information), and melting point determination. α-D-Fructofuranose β-D-fructofuranose 1,2′:2,3′-dianhydride was a gift of the former Institute für Technologie der Kohlenhydrate e.V., Braunschweig, Germany, and was >99% pure as seen by NMR and GC (see Supporting Information). The ion-exchange resin Lewatit S2328 was obtained as a gift from LanXess (Barcelona, Spain). Before use, the resin was conditioned by sequential elution with water, 1 N aqueous HCl, and methanol and then dried (room temperature 90 °C) as previously reported (*25*). 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 (*31*), having the following technical characteristics: dry matter (° Brix) 79.2; pH (50% in demineralized water) 2.80; color (absorbance at 520 nm) 6.04. All other chemicals were obtained from Sigma Chemical (Madrid, Spain), unless otherwise stated. FOS (Beneo-95) used in the TNBS model of rat colitis was provided by Orafti (Tienen, Belgium).

Preparation of the DFA-Enriched Caramel. The caramelization reactions were carried out in an IKA MINIPLANT LR.2-ST modular system equipped with a EUROSTAR Power Control visc P7 with an anchor-shaped rod for mechanical stirring, a 2 L single-wall borosilicate vessel, an HBR 4 digital heating bath, and a PT 100 temperature probe (Mac Technologie, Gretz Armainvilliers, France). Before its use as caramelization promoter, the Lewatit S2328 ion-exchange resin (500 g) was stacked in a glass column (60 × 3 cm) and subsequently eluted (3 mL min^{-1}) with deionized water (2 L), 1 N aqueous HCl (2 L), and MeOH (1 L). Drying was effected first under air current at room temperature for 4 h and then in an oven at 90 °C for 16 h. The resin thus conditioned showed an exchange capacity of 5.39 mequiv of H^{+}/g of dry resin and was stored in a desiccator under P_2O_5. To a 90% (w/v) solution of D-fructose (300 g) in deionized water, the appropriate amount of freshly conditioned Lewatit S2328 ion-exchange resin (30 g, 10% w/v) was added. The solution was mechanically stirred (25 rpm) at 90 °C for 2 h. Ice-cold water (1 L) was then added, the resin was separated by filtration followed by centrifugation at 7000 rpm for 30 min, and the resulting clean solution was concentrated at 40 °C under reduced pressure. The caramel thus obtained had the following technical characteristics: dry matter (° Brix) 88.0; pH (50% in demineralized water) 2.87; color (absorbance at 520 nm) 1.50.

GC−FID Analysis of Difructose Dianhydrides in Caramel Samples. For GC analysis, freeze-dried caramel samples as well as samples of the pure DFA standards used for identification were transformed into their corresponding per-*O*-trimethylsilyl (TMS; nonreducing sugars) or per-O-trimethylsilylated oxime (TMS-oximes; reducing sugars) derivatives as previously reported (*32*). Typically, to 15−20 mg of each sample was added deionized water (1 mL). To 100 μL of the resulting solution was then added 100 μL of internal standard (4 mg mL^{-1} phenyl β-D-glucopyranoside in acetone−water, 1:9 v/v), and the final solution was evaporated to dryness at 60 °C (drying oven). The residue was treated with 1 mL of a solution of hydroxylamine in pyridine (20 mg mL^{-1}) at 60 °C over 50 min, with mixing at intervals. Neat hexamethyldisilazane (200 μL) and trimethylchlorosilane (100 μL) were then added, and the reaction mixtures were kept at 60 °C over a further 40 min period. Formation of a white precipitate was observed during this operation, which was separated by centrifugation (13000 rpm, 5 min) before injection in the GC apparatus.

GC−FID was carried out using an Agilent 6890 Series Plus chromatograph with an EPC injector fitted with a cross-linked 5% phenyldimethylsiloxane column (HP-5; 30 m × 320 μm × 0.25 μm). Operating conditions were as follows: injection port temperature 310 °C; splitting ratio 25:1; injection volume 1 μL of derivatized samples; column oven temperature programmed from 180 to 310 °C at 5 °C min^{-1}, with a 25 min hold at 310 °C; carrier gas helium (constant flow at 1.2 mL min^{-1}); detector port temperature 310 °C. Total acquisition time was 56 min. Response factors (RFs) for D-fructose (1.42), D-glucose (1.11), and eight individual DFAs (α-D-fructofuranose β-D-fructofuranose 1,2′:2,1′-dianhydride (0.75); di-α-D-fructofuranose 1,2′:2,1′-dianhydride (0.68); di-β-D-fructofuranose 1,2′:2,1′-dianhydride (1.15); α-D-fructopyranose β-D-fructopyranose 1,2′:2,1′-dianhydride (0.76); di-β-D-fructopyranose 1,2′:2,1′-dianhydride (0.85); α-D-fructofuranose β-D-fructopyranose 1,2′:2,1′-dianhydride (0.68); β-D-fructofuranose β-D-fructopyranose 1,2′:2,1′-dianhydride (0.78); α-D-fructofuranose β-D-fructofuranose 1,2′:2,3′-dianhydride (0.89)), at concentrations similar to those encountered in the experiments, were determined relative to the internal standard phenyl β-D-glucopyranoside and used for quantification of their relative proportion in caramels. The average of the RF values for the eight DFAs available in pure form (0.78) was applied to the rest of DFA components in the mixtures. See **Figure 3** for the chromatographic profiles (DFA region) of the DFA-enriched caramel and commercial sucrose caramel used in this study.

