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Hypolipidemic Effect in Cholesterol-Fed Rats of a Soluble Fiber-Rich Product Obtained from Cocoa Husks

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A new soluble cocoa fiber product (SCFP), obtained after enzymatic treatment of cocoa husks, was characterized and its potential health effects studied in an animal model of dietary-induced hypercholesterolemia. The SCFP was rich in soluble dietary fiber (DF) and antioxidant polyphenols. Consumption of a cholesterol-rich diet containing the SCFP as a source of DF resulted in lower food intake and body weight gain in comparison with control groups consuming cholesterol-free or cholesterol-rich diets with cellulose as DF. The cholesterol-rich diet caused remarkable hypercholesterolemia. However, the SCFP diminished the negative impact of the cholesterol-rich diet, buffering the decrease of high density lipoprotein-cholesterol, and the increase of total and low density lipoprotein-cholesterol levels, and lipid peroxidation (malondialdehyde levels) induced by the fatty diet. The SCFP also decreased triglyceride levels to values lower than those in the group fed the cholesterol-free diet. These results put forward the potential application of the SCFP as a dietary supplement or functional food ingredient.

KEYWORDS: Soluble cocoa fiber; polyphenols; hypercholesterolemia; lipid peroxidation; body weight

INTRODUCTION

Advances over the last decade in the study of the relationship between diet and health have established that certain dietary patterns promote health, while others increase the risk of chronic diseases such as cardiovascular disease (CVD) or some types of cancer. A healthy diet is rich in dietary fiber (DF), low in saturated and trans fats and cholesterol, with relatively high omega-3 fatty acids; in other words, a diet rich in fruits and vegetables, legumes, whole grains, and fish (*1*).

Soluble and insoluble nondigestible polysaccharides constitute the carbohydrate fraction of DF. Many epidemiological and intervention studies have shown a relationship between the consumption of DF and protection against various Western diseases, specially metabolic disorders such as obesity (*2, 3*), type 2 diabetes mellitus (*4*), and cardiovascular disease (*1, 3–5*), as well as disorders related to the gastrointestinal tract such as constipation, ulcerative colitis, diverticulosis, irritable bowel syndrome, or colorectal and other types of cancer (*1, 5–7*).

These beneficial effects of DF might be globally explained by the mechanisms of action of DF in the digestive tract. Besides a reduced energy intake associated with DF consumption, the satiating effect elicited by insoluble DF (IDF) and viscous, gel-forming soluble DF (SDF), as well as the decreased absorption of other food constituents (carbohydrates, fats, proteins) due to the effect of DF increasing the intestinal bulk and decreasing transit time, which hinders the action of digestive enzymes and the absorption of nutrients in the upper gastrointestinal tract also have to be added. Delayed absorption of glucose and fat is directly related to the antiobesity effects of DF, its influence on postprandial glycemia, and insulinemia, and its hypolipidemic effects. Dilution of intestinal contents together with the fecal bulking effect of DF is of importance in the regulation of the intestinal function and the prevention of related diseases. Furthermore, SDF fermentation by the colonic microflora leads to the formation of short chain fatty acids (SCFA-acetic, propionic and butyric acids) that are associated with the prevention of CVD (through an inhibitory action of absorbed propionate on liver lipogenesis), colorectal cancer, and inflammation (mediated by the trophic effect of butyrate in the intestinal epithelium as well as by its antiproliferative and pro-apoptotic action in neoplasic cells). In parallel, DF as a prebiotic food component promotes a change in the colonic microflora, diminishing potentially detrimental strains and favoring a more healthy bacterial profile and intestinal environment (acidic pH,

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lower conversion of primary bile acids into secondary acids, dilution of potential carcinogens, etc.) (*7*).

All this has prompted the recommendation of increased fiber intake (*8*). A minimum daily consumption of 25 g total DF is advised to prevent pathologic conditions associated with low DF intakes (*8*), although consumption of 30−35 g/d would provide further protective effects against CVD (*5*). In spite of this, DF intake in Western countries is well below these recommendations because of the predominant consumption of refined cereals and animal foodstuffs, low in DF, over plant foods and whole-grain cereal products. To compensate for this reduced intake of DF, there has been an increased demand by consumers of fiber-rich foods and dietary supplements containing DF, which has encouraged food scientists and the food industry to search for new sources of DF.

Different forms of DF have different physiological effects. Particularly, SDF sources exhibit lower postprandial blood glucose response and long-term control of glycemia (*9*). In addition, soluble, viscous fibers delay gastric emptying and intestinal transit, and they have been shown to have an effect on body weight management (*10*). Nevertheless, lipid lowering is possibly the best recognized effect of viscous SDF (*5, 9–11*) to the extent that the US Food and Drug Administration allows CVD risk reduction claims for two viscous fibers, oat β-glucan and psyllium (*12*). Because of the great prevalence of CVD, which is the major cause of death in most Western countries, the search for new sources of soluble fiber as food ingredients is considered as an important research issue.

Cocoa husks constitute a byproduct of the cacao industry, with an estimated content of up to 50% total DF (TDF), mostly insoluble fiber (*13*). However, by application of enzymatic treatments, it is feasible to obtain soluble fiber from agricultural byproducts (*14*). In the present work, a product rich in soluble dietary fiber obtained after enzymatic treatment of cocoa husks with β-glucanase was characterized and its potential beneficial effects assessed in an animal model of dietary-induced hyperlipemia. This soluble cocoa fiber product (SCFP) contained nearly 50% SDF, retained appreciable amounts of polyphenolic compounds with antioxidant activity, and showed remarkable hypocholesterolemic effects, thus proving its potential application as a functional food ingredient.

MATERIALS AND METHODS

Reagents. AAPH (2,2′-azobis(2-amidinopropane)dihydrochloride), ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 1,1,3,3-tetraethoxypropane (TEP), tripyridyltriazine (TPTZ), dinitrophenylhydrazine (DNPH), and fluorescein were purchased from Sigma Chemical Co. (Madrid, Spain). The Folin−Ciocalteau's reagent was from Panreac S.A (Barcelona, Spain). Ultraflo L was from Novozymes (Krogshoejvej, Denmark), and the K-TDFR enzyme kit for dietary fiber analysis was from Megazyme International Ltd. (Wicklow, Ireland). Randomly methylated β-cyclodextrin was from Cyclodextrin Technologies Development Inc. (High Springs, FL). All other reagents were of analytical or of chromatographic grade.

