

Effect of Milk on the Urinary Excretion of Microbial Phenolic Acids after Cocoa Powder Consumption in Humans

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Health effects of cocoa flavonols depend on their bioavailability, which is strongly influenced by the food matrix and the degree of flavanol polymerization. The effect of milk on the bioavailability of cocoa flavonoids considering phase II metabolites of epicatechin has been the subject of considerable debate. This work studies the effect of milk at the colonic microbial metabolism level of the nonabsorbed flavanol fraction that reaches the colon and is metabolized by the colonic microbiota into various phenolic acids. Twenty-one human volunteers followed a diet low in polyphenols for at least 48 h before taking, in a random order, 40 g of cocoa powder dissolved either in 250 mL of whole milk or in 250 mL of water. Urine samples were collected before the intake and during three different periods (0–6, 6–12, and 12–24 h). Phenolic acids were analyzed by LC-MS/MS after solid-phase extraction. Of the 15 metabolites assessed, the excretion of 9 phenolic acids was affected by the intake of milk. The urinary concentration of 3,4-dihydroxyphenylacetic, protocatechuic, 4-hydroxybenzoic, 4-hydroxyhippuric, hippuric, caffeic, and ferulic acids diminished after the intake of cocoa with milk, whereas urinary concentrations of vanillic and phenylacetic acids increased. In conclusion, milk partially affects the formation of microbial phenolic acids derived from the colonic degradation of procyanidins and other compounds present in cocoa powder.

KEYWORDS: Phenolic acids; milk effect; microbiota; cocoa powder; humans; LC-MS/MS

INTRODUCTION

The consumption of cocoa foods has been linked to short- and long-term health benefits, particularly related to cardiovascular diseases (1, 2). Some studies have placed the polyphenolic content of cocoa foods behind these effects, showing that they are important sources of these compounds (3). Flavanols are the most abundant flavonoids in cocoa, occurring as monomers (mainly (–)-epicatechin) and as oligomeric (procyanidins B1, B2, and C1) and polymeric forms (procyanidins) (4). Monomers account for 5–10% of total cocoa polyphenols and oligomers and polymers for ≥90% (5). Requisites such as bioavailability, including microbiota degradation, have been shown as mandatory keys to these expected healthy effects (4, 6).

The bioavailability of flavanols is strongly influenced by several factors, such as their degree of polymerization (7) and the food matrix. Whereas monomers seem to be readily absorbed in the

small intestine, the absorption of dimeric procyanidins in humans seems to be very limited (8, 9), and polymeric procyanidins are not well absorbed in their native form. These unabsorbed procyanidins reach the colon, where they are largely metabolized by the colonic microbiota, producing a complex phenolic acid profile from the qualitative and quantitative points of view (10–13). Once formed, they are absorbed, further metabolized in the liver, and excreted in urine. In vitro studies showed that phenolic acids inhibited the secretion of proinflammatory cytokine involved in atherosclerosis from LPS-induced human PBMC (14), and they showed the highest effect on modulation of NF-κB activity with strong inhibition of LPS-induced NF-κB activity (15). Gut microbiota has been largely related to some aspects of the metabolism and human health (16) due to their ability to recover bioactive substances from foods that would otherwise be washed out of the intestinal tract without benefit (17). In this context, microbial metabolome is very important given that a significant proportion of dietary polyphenols reaches the gut intact or scarcely metabolized, being further metabolized by colonic microbiota (18).