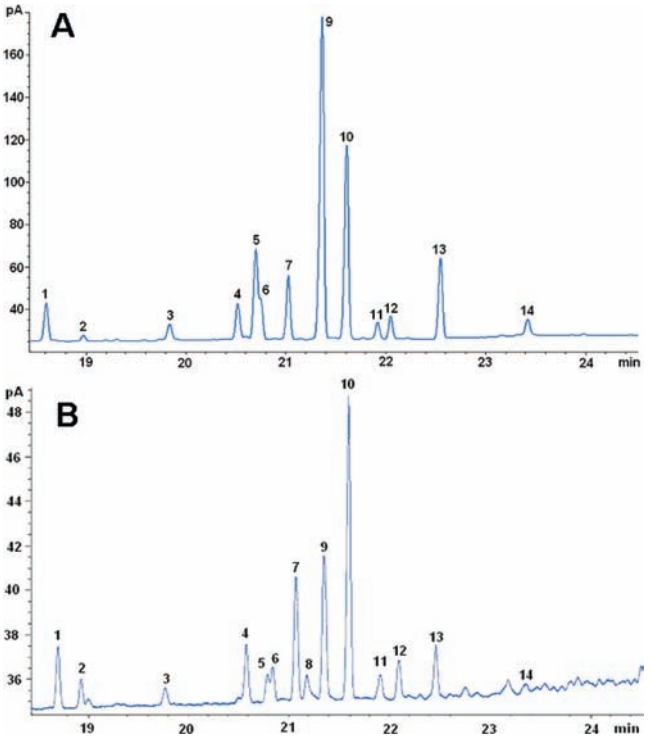

Figure 3. FID−GC profiles of the DFA-enriched caramel (**A**) and the commercial sucrose caramel (**B**) used in this study (DFA region).

Determination of Glycosyl-DFAs in Caramels by Acetylation and Column Chromatography. Caramel samples were acetylated by treatment with Ac_2O−pyridine (1:1, 10 mL/g of caramel) at room temperature for 16 h. The crude acetylated product was dropped into ice−water (100 mL/g of starting caramel) and extracted with CH_2Cl_2 (2 × 50 mL); the organic phase was washed successively with 1 N H_2SO_4 (2 × 50 mL), saturated aqueous $NaHCO_3$ (2 × 50 mL), and water (2 × 50 mL), dried by addition of anhydrous sodium sulfate (2 g), filtered, and finally concentrated. The absence of organic material in the aqueous phase was checked during the separation and washing processes by thin-layer chromatography (TLC; aluminum plates, silica gel 60 F_{254}, E. Merck, 0.25 mm, eluent EtOAc−petroleum ether, 2:1). The acetylation mixture (1 g) was subjected to column chromatography (glass column, 56.5 × 2.5 cm) with silica gel 60 (E. Merck, 230−400 mesh, 30 g) by eluting with a gradient of EtOAc−petroleum ether, 1:1 (200 mL) → 2:1 (200 mL) → 4:1 (200 mL) → neat AcOEt (400 mL). Fractions (10 mL) were collected and checked by TLC (EtOAc−petroleum ether, 2:1) as well as by ESI-MS. Fractions containing glycosyl-DFAs and higher oligosaccharides were further combined separately and concentrated for quantification.

Animals and Diets. Female Wistar rats (180−200 g) were obtained from the Laboratory Animal Service of the University of Granada (Granada, Spain) and maintained in specific pathogen-free conditions. They had free access of water and standard rodent food (Panlab A04 diet) provided by Panlab S.A. (Barcelona, Spain). This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals, as promulgated by the National Institutes of Health, and the care and treatment protocols were approved by the Institutional Animal Care and Use Committee at the University of Granada (Granada, Spain).

Experimental Design. The rats were randomly assigned to five groups: two control groups without treatment (n=10) and three treated groups (n=10) which were given the DFA-enriched caramel, fructooligosaccharides, or the

Table 1. Scoring Criteria of Full-Thickness Distal Colon Sections[a]

mucosal epithelium and lamina propria
ulceration: none (0); mild surface (0−25%) (1); moderate (25−50%) (2); severe (50−75%) (3); extensive−full thickness (more than 75%) (4)
polymorphonuclear cell infiltrate
mononuclear cell infiltrate and fibrosis
edema and dilation of lacteals
crypts
mitotic activity: lower third (0); mild mid third (1); moderate mid third (2); upper third (3)
dilations
goblet cell depletion
submucosa
polymorphonuclear cell infiltrate
mononuclear cell infiltrate
edema
vascularity
muscular layer
polymorphonuclear cell infiltrate
mononuclear cell infiltrate
edema
infiltration in the serosa

[a] Scoring scale: 0, none; 1, slight; 2, mild; 3, moderate; 4, severe. Maximum score: 59.