Cocoa-Derived Product. Nonroasted cocoa (*Theobroma cacao* L.) husks provided by Natra Cacao (Valencia, Spain) were sterilized and ground. Enzymatic hydrolysis was conducted treating cocoa husks (mixed with water, 1:5 w/w) with 0.04% of a commercial enzyme preparation Ultraflo L for 90 min at 60 °C. This is a multiactive β-glucanase with cellulase, xylanase, pentosanase, and arabinase activities. After the reaction time, the enzyme was inactivated by heating at 100 °C for 10 min. The hydrolysis mixtures were centrifuged at 4500*g* for 20 min. The supernatants were further concentrated and dried overnight in a vacuum dryer (Gallenkamp, Leicester, England) at 60 °C and 800 mbar.

Dietary Fiber Determination. Total, soluble, and insoluble dietary fiber contents were determined by the AOAC enzymatic-gravimetric method (*15*) using the commercial enzymatic kit from Megazyme. Duplicate samples of 1 g were subjected to sequential enzymatic digestion by heat stable α-amylase, protease, and amyloglucosidase to remove protein and starch. Soluble fiber was precipitated with ethanol. Soluble and insoluble dietary fiber fractions were hydrolyzed as described in ref *13*; the constituent neutral sugars were analyzed by HPLC, and uronic acids were spectrophotometrically analyzed. Total dietary fiber (TDF) was calculated as the sum of soluble and insoluble dietary fiber fractions after correcting for ash and undigested protein. Ash was determined gravimetrically after calcination of the DF residues at 525 °C for 5 h and protein by quantifying total nitrogen by Kjeldhal.

Total Polyphenols, Flavonoids, and Antioxidant Capacity. The soluble cocoa fiber product (SCFP) was extracted with acidic 50% aqueous methanol followed by extraction with 70% aqueous acetone as described elsewhere (*16*). Total polyphenols were quantified in the extracts by using the Folin−Ciocalteau's reagent and catechin as standard. Additionally, the polyphenolic profile was determined by HPLC (*17*).

The antioxidant capacity of SCFP was analyzed by different methods. In all cases, Trolox was used as standard and the results expressed as μmol of Trolox equivalents (TE) per gram of SCFP dry matter.

The reducing power of samples was determined by the ferric reducing/antioxidant power (FRAP) assay (*18*). Briefly, freshly prepared FRAP reagent (containing 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$, and 0.3 M acetate buffer, pH 3.6), was mixed with test samples, water (blank), or Trolox (standard). Absorbance readings at 595 nm were taken after 30 min of reaction at 37 °C using a Beckman DU640 spectrophotometer (Beckman Instruments Inc., Fullerton, CA). Increases in absorbance are due to Fe^{3+} reduction by antioxidants and the subsequent formation of a colored TPTZ-Fe^{2+} complex with an absorbance maximum at 595 nm.

The free-radical scavenging capacity of samples was determined by the method of Re et al. (*19*). The stable radical cation $ABTS^{\bullet+}$ was formed by overnight reaction of ABTS with potassium persulfate at room temperature in the dark. Decrease of $ABTS^{\bullet+}$ absorbance at 734 nm in the presence of the antioxidant was monitored during 6 min. The area under the absorbance curve taken between 0 and 6 min was used for calculations.

Finally, both the lipophilic (L-ORAC) and hydrophilic (H-ORAC) ORAC assays were used to determine the antioxidant capacity of SCFP (*20*). Briefly, AAPH was used to generate peroxyl radicals that oxidize fluorescein, causing a decrease in fluorescence (excitation wavelength 485 nm and emission wavelength 528 nm) that is measured every minute for 1 h at 37 °C in a multiwell plate reader. Trolox was used as standard in both hydrophilic and lipophilic assays. In the lipophilic assay, samples and Trolox are prepared in 7% randomly methylated cyclodextrins. The results are calculated using the differences of the areas under the curve between the blank and samples. Total antioxidant capacity was calculated by summing up L-ORAC and H-ORAC.

Animals and Diets. Thirty male Sprague−Dawley rats (10 weeks old) were obtained from the School of Medicine, Universidad Autónoma (Madrid, Spain). Animals were placed individually in stainless steel wire-bottomed metabolic cages housed in a room under controlled conditions (19−23 °C, 50−60% humidity and 12 h light/darkness cycles) at the animal facility of the School of Pharmacy, Universidad Complutense, where the experiment was conducted.

Diets were prepared from a Fiber Free AIN-93 M Purified Rodent Diet (Panlab S.L., Barcelona, Spain) that provides the nutrients required by adult rats according to the National Research Council guidelines (*21*). Rats were randomly assigned to three different dietary groups (10 animals per group). One group received a control, cholesterol-free diet containing 10% cellulose (normocholesterolemic control, NC); a second control group (hypercholesterolemic control, HC) received a cholesterol-rich diet, also containing cellulose as a source of DF; the third group was fed a hypercholesterolemic test diet containing the SCFP (SCFP group) and no cellulose. The composition of the diets is given in **Table 1**. Both hypercholesterolemic diets were prepared from the basal diet supplemented with 10 g of cholesterol and 2 g of sodium

Table 1. Composition of the Experimental Normocholesterolemic Control (NC), Hypercholesterolemic Control (HC), and SCFP Test Diets (g/kg Dry Weight)[a]

	NC	HC	SCFP
casein	140	140	140
dextrose	155	155	155
sucrose	100	100	100
fat	40	40	40
t-BHQ[b]	0.008	0.008	0.008
mineral mix.	35	35	35
vitamin mix.	10	10	10
L-Cys	1.8	1.8	1.8
cholin bitartrate	2.5	2.5	2.5
cholesterol		10	10
sodium cholate		2	2
cellulose	100	100	
starch	415.692	403.692	288.692
cocoa product			215
energy (kJ/100 g)	159.1	160.9	148.5

[a] Hypercholesterolemic diets were supplemented with 10 g of cholesterol and 2 g of sodium cholate per kg of diet, at the expense of starch. [b] *tert*-Butylhydroquinone.

cholate per kg of the basal diet, at the expense of starch. The test diet was prepared from the Fiber-Free AIN-93 M basal diet supplemented with the powdered SCFP added as a source of DF and polyphenols at the expense of starch. Considering the DF content of the SCFP, 215 g of this product in 1 kg of basal diet provided 10% DF. The energy content of the diets was comparable, yet slightly lower in the experimental diet. Food and water were available ad libitum.