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The other important aspect that could modulate the bioavailability of polyphenols is the food matrix. Several studies have provided conflicting evidence about the effect of milk on the bioavailability of polyphenols, specifically flavanols, when looking at their phase II conjugate forms, from different dietary sources such as cocoa or tea (19–22). In this context, a recent study clarified the controversy, suggesting that the possible influence of milk on cocoa flavonoid absorption, and consequently on the urinary excretion of phase II metabolites, is more relevant for drinks with lower flavan-3-ol content, which is typical of many commercial cocoas, than for drinks with higher content (23). Therefore, bearing in mind that milk significantly lowered the excretion of urinary phase II metabolites of flavan-3-ol (23), there is a need to study the effect of milk on the colonic microbial metabolism of the nonabsorbed flavanol fraction that reaches the colon. As far as we know, little information is available about the evaluation of the impact of milk on phenolic acid excretion after consumption of flavan-3-ol-rich sources such as cocoa products. Studies related to the interaction between milk and cocoa polyphenol absorption are important because Spain has the largest consumption of cocoa powder products per person (1668 g/person/year), followed by Norway (1647 g/person/year) and Sweden (1288 g/person/year) [reports of ACNielsen, Euro-monitor International, and Caobisco Association of the Chocolate biscuit and confectionery industries of the European Union (EU)], representing approximately 28% of the total cocoa consumption in this country (24). In addition, cocoa powder is mainly consumed with milk and during breakfast, and this represents the main source of flavonoids in the young population (Family Food Panel, Spain 2005–2006, Taylor Nelson Sofres).

In this context, the aim of this study was to evaluate the effect of milk at a colonic microbial level, studying the urinary excretion of microbial-derived phenolic acids after the intake of a standard portion of cocoa powder with either water or milk in an acute intervention using a targeted quantitative procedure.

MATERIALS AND METHODS

Standards and Reagents. The following compounds (% purity, when available) were used. 3,4-Dihydroxyphenylpropionic acid ($\geq 98\%$), caffeic acid ($\geq 95\%$), ferulic acid ($\geq 98\%$), *p*-coumaric acid ($\geq 98\%$), 3,4-dihydroxyphenylacetic acid (98%), 3-methoxy-4-hydroxyphenylacetic acid (99%), 3-hydroxyphenylacetic acid ($\geq 97\%$), phenylacetic acid ($\geq 98\%$), protocatechuic acid ($> 97\%$), 4-hydroxybenzoic acid ($\geq 98\%$), 3-hydroxybenzoic acid ($\geq 98\%$), hippuric acid (98% purity), ethyl gallate ($\geq 96\%$), creatinine, and β -glucuronidase/sulfatase (from *Helix pomatia*) were purchased from Sigma-Aldrich (St. Louis, MO). 4-Hydroxyhippuric acid ($> 99\%$) was purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Vanillic acid and *m*-coumaric acid were purchased from Extrasynthese (Genay, France). HPLC grade solvents methanol, acetonitrile, and formic acid were purchased from Scharlau (Barcelona, Spain). Hydrochloric acid was purchased from Panreac (Barcelona, Spain).

Subjects and Study Design. Twenty-one nonsmoking healthy volunteers (9 women and 12 men) between 18 and 50 years old with a body mass index of 21.6 ± 2.1 were recruited. None reported any history of cardiovascular risk factors, heart disease, or other medical disease, nor were receiving any medication or vitamin supplement. All gave written informed consent before their inclusion in the trial, and the Institutional Review Board of the Hospital Clinic of Barcelona (Spain) approved the study protocol.

The study was an open, prospective, randomized, crossover feeding trial. Participants were instructed to abstain from polyphenol-rich foods for at least 48 h before and during the intervention day. After overnight fasting, the 21 subjects were provided randomly with a single dose of 40 g of cocoa powder with 250 mL of water (hereafter termed CW) or with 250 mL of milk (hereafter termed CM). This protocol was repeated after 1 week following a crossover experimental design. Test meals were

prepared each day of the study following a standardized procedure. Four hours after the cocoa intake, a light meal of bread and cheese was provided. Urine samples were collected before ingestion and in the periods of 0–6, 6–12, and 12–24 h. They were stored at -80°C until analysis.