commercial sucrose caramel, incorporated in the drinking water and prepared daily at the concentration of 100 mg mL^{-1}, being the average rat intake approximately of 1 g/day (the mean water intake was 9.8 ± 0.7 mL per rat per day, without showing differences among groups). This dose is equivalent to that used in previous studies with other prebiotics in the same experimental model of rat colitis (*11*, *12*). Two weeks after starting the experiment, 10 rats from the control group and all treated groups were fasted overnight and were rendered colitic by the method described previously (*13*). Briefly, they were anaesthetized with halothane and given 10 mg of TNBS dissolved in 0.25 mL of 50% ethanol (v/v) by means of a Teflon cannula inserted 8 cm through the anus. Rats from the noncolitic group were administered intracolonically 0.25 mL of phosphate-buffered saline instead of TNBS. After colitis induction, rats were maintained on the same test compound intake for an additional 1 week and then killed with an overdose of halothane. Food and water intake, as well as weight evolution, was recorded daily throughout the treatment period.

Sampling Procedure and Evaluation of the Macroscopic Damage. Once the rats were sacrificed, the colon was removed aseptically, placed on an ice-cold plate, and longitudinally opened, and the luminal contents were collected for the measurements of microbiological concentrations and short chain fatty acid (SCFA) production. Afterward, the colonic segment was cleaned of fat and mesentery and blotted on filter paper; each specimen was weighed and its length measured under a constant load (2 g). The colon was scored for macroscopically visible damage on a 0−10 scale by two observers unaware of the treatment, according to the criteria previously reported (*13*), which takes into account the extent as well as the severity of colonic damage. The colon was subsequently divided into different segments and stored at −80 °C until their use for biochemical determinations. All biochemical measurements were completed within 1 week from the time of sample collection and were performed in duplicate.

Histological Analyses. Representative whole gut specimens were taken from a region of the inflamed colon corresponding to the adjacent segment to the gross macroscopic damage and were fixed in 4% buffered formaldehyde. Cross sections were selected and embedded in paraffin. Equivalent colonic segments were also obtained from the noncolitic group. Four to five full-thickness sections of 5 μm were taken at different levels and stained with hematoxylin and eosin. The histological damage was evaluated on a 0−59 scale by a pathologist observer, who was blinded to the experimental groups, according to the criteria described in **Table 1**.

Myeloperoxidase Activity Determination. MPO activity was measured according to the technique described by Krawisz et al. (*33*); the results were expressed as MPO units/g of wet tissue; one unit of MPO activity was defined as that degrading 1 μmol of hydrogen peroxide/min at 25 °C.

Glutathione Content Determination. Total glutathione (GSH) content was quantified with the recycling assay described by Anderson (*34*), and the results were expressed as nanomoles per gram of wet tissue.

Tumor Necrosis Factor α and Interleukin 1β Protein Assays. Colonic samples for TNFα and IL-1β determinations were immediately weighed, minced on an ice-cold plate, and suspended (1:5 w/v) in a lysis buffer containing 20 mM HEPES (pH 7.5), 10 mM ethylene glycol bis-(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 40 mM β-glycerophosphate, 2.5 mM magnesium chloride, 1% Igepal, 1 mM dithiothreitol, 500 μM phenylmethanesulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL iodoacetamide, and 2 mM sodium orthovanadate. The tubes were placed in an orbital rotor (4 °C) for 20 min and centrifuged at 9000*g* for 10 min at 4 °C; the supernatants were frozen at −80 °C until assay. TNFα and IL-1β were quantified by enzyme-linked immunoabsorbent assay (R&D Systems Europe, Abingdom, U.K.), and the results were expressed as picograms per gram of wet tissue.

Inducible Nitric Oxide Synthase Analysis. The colonic expression of iNOS was analyzed by Western blotting as previously described (*13*). The dilution of the primary antibody was 1:2000 (Transduction Laboratories, Becton Dickinson Biosciences, Madrid, Spain) and was incubated overnight at 4 °C followed by peroxidase-conjugated anti-rabbit IgG antibody (1:3000) for 1 h. Control of protein loading and transfer was conducted by detection of the α-actin concentrations.

Microbiota Analysis. The microbiological studies were performed on the luminal content samples, which were weighed, homogenized, and serially diluted in sterile peptone water. Serial 10-fold dilutions of homogenates were plated on specific media for *Lactobacillus* (MRS media, Oxoid) or *Bifidobacterium* (MRS media supplemented with 0.5 mg/L dicloxacilin, 1 g L^{-1} LiCl, and 0.5 g L^{-1} L-cysteine hydrochloride) and incubated under anaerobic conditions in an anaerobic chamber for 24−48 h at 37 °C. In addition, enterobacteria were also determined by using specific Count Plates Petrifilm (3M, St. Paul, MN). After incubation, the final count of colonies was reported as log colony-forming units (CFU) per gram of material.