After a period of adaptation to the diets and metabolic cages that lasted four days, a three-week experimental period followed. Food intake and body weight were monitored daily throughout this time. Feces were collected daily, weighed fresh, and after freeze-drying, milled to a particle size less than 1 mm. Protein and fat were measured in lyophilized feces. At the end of the experimental period, rats were sacrificed after an overnight fast. Troncal blood was collected, centrifuged (1500 rpm, 10 min, 4 °C), serum separated, and kept at −80 °C until analysis. Animal handling and sacrificing were performed in accordance with the National Research Council guidelines for the use and care of laboratory animals (*21*).

Analytical Methods. *Analysis of Blood Lipids.* Serum lipids were analyzed immediately after collection of samples. Triglycerides (TG), total cholesterol (T-chol), and HDL-cholesterol were determined as described elsewhere (*16*). LDL-cholesterol was calculated using the Friedewald equation as follows: LDL-cholesterol = Total cholesterol − HDL-cholesterol − (TG/5) (*16*). The atherogenic index (AI) was calculated as AI = (Total cholesterol − HDL-cholesterol)/HDL-cholesterol).

Antioxidant Capacity of Serum Samples. The antioxidant capacity of rat sera was analyzed by the two complementary methodologies described above, the FRAP method and the TEAC assay, in order to determine their reducing power and free radical scavenging capacities, respectively. Results were expressed as μM TE.

MDA Determination. Malondialdehyde (MDA) was measured in serum as a biomarker of lipid peroxidation. MDA was determined by HPLC as its hydrazone after protein precipitation and MDA derivatization with dinitrophenylhydrazine according to the method of Mateos et al. (*22*). HPLC-DAD analyses were performed on an Agilent 1100 liquid chromatographic system. A Nucleosil 100 RP-18 column (4.0 × 125 mm, 5-μm particle size, Agilent) preceded by a Lichrospher guard column of the same material (4.0 × 4.0 mm) was used. The column was isocratically eluted with a mixture of 0.2% (v/v) acetic acid in deionized water and acetonitrile (62:38, v/v) at a flow rate of 0.6 mL/min at room temperature. Chromatograms were acquired at 310 nm. An Agilent Chemstation software system controlled all the equipment and carried out data processing. Standard MDA was prepared by acidic hydrolysis of 1,1,3,3-tetraethoxypropane (TEP) in 1% sulfuric acid. Concentrations were expressed as nmol MDA per mL plasma.

Analysis of Feces. The water content of feces was measured after freeze-drying the fresh fecal samples and the results expressed as the percentage of the total fecal weight. Nitrogen in fecal samples was determined by thermal conductivity (Dumas method) using an automated nitrogen analyzer (LECO FP-2000, St Joseph, MI). Protein was calculated as N × 6.25. Fat in feces was evaluated gravimetrically after extraction with light petroleum with a Soxtec System HT equipment (Tecator, Höganas, Sweden). Protein and fat content of feces are expressed as the percentage of dry feces. Polyphenols in feces were quantified in the aqueous methanol−acetone extracts obtained as described above. Total polyphenols were determined spectrophotometrically using the Folin−Ciocalteau reagent and catechin as standard.

Statistical Analysis. Statistical analysis of data was performed by one-way ANOVA followed by the Bonferroni test when variances were homogeneous or by the test of Tamhane when variances were not homogeneous. Previously, homogeneity of variances was tested by the test of Levene. The level of significance was $p < 0.05$. An SPSS version 12.0 program was used.

Table 2. Chemical Composition of the SCFP (g/kg Dry Matter)[a]

	g/kg (dry matter)		
	mean	SD	% relative to TDF
insoluble dietary fiber	*26.3*	*3.7*	*5.1*
soluble dietary fiber	*486*	*5.9*	*94.8*
uronic acids	238	21	46.4
neutral sugars	121		23.7
galactose	97	8	
mannose	17	1.3	
glucose	5	0.4	
arabinose	2	0.2	
total dietary fiber	*512.3*		*100*

[a] The values are expressed as the mean ± SD ($n = 3$).

Table 3. Total Polyphenols (mg/g), Procyanidin Profile (mg/g), and Antioxidant Capacity (μmol TE/g) of the Soluble Cocoa Fiber Product[a]

	mean	SD
total polyphenols content[b]	23.9	0.11
total procyanidins content[c]	0.32	0.01
procyanidin B1	0.093	0.002
catechin	0.088	0.002
procyanidin B2	0.039	0.007
epicatechin	0.100	0.003
antioxidant capacity[d]		
FRAP	112.6	3.9
TEAC	83.3	0.9
ORAC Total	569	76
H-ORAC	552	75
L-ORAC	17	1

[a] The values are expressed as the mean ± SD ($n = 3$). [b] Measured by Folin−Ciocalteu's method. [c] Measured by HPLC. [d] μmol TE/g dry matter.

RESULTS

Dietary Fiber, Polyphenolic Content, and Antioxidant Capacity of the Cocoa-Derived Product. The enzymatic treatment of cocoa husks yielded a product with only minor amounts of insoluble fiber (2.6 g/100 g dry matter) and rich in soluble dietary fiber (48.6 g/100 g dry matter) (**Table 2**). The analysis of neutral sugars in the SDF fraction showed appreciable amounts of galactose and mannose residues, suggestive of the presence of hemicellulosic galactomannans, yet the major constituents of the SDF were pectic polysaccharides as shown by the high uronic acid content, accounting for 49% of the SDF.