The CM and CW macronutrient composition (in 250 mL), respectively, was as follows: carbohydrates, 30.75 and 58.40 g; fat, 10.91 and 2.16 g; protein, 13.54 and 5.64 g; energy, 275.35 and 276.6 kcal. The phenolic composition (mean \pm SD) of the cocoa powder was 0.71 ± 0.09 mg/g of (–)-epicatechin, 0.21 ± 0.01 mg/g of (+)-catechin, 0.64 ± 0.06 mg/g of procyanidin B2, 33.87 $\mu\text{g/g}$ of isoquercitrin, 5.74 $\mu\text{g/g}$ of quercetin, 4.33 $\mu\text{g/g}$ of quercetin-3-glucuronide, and 36.32 $\mu\text{g/g}$ of quercetin-3-arabinoside (8). The milk had no phenolic compounds.

Extraction of Phenolic Acids from Urine. The extraction of phenolic acids from the urine was carried out following a validated and published method (8). Briefly, after hydrolysis of the urine samples with β -glucuronidase/sulfatase, they were loaded into preconditioned Oasis MCX 96-well plates for solid-phase extraction (Waters, Milford, MA), washed, and extracted with methanol. Then, they were evaporated to dryness and reconstituted with 100 μL of mobile phase.

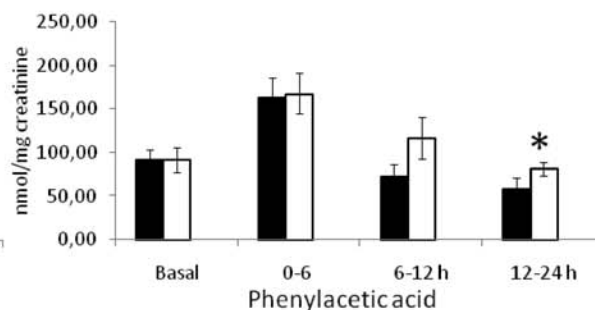
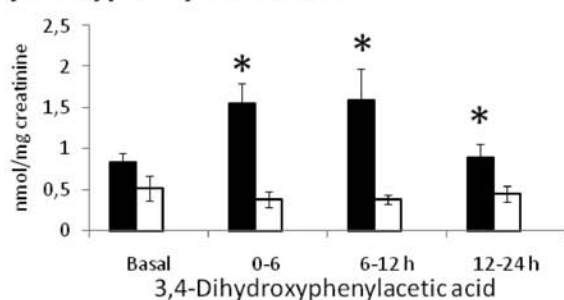
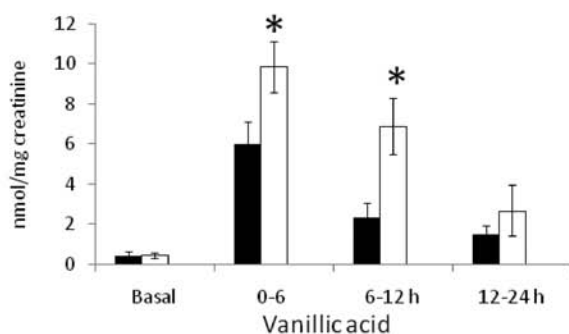
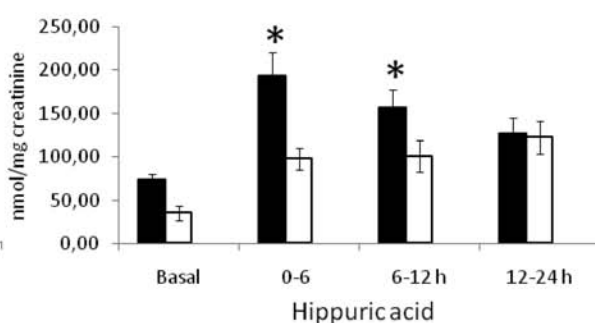
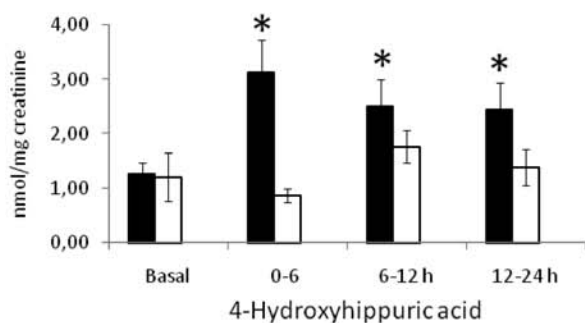
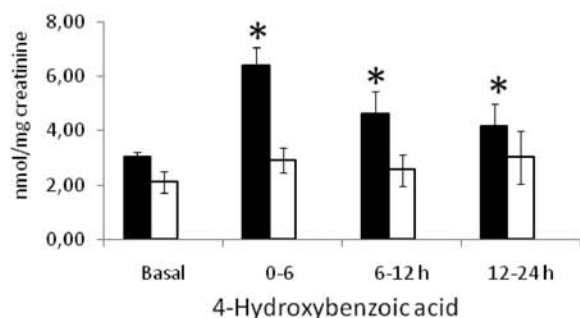
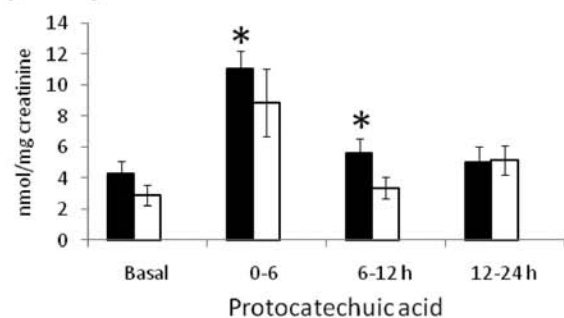
LC-MS/MS. The analyses were performed with liquid chromatography–tandem mass spectrometry (LC-MS/MS). LC-PAD analyses were performed using a Perkin-Elmer series 200 (Norwalk, CT) equipped with a quaternary pump and a refrigerated autosampler plate. An Applied Biosystems API 3000 triple-quadrupole mass spectrometer (PE Sciex, Concord, ON, Canada) equipped