Short Chain Fatty Acid Determinations. To quantify the SCFA concentrations in the colonic luminal contents, the samples were homogenized with 150 mM $NaHCO_3$ (pH 7.8) (1:5 w/v) in an argon atmosphere. Samples were incubated for 24 h at 37 °C and stored at −80 °C until the extraction. To extract the SCFAs, 50 μL of the internal standard 2-methylvaleric acid (100 mM), 10 μL of sulfuric acid, and 0.3 mL of chloroform were added to 1 mL of the homogenate and, then, centrifuged at 10000*g* for 5 min at 4 °C. The supernatants were dried with sodium anhydrous sulfate and centrifuged at 10000*g* for 5 min at 4 °C. Later, 1 μL of the supernatant was split inoculated (1:33) into a gas chromatograph (Perkin-Elmer Autosystem GC-FID) equipped with a capillary column (CPWAX 52CB 60 m × 0.25 mm, 0,25 μm; Varian). Nitrogen was used as the carrier and the makeup gas, with a flow rate of 1 mL/min. The injection temperature was 250 °C. Acetate, propionate, and butyrate concentrations were automatically calculated from the areas of peaks using the Star Chromatography WorkStation program (version 6, Varian), which was online connected to the GC−FID chromatograph.

Statistical Analysis. All results are expressed as the mean ± SEM. Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) and post hoc least significance tests. Nonparametric data (score) are expressed as the median (range) and were analyzed using the Mann−Whitney *U*-test. All statistical analyses were carried out with the Statgraphics 5.0 software package (STSC, Maryland), with statistical significance set at $P < 0.05$.

RESULTS AND DISCUSSION

The discovery of DFAs in caramel and the accumulated evidence on the prebiotic properties of some individual DFA representatives raise the question whether or not caramel itself might exhibit prebiotic properties (*35*). On the other hand, if DFAs and their glycosylated derivatives, the glycosyl-DFAs, are the active prebiotic species, increasing their relative abundance in caramel might have further beneficial effects. It must be noted that DFAs in caramel embody a complex family of up to 14 diastereomers (**Figure 1**), of which only compound **1** (DFA III), industrially available since 2004 (*36*), has been thoroughly

investigated for its nutritional behavior (*17*−*20*). The fact that products containing up to 40% of this DFA mixture, generated by pyrolysis of sucrose or inulin (*21*−*23*, *37*), have shown a quite significant prebiotic potential seems to point to the presence of more active prebiotic DFA components or the existence of favorable synergic effects. To check this hypothesis, we have prepared a DFA-enriched caramel containing up to 70% of the ensemble of DFAs, glycosyl-DFAs, and higher fructooligosaccarides by acid resin-promoted caramelization of D-fructose. This product has been evaluated in the TNBS model of rat colitis, and their effects were compared with those obtained with a commercial sucrose caramel. In addition, commercial FOS has been included in our study as a control.

Chemical Characterization of Caramels. In a previous work (*25*), a thorough investigation of the kinetics of the Lewatit S2328 ion-exchange resin-promoted caramelization reaction of D-fructose (10−50 g) as a function of the initial concentration of the monosaccharide, catalyst proportion, and temperature was reported. It was concluded that high (80−95% w/v) D-fructose concentrations, 10−20% (w/w) catalyst proportions, 80−90 °C, and relatively short (< 3 h) reaction times represented optimal condition in view of efficient conversions into DFAs, glycosyl-DFAs, and higher fructooligosaccharides (> 60% altogether) while keeping the proportion of 5-hydroxymethyl-2-furaldehyde (HMF), the main volatile resulting from fructose dehydration (*38*), in the final product below 2%. For scaling up purposes, we have now used 90% (w/v) D-fructose solutions in deionized water, 10% (w/w) D-fructose:acid resin ratio, 90 °C, and 2 h reaction time. Caramelization was effected in a laboratory reactor system in the 100−500 g scale with strict control of temperature and mechanical stirring conditions, leading consistently to 70 ± 3% conversions of the starting sugar into DFAs (32 ± 1%), glycosyl-DFAs, and higher fructooligosaccharides (38 ± 2%), with 26 ± 3% remaining D-fructose.

The commercial soft caramel used in our study was obtained from the treatment of sucrose at 160 °C in the presence of 0.1% AcOH. Caramelization of sucrose involves hydrolysis of the disaccharide into the constitutive monosaccharides as the first step. It has been shown that the D-fructose moiety then undergoes dimerization to give DFAs as a main reaction pathway, though the formation of a small proportion of higher fructooligosaccharides cannot be discarded (*39*). Thermal activation of D-glucose, on its side, leads to reversion (self-glycosylation) reactions to produce highly branched glucooligosaccharides. Alternatively, D-fructose and D-glucose units can glycosylate in situ the DFAs, leading to glycosyl- (fructosyl- and glucosyl-) DFAs. The dry matter composition of the different fractions, determined following the protocol previously reported (*16*), afforded 17 ± 2% D-fructose, 27 ± 3% D-glucose, 14 ± 3% DFAs, and 36 ± 3% for the ensemble of glycosyl-DFAs, reversion glucooligosaccharides, and higher fructooligosaccharides.