The polyphenolic content of the SCFP was relatively low (2.4 g/100 g) (**Table 3**). The monomeric flavanols epicatechin and catechin were the main phenolics, together with appreciable amounts of dimeric procyanidins. In spite of the low polyphenolic content of the cocoa fiber product, its antioxidant capacity was remarkable as can be seen in **Table 3**, especially as determined by the hydrophilic ORAC method. These values

Table 4. Total Food Intake, Body Weight Gain, Glycemia, and Fecal Excretion of Rats[a]

	NC		HC		SCFP	
	mean	SD	mean	SD	mean	SD
total food intake (g/21d)	426.4	26.1 a	522.3	30.4 b	377.2	31.7 c
body weight gain (g/21d)	80.9	19.9 ab	100.4	20.0 a	51.0	17.3 b
alimentary efficiency [b]	0.18	0.05 ab	0.19	0.05 a	0.14	0.05 b
feces (wet weight) (g/d)	3.82	0.88 a	7.28	0.99 b	4.45	0.28 a
feces (dry weight) (g/d)	2.19	0.32 a	3.47	0.48 b	1.77	0.16 a
% Water in feces	56.20	3.28 a	63.88	1.39 b	70.24	1.50 c

[a] Data in a row with different letters are statistically different ($p \leq 0.05$). Mean values ± standard deviations of 10 animals per group. [b] Alimentary efficiency = body weight gain × food intake^{-1}.

were comparable with those reported for other foods and food ingredients with well-known antioxidant activity such as fruits (e.g., strawberry, 82 and 202 μmol TE/g d.m. as determined by the TEAC and FRAP methods, respectively), nuts (e.g., walnuts, 154 and 115 μmol TE/g d.m. as determined by the TEAC and FRAP methods, respectively), or other antioxidant products obtained from industrial wastes and used as functional ingredients such as red grape pomace (124 and 264 μmol TE/g d.m., again by the TEAC and FRAP methods, respectively) (*23*). The ORAC value for the SCFP was lower than that found in baking chocolate (1040 μmol TE/g) yet much higher than the values reported for milk chocolate candy bars or dark chocolate (81.7 and 225 μmol TE/g, respectively) (*20, 24*), clearly showing the relevance of the SCFP as a potential source of soluble fiber with associated antioxidant activity.

Animal Experiment. The three diets were well tolerated by the animals that adapted easily to the powdered food and showed no apparent external evidence of potential alterations due to the cholesterol-rich diets.

Food Intake and Body Weight Gain. Food intake was significantly higher in the hypercholesterolemic control (HC) group than in the animals fed the cholesterol-free control diet (**Table 4**), suggesting stimulated food consumption as a consequence of the higher fat (cholesterol) content of the hypercholesterolemic diet. However, supplementation of the cholesterol-rich diet with SCFP led to a significant reduction of food intake, with values even lower than those in the normocholesterolemic control (NC) group in spite of the slightly lower energy content of the SCFP diet (**Table 1**). This lower food consumption could be indicative of a satiating effect of SCFP.

These differences in the food consumption pattern led to important differences in body weight gain at the end of the experimental period (**Table 4**). Consistent with the higher food intake, the HC group showed the highest weight gain. Similarly, the SCFP group had the lowest pondered growth. However, it is worth noting the different linear relationship between food intake and weight increase among the three groups. Thus, an approximately 20% higher food (and energy) intake by the HC group resulted in a 24% higher final body weight in comparison with the NC animals, yielding a similar alimentary efficiency in the two control groups. However, the SCFP group that also consumed a cholesterol-rich diet, with an approximately 30% lower food intake than the HC group (equivalent to approx 33% lower energy intake) showed a total growth half-that of the hypercholesterolemic animals. When the SCFP group was compared to the NC one consuming the basal, cholesterol-free diet, a 12% lower food consumption by the SCFP animals corresponded to nearly 40% lower weight increase than in the NC group. Therefore, the alimentary efficiency of this SCFP diet was even lower than that of the control diet, and thus, consumption of a fatty, cholesterol-rich diet together with SCFP as a source of DF resulted in a decreased body weight gain. This would suggest that together with the satiating action, other mechanisms might be involved in the SCFP effect reducing weight gain.

Table 5. Intake and Excretion of Protein and Fat in Normocholesterolemic Control (NC), Hypercholesterolemic Control (HC), and SCFP Groups[a]

	NC		HC		SCFP	
	mean	SD	mean	SD	mean	SD
		Protein				
intake (g/21d)	64.73	3.66 a	73.12	4.26 b	63.37	5.32 a
fecal excretion (g/21d)	9.23	1.35 a	15.28	2.13 b	13.58	1.20 b
fecal excretion (%)	20.09	1.19 a	20.95	3.49 a	36.54	1.69 b
ADC[b]	85.69	0.78 a	79.04	1.19 b	78.44	1.81 b
PER[c]	1.26	0.35 a	1.39	0.33 a	0.79	0.32 b
		Fat				
intake (g/21d)	18.49	1.04 a	27.16	1.58 b	20.75	1.74 c
fecal excretion (g/21d)	1.00	0.15 a	5.74	0.80 b	3.21	0.28 c
fecal excretion (%)	2.17	0.29 a	7.87	0.72 b	8.63	0.78 b
ADC[b]	94.58	1.30 a	78.80	1.21 b	84.44	1.31 c

[a] Data in a row with different letters are statistically different ($p \leq 0.05$). Mean values ± standard deviations of 10 animals per group. [b] ADC: apparent digestibility coefficient = [(intake − fecal excretion) × intake^{-1}] × 100. [c] PER: protein efficiency ratio = body weight gain × protein intake^{-1}.