with a Turbo IonSpray ionizing in negative mode was used. A Phenomenex Luna C₁₈ analytical column [50 \times 2.0 mm i.d., 5 μm] (Torrance, CA) with mobile phases A (95% water, 5% acetonitrile, and 0.1% formic acid) and B (100% acetonitrile and 0.1% formic acid) was used. The gradient, at a flow rate of 400 $\mu\text{L}/\text{min}$, was as follows: 0–1 min, 4–40% B; 1–3 min, 40–100% B; 3–5 min, 100% B; 6–10 min, 4% B (8). Finally, the column was washed and re-equilibrated for 6 min. The injected volume was 15 μL . MS/MS parameters used were as follows: capillary voltage, -3700 V; focusing potential, -200 V; entrance potential, -10 V; declustering potential, -50 V; nebulizer gas, 10 (arbitrary units); curtain gas, 12 (arbitrary units); collision gas, 5 (arbitrary units); auxiliary gas temperature, 400°C ; auxiliary gas flow rate, 6000 cm^3/min . The collision energy for each compound was as published previously (8). For quantification of phenolic acids, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific for each compound as follows: 3,4-dihydroxyphenylpropionic acid (181/137); caffeic acid (179/135); ferulic acid (193/134); *m*- and *p*-coumaric acids (163/119); 3,4-dihydroxyphenylacetic acid (167/123); 3-methoxy-4-hydroxyphenylacetic acid (181/137); 3-hydroxyphenylacetic acid (151/107); phenylacetic acid (135/91); protocatechuic acid (153/109); 3- and 4-hydroxybenzoic acids (137/93); 4-hydroxyhippuric acid (194/100); vanillic acid (167/152). A dwell time of 80 ms was used for each MRM transition. Hippuric acid was analyzed by PAD at 240 nm (8).

Human urine creatinine concentrations were measured by a colorimetric assay using picric acid (25).