Relative Abundance of Individual DFA Diastereomers in DFA-Enriched and Commercial Caramel. Gas chromatography analysis of derivatized samples (see Experimental Procedures) evidenced significant variations in the relative abundance of the 14 DFA diastereomers between the commercial sucrose caramel and the DFA-enriched product obtained by acid resin-promoted caramelization of D-fructose. Thus, while difructofuranose compounds are predominant in the sucrose caramel, with the α-D-fructofuranose β-D-fructofuranose 1,2′:2,1′-dianhydride (**10**, DFA I) as the major representative, frutopyranose-containing isomers became the most abundant components in the case of the DFA-enriched caramel obtained from D-fructose, the α-D-fructofuranose β-D-fructofuranose 1,2′:2,1′-dianhydride (**9**) being the prevailing structure (**Figure 4**). This is consistent with a displacement toward a thermodynamic distribution in the acid resin-catalyzed caramelization reaction as compared with classical thermal caramelization under homogeneous conditions (*15*).

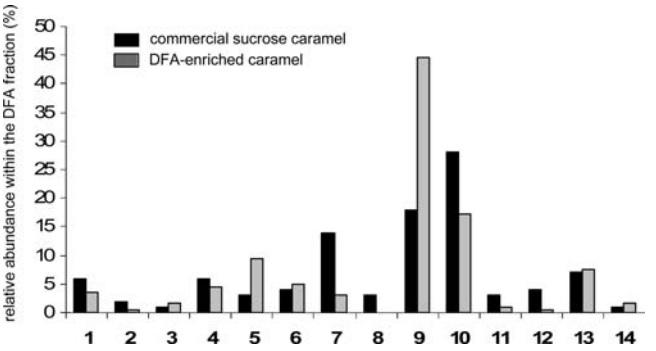

Figure 4. Relative proportions of the individual DFA isomers in the commercial sucrose caramel used in our study (total DFA 14%) and the DFA-enriched caramel obtained by caramelization of 90% (w/v) D-fructose in deionized water at 90 °C in the presence of Lewatit S2328 (H^+) resin (10% w/w) during 2 h (total DFA 32%; total glycosyl-DFA content 38%). Note that the D-fructose−D-glucose mixed dianhydride **8** is absent in the second one.

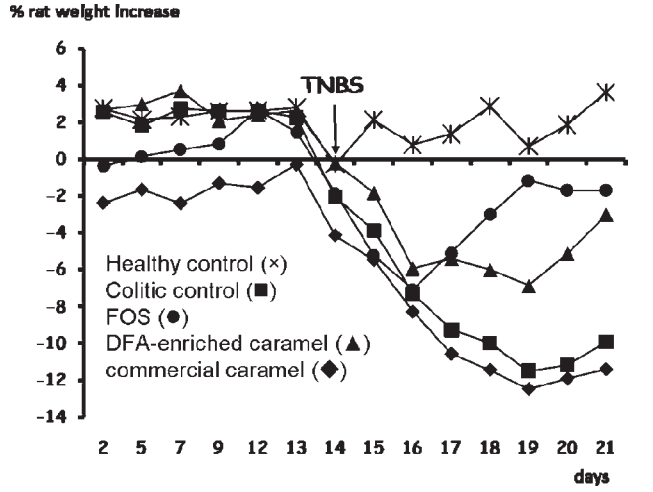

Figure 5. Effects of fructooligosaccharides (FOS) (1 g/day), DFA-enriched caramel (1 g/day), or commercial caramel (1 g/day), administered for 2 weeks before colitis induction and 1 week thereafter, on rat weight increase evolution.

DFA-Enriched Caramel Supplementation Ameliorates TNBS-Induced Colonic Damage. The pretreatment of colitic rats with the DFA-enriched caramel resulted in the amelioration of the inflammatory process induced by TNBS when compared to the untreated control group as well as to the commercial sucrose caramel-treated group, showing efficacy similar to that shown after FOS administration to colitic rats. The beneficial effects were already evidenced in the course of the treatment after colitis induction, since the groups of colitic rats treated with the DFA-enriched caramel or FOS showed a faster weight recovery than the other two colitic groups (**Figure 5**). Once rats were sacrificed, the antiinflammatory effects exerted by the DFA-enriched caramel or by FOS were evidenced macroscopically by a significantly lower colonic damage score compared to control rats ($P < 0.05$; **Table 2**), corresponding to a significant reduction in the extent of colonic necrosis and/or inflammation induced by the administration of TNBS/ethanol. In addition, these treatments were able to reduce the colonic weight/length

Table 2. Preventive Effects of Fructooligosaccharides (FOS) (1 g/day), DFA-Enriched Caramel (1 g/day), or Commercial Caramel (1 g/day) on Colonic Macroscopic Damage Score, Weight/Length Ratio, Myeloperoxidase (MPO) Activity, and Total Glutathione (GSH) Content in TNBS Experimental Colitis in Rats[a]

group (n = 10)	damage score (0−10)	weight/length (mg/cm)	MPO activity (units/g)	GSH content (nmol/g)
noncolitic	0	69.5 ± 1.9 a	6.3 ± 0.9 a	902.6 ± 115.2 a
TNBS control	8 (7−9) a	187.3 ± 16.5 b	326.1 ± 48.2 b	211.3 ± 41.1 b
FOS	6 (5−8) b	139.2 ± 12.5 c	217.4 ± 37.1 c	355.1 ± 39.8 c
DFA-enriched caramel	6 (5−8) b	140.1 ± 6.2 c	187.0 ± 31.1 c	496.6 ± 117.0 c
commercial caramel	8 (6−9) a	173.8 ± 10.3 b	244.1 ± 25.4 b,c	232.1 ± 43 b

[a] Damage score for each rat was assigned according to the criteria previously described (*13*), and data are expressed as median (range); medians in the column without a common letter differ, $P < 0.01$ (Mann−Whitney U-test). Colon weight/length, MPO activity, and GSH content data are expressed as mean ± SEM; means in a column without a common letter differ, $P < 0.05$ (Bonferroni t-test).