Fecal Bulking. The highest fecal output corresponded to the HC animals, with a total fresh weight of feces nearly twice that in the NC group (**Table 4**). The dry weight was also higher in the HC animals than in the group consuming the cholesterol-free control diet, as was the water content of feces. Considering that the two cholesterol-free and cholesterol-rich control diets contained 10% cellulose as DF, the differences observed in the fecal output should be ascribed to the higher fat content in the hypercholesterolemic diet affecting the digestibility and absorption of dietary components.

Incorporation of the SCFP into the diet, with a 10% DF (9.2% SDF) instead of the insoluble cellulose, resulted in a higher water content of feces in comparison with the two control groups. The total fresh and dry weights of feces were similar to those excreted by the NC animals, although the dry mass tended to be smaller because of the higher humidity of feces in the SCFP group. On the contrary, fecal output was lower in this group than in the HC one.

Protein and Fat Excretion. Protein and fat intake and excretions are shown in **Table 5**. SCFP contributed with 28 g of protein per kilogram of diet, and thus, the protein intake was similar to that in the NC group in spite of the lower total food consumed by the SCFP animals. The highest protein consumption corresponded to the HC animals in accordance with their higher food intake. Net fecal protein excretion was higher in the SCFP group than in the NC one, yet similar to the HC. However, the percentage of protein excreted in feces by the animals consuming the SCFP diet almost doubled that in the other groups, which might be attributed to the low total fecal output in this group, although the contribution of the fecal bacterial mass to this increased nitrogen excretion should also be considered, taking into account the fermentable nature of the soluble fiber in the SCFP and the presumable increase in the colonic microflora. Nonetheless, the apparent digestibility coefficient (ADC) in the SCFP group was statistically lower than that in the NC group but similar to the HC one. Therefore, an effect of the excess cholesterol in the diet rather than the viscous soluble fiber or the polyphenols in SCFP hindering protein digestion and/or absorption cannot be ruled out in view

of the similar ADC in the HC group. However, the protein efficiency ratio (PER) was lower in the SCFP animals since despite a similar protein intake, body weight gain was lower in this group than in the control animals as seen above.

Fat intake was higher in the hypercholesterolemic controls followed by the group consuming the cholesterol-rich SCFP diet and the cholesterol-free control diet (**Table 5**). The SCFP, with merely 1.4% fat content, only contributed with 3 g of fat per kg of diet. The percentage and the total amount of fat excreted in feces were lower in the NC animals fed the cholesterol-free diet than in the two groups consuming the cholesterol-rich diets. In these hypercholesterolemic groups, the percentage of fecal fat was similar, although it tended to be higher in the group supplemented with the SCFP. This might be suggestive of an effect of the soluble, viscous fiber in the cocoa product increasing fecal fat excretion. Conversely, the net amount of fat excreted in the feces over the experimental period was lower in the SCFP animals than in the HC rats, partly due to the lower fat intake. When calculating the ADC of fat, the highest value corresponded to the NC group consuming the standard diet with no added cholesterol, while the lowest value was found in the HC animals and not in the SCFP group as it would have been expected should the soluble fiber in this diet have an effect increasing fat excretion.

Serum Lipid Profile. The concentrations of total, LDL- and HDL-cholesterol are shown in **Figure 1**, together with the TG levels and the atherogenic index (AI) calculated for the three diets. Consumption of the cholesterol-rich control diet resulted in increased concentrations of total and LDL-cholesterol, with values nearly 4 and 9 times higher, respectively, than those in the NC animals (**Figures 1B** and **1C**). TG levels were not modified by the hypercholesterolemic diet (**Figure 1A**), contrary to what occurred to the HDL-cholesterol fraction that was significantly diminished (**Figure 1D**). This was reflected in the AI that was 10 times higher than that in the control group (**Figure 1E**). When the SCFP was introduced into such an atherogenic diet, a reduction of 58% of total cholesterol concentrations and up to 68% in the LDL-cholesterol levels in comparison with HC consuming cellulose as a source of DF was observed. Therefore, supplementation of a cholesterol-rich diet with the SCFP had a marked hypocholesterolemic effect, yet total and LDL-cholesterol levels of normocholesterolemic animals were not attained. However, HDL-cholesterol concentrations in the SCFP group were increased in comparison with those of the HC group, recovering the levels of the NC animals consuming the cholesterol-free diet. SCFP also showed a remarkable hypotriglyceridemic action, with a 40% reduction of serum TG levels as compared with both normo- and hypercholesterolemic controls. It is worth noting that the TG levels in the SCFP group were significantly lower than those in the normal control animals. This remarkable hypolipidemic effect of SCFP was reflected in the AI that was nearly 5 times lower than that in the hypercholesterolemic diet, thus indicating a reduced atherogenic risk of fatty diets when consumed together with SCFP. Again, values of the control, cholesterol-free diet were not achieved.

Antioxidant Capacity and MDA Levels in Serum. Consumption of the SCFP diet did not modify the total antioxidant capacity of serum in fasting animals, with FRAP and ABTS values comparable to those of the NC and HC groups (**Figure 2**). Supplementation of the diet with the SCFP only added 5.14 g of polyphenols per kg of diet, an amount too small to significantly contribute to the antioxidant defenses in a fasting, not postprandial state. Considering the limited bioavailability