Statistical Analysis. The SPSS Statistical Analysis System, ver. 15.0 (SPSS), was used to perform the statistical analysis. Because the data were nonparametric (Kolmogorov test) and presented nonhomogeneous variances (Levene test), the Wilcoxon test for related samples was used to compare changes in outcome variables in response to (A) both tested meals at each time period and (B) differences between time periods in each tested meal. Concentration levels were expressed as means (nmol/mg creatinine) \pm standard error of the mean (SEM). Differences with $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

In this work we evaluated the excretion of 15 phenolic acids, which had been described in the microbial degradation pathway of flavanols and is well-known in the literature (10–13, 18, 26). The effect of milk on the urinary excretion of microbial-derived phenolic acids after 40 g of cocoa powder consumed with 250 mL of water or 250 mL of whole milk is presented in **Figure 1**. Of the 15 studied phenolic acids, 9 showed significant differences as a function of the consumption of CW or CM (**Figure 1** and **Table 1**).

Hydroxyphenylacetic acids**Hydroxybenzoic acids**

■ Cocoa with water
□ Cocoa with milk

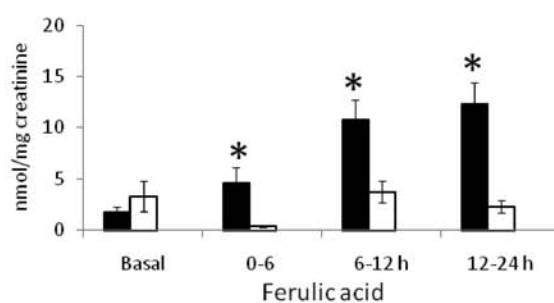
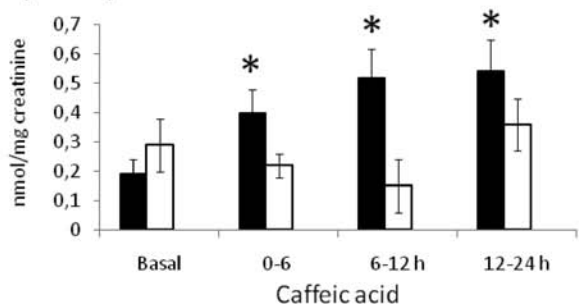
Hydroxycinnamic acids

Figure 1. Urinary excretion (mean \pm SEM) of the nine phenolic acids significantly affected by the consumption of cocoa powder with water or with milk. Bars with an asterisk are significantly different ($P < 0.05$; Wilcoxon's test; $n = 21$) in the same time period.

Table 1. Urinary Excretion of Phenolic Acid Metabolites after Consumption of Cocoa Powder with 250 mL of Water or Cocoa Powder with 250 mL of Milk^a