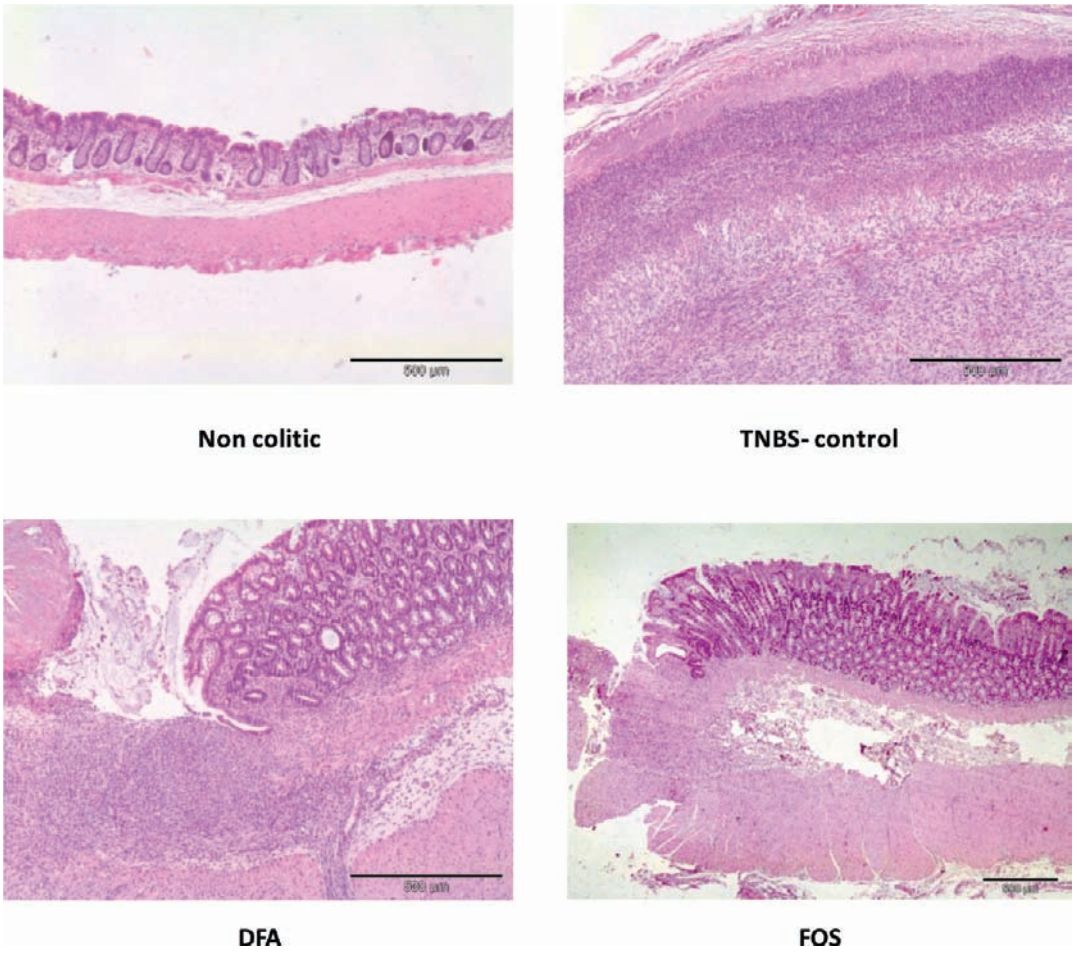

Figure 6. Histological sections of colonic mucosa from colitic rats 1 week after TNBS instillation stained with hematoxylin and eosin. The noncolitic shows the normal histology of the rat colon. The TNBS control shows complete destruction of the mucosa, which has been substituted by inflammatory granulation tissue; there is evident edema and intense diffuse transmural inflammatory infiltrate. DFA-enriched caramel (1 g/day) and FOS (1 g/day) treated groups show amelioration in the inflammatory process and "restoration" of the mucosal tissue with the presence of mucin-replenished goblet cells.

ratio, which was significantly increased due to the inflammatory process (**Table 2**).