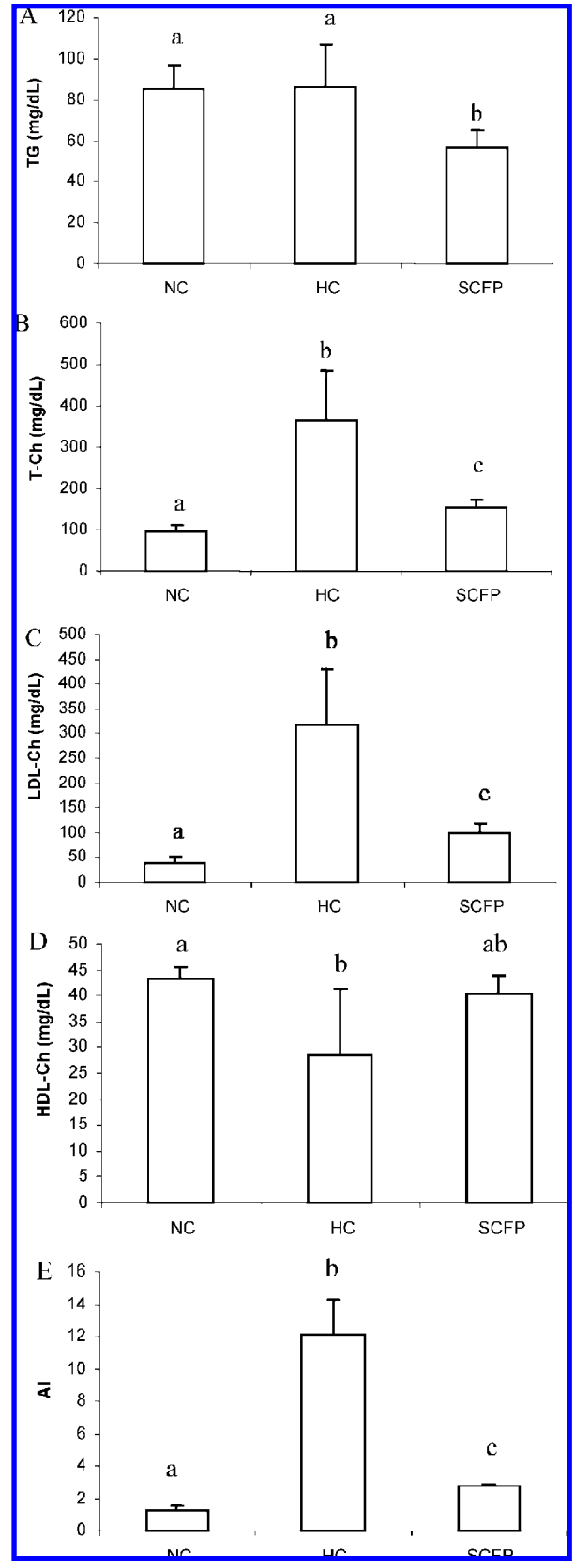

Figure 1. Lipid profile in serum of rats fed the normocholesterolemic control (NC), hypercholesterolemic control (HC), or hypercholesterolemic-SCFP diets. (**A**) Triglycerides; (**B**) total cholesterol; (**C**) LDL-cholesterol; (**D**) HDL-cholesterol; (**E**) atherogenic index [AI = (total cholesterol − HDL-cholesterol) × HDL-cholesterol^{-1}]. Different superscript letters denote statistically significant differences among groups ($p \leq 0.05$).

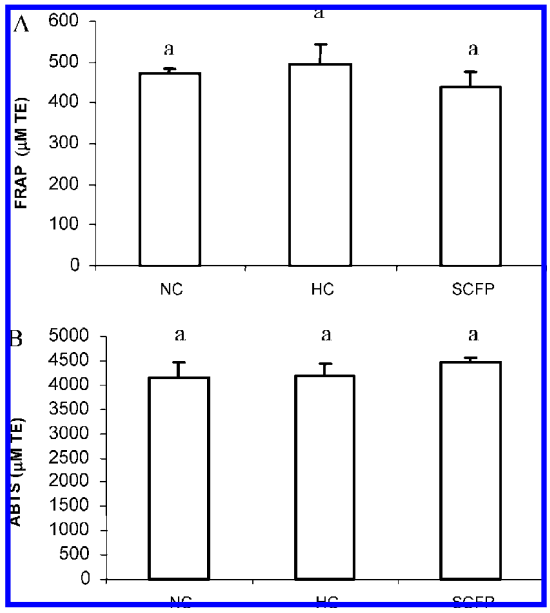

Figure 2. Total antioxidant capacity of serum from rats fed the normocholesterolemic control (NC), hypercholesterolemic control (HC), or hypercholesterolemic-SCFP diets. (**A**) FRAP assay; (**B**) ABTS assay. Different superscript letters denote statistically significant differences among groups ($p \leq 0.05$).

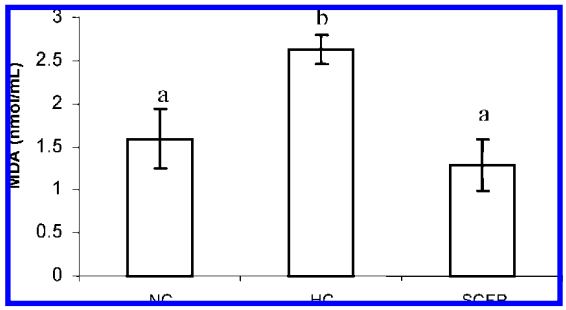

Figure 3. Serum malondialdehyde (MDA) levels in rats fed the normocholesterolemic control (NC), hypercholesterolemic control (HC), or hypercholesterolemic-SCFP diets. Different superscript letters denote statistically significant differences among groups ($p \leq 0.05$).

of polyphenolic compounds and the relatively short half-lives of absorbed polyphenols, the lack of effect of the SCFP diet on serum antioxidant capacity of fasting animals was predictable.

The fact that none of the experimental diets (either cholesterol-free or cholesterol-rich diets) significantly affected the serum antioxidant capacity would suggest that the higher serum lipid concentrations observed in the hypercholesterolemic animals would not undermine the serum antioxidant defenses. Nonetheless, serum concentrations of MDA were increased in the HC animals (**Figure 3**), indicating a higher lipid peroxidation in this model of dietary-induced hypercholesterolemia. The consumption of the SCFP resulted in decreased levels of MDA as a biomarker of lipid peroxidation, reaching values similar to those of the NC animals. As seen above, SCFP lowered total and LDL-cholesterol concentrations in serum, although not to values of control, healthy animals. In spite of this high circulating levels of total and LDL-cholesterol, these lipids appear to be less prone to oxidation, which would suggest a protective effect of the SCFP against lipid peroxidation.