phenolic acid	cocoa powder with water (nmol/mg of creatinine)				cocoa powder with milk (nmol/mg of creatinine)			
	baseline	0–6 h	6–12 h	12–24 h	baseline	0–6 h	6–12 h	12–24 h
hydroxyphenylpropionic acids								
3,4-dihydroxyphenylpropionic acid	5.96 ± 1.07 a	16.84 ± 2.81 b	7.92 ± 1.49 a	6.62 ± 1.67 a	3.37 ± 0.96 a	16.9 ± 4.00 b	5.19 ± 1.17 a	8.1 ± 1.21 c
hydroxyphenylacetic acids								
3,4-dihydroxyphenylacetic acid	0.84 ± 0.11 ac	1.55 ± 0.25 b	1.60 ± 0.37 b,c	0.89 ± 0.17 a	0.56 ± 0.15 a	0.38 ± 0.10 a	0.38 ± 0.05 a	0.45 ± 0.10 a
3-methoxy-4-hydroxyphenylacetic acid	4.37 ± 0.50 a	7.67 ± 1.05 ab	11.30 ± 1.63 b	8.45 ± 0.94 a	3.99 ± 0.31 a	6.95 ± 0.57 b	9.30 ± 0.74 b	8.05 ± 1.15 b
3-hydroxyphenylacetic acid	3.45 ± 0.73 a	5.25 ± 2.04 a	7.34 ± 2.23 a	10.1 ± 3.42 b	3.70 ± 1.16 ab	1.71 ± 0.23 a	4.12 ± 0.62 b	7.36 ± 1.61 b
phenylacetic acid	91.35 ± 11.36 a	163.77 ± 22.10 b	71.90 ± 14.78 ac	58.85 ± 11.94 c	91.80 ± 14.34 ab	167.72 ± 23.80 a	116.64 ± 24.47 b	81.37 ± 7.83 b
hydroxybenzoic acids								
protocatechuic acid	4.33 ± 0.77 a	11.07 ± 1.19 b	5.58 ± 0.97 a	5.05 ± 0.99 a	2.90 ± 0.65 a	8.88 ± 2.20 b	3.36 ± 0.69 a	5.18 ± 0.92 c
4-hydroxybenzoic acid	3.02 ± 0.20 a	6.39 ± 0.66 b	4.60 ± 0.85 a	4.16 ± 0.84 a	2.10 ± 0.40 a	2.92 ± 0.47 a	2.56 ± 0.58 a	3.01 ± 0.97 a
3-hydroxybenzoic acid	0.11 ± 0.03 a	0.24 ± 0.03 b	0.56 ± 0.24 abc	0.55 ± 0.17 c	0.18 ± 0.10 a	0.25 ± 0.41 a	0.41 ± 0.26 a	0.60 ± 0.28 b
4-hydroxyhippuric acid	1.26 ± 0.20 a	3.13 ± 0.60 b	2.51 ± 0.50 b	2.44 ± 0.51 b	1.21 ± 0.44 ab	0.87 ± 0.12 a	1.76 ± 0.30 b	1.39 ± 0.33 a
hippuric acid	73.05 ± 6.35 a	193.16 ± 27.89 b	156.14 ± 21.93 bc	126.55 ± 18.00 c	35.43 ± 8.32 a	97.92 ± 12.15 b	100.62 ± 18.63 b	122.82 ± 18.83 b
vanillic acid	0.41 ± 0.24 a	5.96 ± 1.15 b	2.29 ± 0.74 c	1.49 ± 0.41 c	0.43 ± 0.15 a	9.85 ± 1.27 b	6.88 ± 1.40 b	2.67 ± 1.26 c
hydroxycinnamic acids								
caffeic acid	0.19 ± 0.05 a	0.40 ± 0.08 b	0.52 ± 0.10 b	0.54 ± 0.11 b	0.29 ± 0.09 abc	0.22 ± 0.04 a	0.15 ± 0.09 b	0.36 ± 0.09 c
ferulic acid	1.72 ± 0.52 a	4.62 ± 1.48 a	10.72 ± 1.97 b	12.35 ± 2.07 b	3.27 ± 1.49 ab	0.39 ± 0.06 a	3.73 ± 1.03 b	2.31 ± 0.57 b
<i>p</i> -coumaric acid	0.004 ± 0.001 a	0.009 ± 0.003 b	0.004 ± 0.002 a	0.005 ± 0.002 a	0.004 ± 0.001 a	0.007 ± 0.002 b	0.003 ± 0.001 a	0.004 ± 0.002 a
<i>m</i> -coumaric acid	0.007 ± 0.001 a	0.024 ± 0.006 a	0.100 ± 0.060 a	0.014 ± 0.007 a	0.007 ± 0.003 a	0.006 ± 0.006 a	0.040 ± 0.015 a	0.013 ± 0.004 a

^a All values are means ± SEMs; *n* = 21. Values in the same row and in the same intervention with different letters are significantly different, *P* < 0.05 (Wilcoxon's test).

With respect to the group of hydroxyphenylacetic acids, their colonic formation could occur mainly from the direct degradation of procyanidins (13). Among the group of hydroxyphenylacetic acids, 3,4-dihydroxyphenylacetic and phenylacetic acids showed significant changes as a function of the food matrix. Significantly lower levels of 3,4-dihydroxyphenylacetic acid were observed after CM when compared with CW in all of the urine fractions after intake. The major differences (~76%) were observed at 0–6 and 6–12 h fractions, and a lower difference (49%) was also observed at 12–24 h (Figure 1). Therefore, these results showed different excretion kinetics for this compound between the two intakes, with no increase in the excretion when CM was taken but increasing at 0–6 h and remaining constant up to 12 h after the consumption of CW (Table 1). This could mean that procyanidins from CW could be bioavailable for the intestinal microbiota, but not from CM, and degraded easily and rapidly, forming the 3,4-dihydroxyphenylacetic acid.