The histological analysis confirmed the intestinal antiinflammatory effect exerted by the novel caramel (**Figure 6**). Colonic samples from the TNBS control group revealed extensive ulceration and inflammation involving all of the intestinal layers, giving a score value of 29 (24−34) expressed as median (range). These segments were characterized by diffuse leukocyte infiltration, mainly composed of neutrophils, in the mucosal layer, which was accompanied by important necrosis of epithelial cells. In fact, most of the rats showed epithelial ulceration of the mucosa affecting between 50% and 75% of the surface. The inflammatory process was associated with crypt hyperplasia and dilation and with goblet cell depletion. In the case of the colonic specimens from colitic rats treated with commercial sucrose caramel, the histological analysis revealed an insignificant recovery of the colonic tissue damage. Thus, although a moderate reduction in the extension of the ulcerated area was evidenced, it remained always higher than 50%, showing mean score values of 26 (16−27). A slight decrease in neutrophil infiltration was also noted in these animals. In contrast, the samples obtained from colitic rats treated with DFA-enriched caramel revealed a pronounced recovery in the intestinal architecture as compared with controls, with a score value of 20 (18−21) ($P < 0.05$ versus TNBS control group). Most of the samples showed a partial restoration of the epithelial cell layer, epithelial ulceration of the mucosa typically affecting less that 50% of the surface. The goblet cell depletion was also less severe. Actually, they seemed replenished

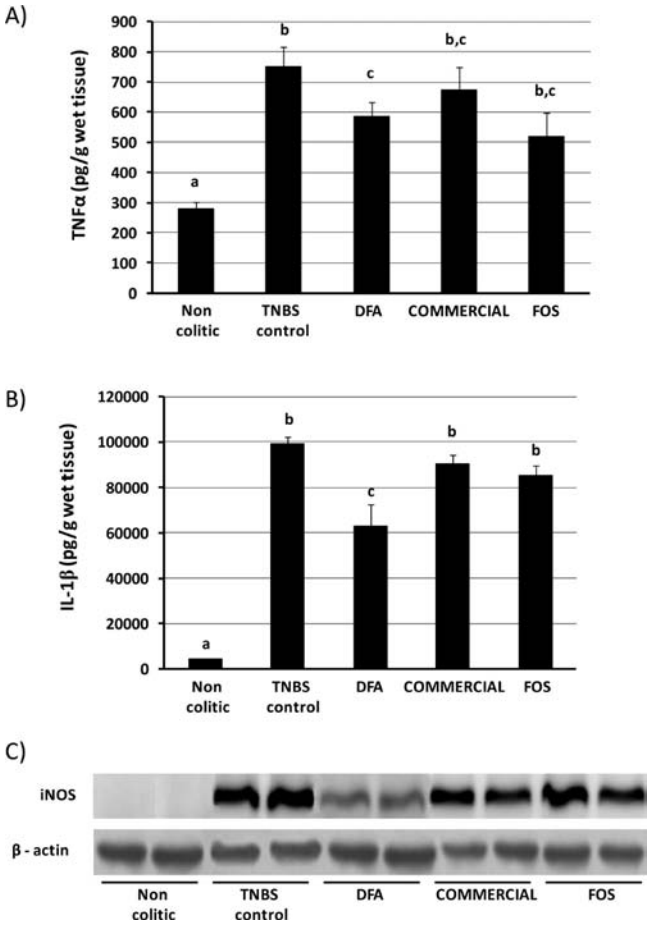

Figure 7. Preventive effects of fructooligosaccharides (FOS) (1 g/day), DFA-enriched caramel (1 g/day), or commercial caramel (1 g/day), in TNBS colitis in rats, on the production of TNFα (**A**) and IL-1β (**B**) quantified by ELISA (n = 10) (means ± SEM) and on colonic iNOS expression (**C**) examined by Western blot. Data are expressed as mean ± SEM. Superscripts with different letters differ: $P < 0.05$.

with their mucin content, and no dilated crypts were observed. The improvement in colonic histology was accompanied by a reduction in the inflammatory infiltrate mainly in the submucosa, although neutrophils were also the predominant cell type. The colonic specimens from colitic rats treated with FOS showed characteristics similar to those described for the DFA-enriched caramel-treated group (**Figure 6**). These samples were assigned a mean score value of 18 (13−22), which was significantly lower than in TNBS-control rats ($P < 0.05$).

DFA-Enriched Caramel Supplementation Improves the Biochemical Inflammatory Markers in the Inflamed Colon. Biochemically, the colonic inflammation induced by TNBS was characterized by higher MPO activity, as a marker of neutrophil infiltration to the inflamed tissue (*34*), and reduced GSH content, which is indicative of the oxidative insult that occurs in these intestinal conditions (*13*) (**Table 2**). In addition, increased concentrations of the proinflammatory cytokines TNFα and IL-1β (*40*,*41*) (**Figure 7**), as well as enhanced iNOS expression, which has been associated with the deleterious effects that NO overproduction has on intestinal function (*42*), were observed in the colonic specimens from TNBS control rats in comparison with noncolitic animals (**Figure 7**). Supplementation with DFA-enriched caramel resulted in a significant reduction in the colonic MPO activity and a partial recovery in GSH depletion, together with a significant decrease in TNFα and IL-1β production (**Figure 7**) and lower iNOS expression when compared with control colitic rats (**Figure 7**). These beneficial effects were not observed in the case of the commercial sucrose caramel, which did not show any significant modification on these inflammatory markers, strongly supporting the hypothesis that high proportions of DFAs and oligosaccharides thereof are necessary to elicit the antiinflammatory activity (**Table 2**, **Figure 7**). Finally, FOS administration also resulted in the improvement in all of these biochemical markers, except for iNOS expression, showing an efficacy similar to that observed in DFA-enriched caramel-treated colitic rats (**Table 2** and **Figure 7**).