DISCUSSION

Every year, tons of cocoa husks are disposed of as waste from cocoa manufacturing, representing a major difficulty in dealing with these high quantities of industrial byproduct. Considering the interest in functional foods and the potential health benefits of dietary fiber in the human diet, this cocoa byproduct has been used as a ready source of inexpensive IDF. However, most fiber-rich materials available in the market are insoluble fibers, being of interest to increase the offer of products rich in SDF. Enzymatic treatment of cocoa husks with β-glucanase yielded an added-value product rich in soluble dietary fiber, especially pectic substances. This product retained polyphenolic compounds with remarkable antioxidant capacity, which can be considered high when compared with other cocoa products such as milk chocolate and dark chocolate, with 80 and 225 μmol TE per gram of dry matter, respectively, when analyzed by the ORAC method (*24*), values much lower than those determined in the SCFP (552 μmol TE/g). Similarly, FRAP and TEAC values of the SCFP were higher than those reported in other cocoa products and foods such as cereals, legumes, vegetables, or fruits (*13, 23*).

Considering the recognized health effects associated with SDF (*5, 9–11*) and the presence of antioxidant polyphenols, it was of interest to study the potentially beneficial effects associated with the SCFP consumption. To this aim, we used an animal model of dietary-induced hypercholesterolemia. Hypercholesterolemic animals were selected for this study because of the established role of DF in the reduction of CVD risk factors (*4, 5*). In this model, feeding rats a diet rich in cholesterol evoked an increase in risk factors for atherosclerosis and CVD such as dyslipidemia (high TG, total and LDL-cholesterol, and low HDL-cholesterol) and lipid peroxidation (increased MDA levels). It also stimulated food intake with a concomitant increase in body weight gain, although not statistically different from controls, probably due to the higher fat and protein excretion. This general profile elicited by an unhealthy dietary pattern (atherogenic diet) was partly or totally reverted when such a diet was consumed together with a cocoa-derived product providing SDF in amounts close to the IDF content of the hypercholesterolemic control diet (10% cellulose). In the SCFP-fed animals, serum total and LDL-cholesterol were drastically reduced, HDL-cholesterol was recovered to values similar to those in normocholesterolemic animals, and TG levels were diminished to concentrations even below those of the NC group. In addition, lipid peroxidation was also reduced to normal, thus diminishing several important risk factors for CVD. Moreover, the reduced food intake and the low body weight gain in animals consuming the SCFP diet suggests that this cocoa product might have application in weight-management programs helping to control excess body weight, which constitutes one of the major risk factors not only for CVD, but also for type 2 diabetes, hypertension, cancer, and other chronic diseases, and thus is one of the most crucial risk factors for all-cause morbidity and mortality (*25*).

Several works have reported the weight-gain reduction associated with the consumption of fiber-rich foods, particularly whole-grain products, as part of a healthy lifestyle (*2*). The effectiveness in weight control of DF supplements such as chitosan or psyllium is less evident, yet other SDF components such as glucomannan, guar gum, pectins, or a SDF product composed of α-cyclodextrins seem to be effective in reducing weight gain (*3, 25*). The DF in the SCFP used in the current study consisted mainly on pectins, with significant amounts of galactomannans.

DF may exert its influence on food intake and weight gain through different mechanisms. The satiating effect elicited by both IDF and SDF is not only a consequence of mechanical

intestinal distension by the bulking action of nondigested and unabsorbed food components and water. Also, delayed starch digestion and glucose absorption reduce glycemic and insulin responses, prolonging satiety and reducing energy consumption. Moreover, a low insulin response would favor lipid oxidation and lipolysis over hepatic lipogenesis and tissue fat storage, which would affect body weight gain (*26*). DF can also affect food intake by modulating the production of gut hormones involved in appetite regulation such as ghrelin (an orexigenic peptide) or the anorexigenic hormones glucagon-like peptide-1 (GLP-1), peptide YY (PYY), or the adipocyte-produced leptin, hormones that would also affect lipid metabolism and energy expenditure, thus influencing body weight (*27, 28*). A satietogenic effect of soluble, fermentable fibers has also been related to the action of short chain fatty acids, which might enhance the production of the satietogenic hormones GLP-1 and PYY (*27*). As mentioned above, the SCFP used in this study was rich in soluble, fermentable polysaccharides (galactomannans and pectins), although its fermentability has not been determined. However, it has to be considered that the consumption of a high-fat diet can also affect the orexigenic and anorexigenic responses (*28*), which may be responsible for the overeating observed in the HC group. However, such hyperphagic behavior was not observed in the SCFP group, also consuming a cholesterol rich diet. To fully comprehend the mechanisms underlying the observed effects of SCFP reducing food intake and body weight gain in animals fed a fat-rich diet, a different study design should have been contemplated to determine the influence of SCFP supplementation of high-fat vs low-fat diets, including the study of hormones regulating food intake. This was not the objective of the present study, but its interest in view of the results obtained merits future research.

Concerning the effect of the SCFP on serum lipids, the observed hypocholesterolemic action might be attributed to the high content of soluble, viscous DF of SCFP. It is accepted that many beneficial health effects of SDF, including the hypocholesterolemic properties, are related to its high viscosity (*9*). The SCFP used in this study has shown *in vitro* to have a high oil retention capacity (4.7 g oil per g of SCF, unpublished results). Soluble fibers such as that in SCFP bind to bile acids (BA) and dietary fats, and interfere with lipid absorption in the small intestine. Viscous fibers form an emulsion with fat that limits the action of pancreatic lipase to the surface of the emulsion, hence reducing the hydrolysis of TG as well as lipid absorption, increasing fat elimination in feces. The fact that HC animals consuming cellulose (a nongel-forming IDF with lower oil retention capacity than soluble fibers) had a higher fecal fat excretion than the SCFP group might be misleading, suggesting a better fat digestion and/or absorption in the SCFP animals. However, nonabsorbed fat retained by the soluble fiber in the gel emulsion might be partially degraded by the colonic microflora, thus accounting for the lower fecal fat and higher apparent fat digestibility in this group in comparison with those in the HC. Soluble fibers are a substrate for the fermentative bacteria in the large intestine, thus increasing the bacterial mass in the colon. Although we have not studied the degradation of dietary fat in the large intestine, it could be hypothesized that the increased fermentative capacity of the colonic microflora subsequent to the consumption of the fermentable fiber in the SCFP might result in enhanced lipid degradation and decreased fat excretion. This is in agreement with results reported by other authors (*3, 29, 30*) who found increased fecal excretion of total lipids, cholesterol, and bile acids after DF intake, although not all the fecal fat made up for the unabsorbed dietary fat. It has to be mentioned that neither the HC nor the SCFP animals, with 21% and 15% of the ingested fat eliminated in feces, respectively, showed apparent signs of steatorrhea.

One of the best-known health properties of DF relates to its role as a hypolipidemic agent, with many animal (*3, 16, 26, 29–32*) as well as human intervention and prospective (*4, 5, 11, 12*) studies in the literature reporting a decrease in plasma cholesterol and TG associated with the intake of DF as observed in the present animal study with the SCFP. Several mechanisms of action have been reported to explain the hypocholesterolemic role of DF, especially of SDF. Besides lipid malabsorption due to the emulsifying effect of viscous fiber disrupting lipid micelles and binding BA and dietary fat, a reduced hydrolysis of TG would further decrease cholesterol absorption due to a lower release of free fatty acids in the small intestine, which is required for cholesterol transport from micelles to enterocytes (*32*). Increased sterol loss in feces would have a direct effect on hepatic and plasma cholesterol homeostasis, especially the disruption of the enterohepatic recirculation of BA. This will suppress the feedback inhibition by BA of the activity of cholesterol 7α-hydroxylase, the rate limiting enzyme of BA synthesis (*31*), resulting in an increased conversion of hepatic cholesterol into BA. The activity and gene expression of this enzyme have been shown to be induced in cholesterol-fed animals consuming different sources of DF (*30*). When the SDF intake is high enough to reduce hepatic cholesterol pools, down-regulation of acyl CoA/cholesterol acyl transferase (ACAT) (*29*) and induced activity of hydroxymethyl glutaryl CoA (HMG-CoA) reductase (*29, 30*) in order to replenish cholesterol pools have also been observed. However, under normal hepatic cholesterol levels HMGCoA reductase would not be affected by SDF, and thus, it has been suggested that the hypocholesterolemic action of DF is not mediated by the inhibition of hepatic cholesterol synthesis (*32*). The up-regulation of apolipoprotein B/E receptor expression in hepatocytes as well as increased receptor-mediated catabolic rates have been shown by different authors (*29*) with a concomitant increased clearance of LDL. Finally, as for the effect of DF reducing plasma TG, this may be mediated by a decreased *de novo* hepatic lipogenesis as a result of reduced activity of lipogenic enzymes such as fatty acid synthase (FAS), acetyl CoA carboxylase, or ATP citrate lyase (*33*). This has been related to the absorption of propionate, a short chain fatty acid resulting from SDF fermentation in the colon that has been reported to decrease FAS mRNA concentration in cultured hepatocytes. As mentioned above, a potential influence of the SCFP on hormones regulating food intake and their implication on thermogenesis and fatty acid oxidation (*28*) should not be overlooked.

However, although the observed hypolipidemic effects of the SCFP are most likely attributable to its high content of soluble, viscous DF, the potential contribution of the polyphenolic compounds in SCFP cannot be ruled out. Many animal and human intervention studies have shown that consumption of chocolate and cocoa products has positive effects on different parameters associated to cardiovascular health such as endothelial function, platelet activation, and eicosanoid synthesis as well as on serum lipid and lipoprotein profiles and on biomarkers of lipid peroxidation (*1, 16, 22, 34, 35*). Similar to what has been found in the present study, increased HDL-cholesterol levels and decreased TG and total and LDL-cholesterol serum concentrations have been reported after the intake of dark chocolate or cocoa extracts by animals and humans (*16, 34, 35*). In spite of the limited bioavailability of cocoa polyphenols and the relatively low content of polyphenolic compounds in the

SCFP (2.4 g/100 g), these antioxidant phenols may be in part responsible for some of the observed effects elicited by the SCFP. Indeed, the reduced lipid peroxidation (low MDA levels) in these animals in comparison with the HC group might be ascribed to the effect of cocoa polyphenols protecting serum lipids from oxidation. This is in agreement with results reported by other authors in hypercholesterolemic rabbits (*35*) as well as with previous results from our laboratory obtained in both normo- and hypercholesterolemic rats fed a cocoa product rich in IDF, also with low polyphenolic content (*13, 16*). In these and other long-term feeding studies (*34, 35*), antioxidant capacity of serum was not modified by cocoa intake, which agrees with the results obtained in the present work; nonetheless, improved resistance of LDL to oxidation and reduced lipid peroxidation were reported in the mentioned studies, suggesting an effect of cocoa polyphenols irrespective of the unaffected fasting total antioxidant capacity of plasma.

In summary, the cocoa product obtained after enzymatic treatment of cocoa husks, rich in SDF and with appreciable amounts of antioxidant polyphenols, brought about remarkable hypocholesterolemic and hypotriglyceridemic responses in animals fed an atherogenic diet, also decreasing lipid peroxidation, thus diminishing several risk factors for CVD. The SCFP showed interesting effects, reducing food intake and body weight gain. The mechanisms of action of the SCFP eliciting these responses, of clear interest in the management of bodyweight related disorders, and their potential impact on the observed hypolipidemic effect require further investigations.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid; BA, bile acids; CVD, cardiovascular disease; DF, dietary fiber; FRAP, ferric reducing/antioxidant power; HC, hypercholesterolemic controls; HDL, high density lipoproteins; IDF, insoluble dietary fiber; LDL, low density lipoproteins; MDA, malondialdehyde; NC, normocholesterolemic controls; ORAC, oxygen radical absorbance capacity; PP, polyphenols; SCFA, short chain fatty acids; SCFP, soluble cocoa fiber product; SDF, soluble dietary fiber; TBARS, thiobarbituric acid reactive substances; TDF, total dietary fiber; TEAC, Trolox equivalent antioxidant capacity; TG, triacylglycerides; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid.

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