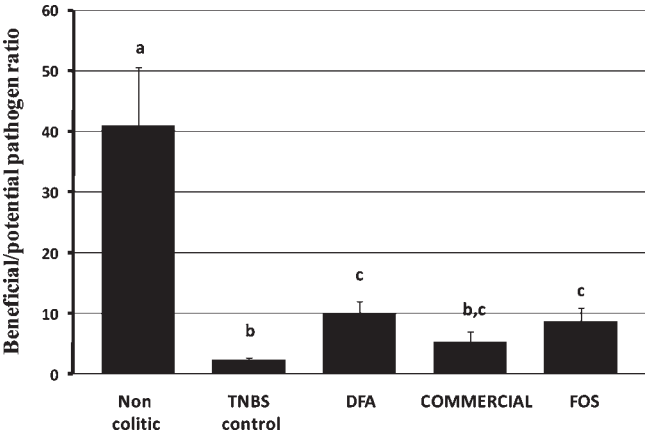

Figure 8. Effects of fructooligosaccharides (FOS) (1 g/day), DFA-enriched caramel (1 g/day), or commercial caramel (1 g/day), in TNBS rat colitis, on beneficial/potential pathogen bacteria ratio in the colonic luminal contents. Data are expressed as mean ± SEM. Superscripts with different letters differ: $P < 0.05$.

The Intestinal Antiinflammatory Effects of DFA-Enriched Caramel Supplementation Are Associated with a Prebiotic Effect. The TNBS model of rat colitis is an experimental condition characterized by an altered intestinal immune response associated with a modification in the colonic microbiota (*43*) and SCFA production (*12*). These observations have been confirmed in the present study. Thus, the intracolonic administration of TNBS resulted in a significant reduction in both lactobacilli and bifidiobacteria concentrations in the colonic contents when compared to healthy rats, together with an increase in enterobacteria, thus reducing the ratio between beneficial and potential pathogen bacteria (**Figure 8**). In addition, when the colonic contents from TNBS control rats were incubated for 24 h, reductions in the levels of SCFA (acetate, propionate, and butyrate) were observed compared with noncolitic rats ($P < 0.05$, **Table 3**). The intestinal contents obtained from the colitic rats treated with DFA-enriched caramel or with FOS showed a significant increase in the ratio between beneficial and potential pathogen bacteria (**Figure 8**) as well as higher SCFA production by the colonic contents (**Table 3**) when compared with control colitic rats. In stark contrast, no significant modifications were observed in the microbiota counts or in SCFA content in colitic rats treated with commercial caramel.

In conclusion, the novel DFA-enriched caramel exerts similar prebiotic effects to that shown by FOS, a well-characterized prebiotic, and these effects clearly contribute to its intestinal antiinflammatory activity in the TNBS model of rat colitis. While a high proportion of DFAs and glycosylated DFAs seems to be a prerequisite for a beneficial action (*44*), it is still uncertain whether the observed biological activities are ascribable to particular components or to the combined action of some of them. The potential

Table 3. Effects of Fructooligosaccharides (FOS) (1 g/day), DFA-Enriched Caramel (1 g/day), or Commercial Caramel (1 g/day) on SCFA (Acetate, Propionate, and Butyrate) Production in the Colonic Contents in TNBS Experimental Colitis in Rats[a]

group (n = 10)	acetate (μmol/g content)	propionate (μmol/g content)	butyrate (μmol/g content)	total (μmol/g content)
noncolitic	149.4 ± 21.4 a	66.2 ± 6.6 a	36.9 ± 3.0 a	250.6 ± 29.3 a
TNBS control	64.7 ± 15.6 b	36.0 ± 4.2 b	15.6 ± 1.9 b	116.2 ± 21.5 b
FOS	120.9 ± 15.0 a	40.8 ± 5.6 b	15.5 ± 1.5 b	177.2 ± 18.8 c
DFA-enriched caramel	126.2 ± 23.0 a	48.9 ± 10.6 a,b	24.8 ± 3.7 a	200.6 ± 36.8 a,c
commercial caramel	98.3 ± 8.4 a,b	80.3 ± 8.6 a	19.1 ± 4.7 a,b	155.7 ± 29.8 b,c

[a] Data are expressed as mean ± SEM. Superscripts with different letters differ: $P < 0.05$.

role of the remaining D-fructose, the volatiles resulting from intramolecular dehydration reactions during caramelization, or the melanoidines responsible for the color also deserve to be investigated. Further studies are also required to clearly characterize the beneficial properties that this novel prebiotic may have on human health. Research in that direction is currently sought in our laboratories.

Supporting Information Available: GC profiles of the synthetic DFA standards used for calibration of the analytical method. This material is available free of charge via the Internet at http://pubs.acs.org.

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