The excretion of phenylacetic acid showed a matrix effect in the last time period (12–24 h), although the same tendency was also observed at 6–12 h but was not statistically significant. Contrary to other hydroxyphenylacetic acids, its excretion at 12–24 h was significantly incremented at around 38% when volunteers consumed CM with respect those who consumed CW (Figure 1). Due to the major urinary excretion after CM, we could hypothesize that phenylacetic acid could come from cocoa phenylalanine, at least partially. During the roasting of cocoa there is a formation of biogenic amines (phenylethylamine) from cocoa amino acids (phenylalanine) (27). This phenylethylamine after CM could be absorbed more easily and rapidly than after CW. In the liver, phenylethylamine is oxidized by aldehyde dehydrogenase and aldehyde oxidase, producing phenylacetaldehyde, which is mainly metabolized to phenylacetic acid (28).

For the remaining hydroxyphenylacetic acids, such as 3-methoxy-4-hydroxyphenylacetic acid and 3-hydroxyphenylacetic acid, no significant changes were observed between the consumptions of CW or CM, although the same kinetic tendency was observed (Table 1).

With respect to hydroxybenzoic acids, the urinary levels of protocatechuic, 4-hydroxybenzoic, 4-hydroxyhippuric, hippuric, and vanillic acids were significantly affected by the matrix.

The formation of protocatechuic acid has been described through β -oxidation of 3,4-dihydroxyphenylpropionic acid (12) or through α -oxidation of 3,4-dihydroxyphenylacetic acid (18). A similar kinetic pattern was observed for protocatechuic acid in both intakes, reaching the maximum concentration at 0–6 h (Figure 1). Moreover, protocatechuic acid excretion was affected by milk intake similar to the 3,4-dihydroxyphenylacetic acid. These data also suggest that although the microbial degradation pathway has been partially elucidated, the exact origin of certain specific phenolic acids such as protocatechuic acid remains to be studied. Lower levels of protocatechuic acid were observed after CM when compared with CW at 0–6 and 6–12 h periods (20 and 40%, respectively), whereas no significant differences were detected in the 12–24 h period. Concentrations of protocatechuic acid showed a significant increase in excretion at 0–6 h with CW, but with CM significant increases occurred at 0–6 and 12–24 h.

Microbial dehydroxylation of protocatechuic acid could give rise to 4-hydroxybenzoic acid derivative that may undergo glycination in the liver and kidney being converted into 4-hydroxyhippuric acid (12). Both 4-hydroxybenzoic and 4-hydroxyhippuric acids showed lower excretion after CM when compared with CW in all of the collected fractions (from 28 to 72% of diminution) (Figure 1 and Table 1). When their kinetic patterns were taken into account, the excretion of both compounds did not vary in the time when CM was taken. However, variations in excretion were observed for 4-hydroxybenzoic acid when CW was consumed, reaching the maximum concentration at 0–6 h. Higher levels of 4-hydroxyhippuric acid were observed in all urine fractions after CW when compared with baseline. Both compounds, 4-hydroxybenzoic and 4-hydroxyhippuric, have been previously reported to arise from the microbial degradation of procyanidin dimer B3 in rats (12). Particularly, 4-hydroxybenzoic acid has also been detected after the administration of wine powder, catechin, and dimer B3 diets to rats (12, 26).

The excretion of hippuric acid, which is most likely derived from the glycination of benzoic acid, was also diminished by the milk. The maximum difference between CM and CW was seen at 0–6 h (49% of diminution after CM), followed by a 36% of diminution at 6–12 h, whereas there was no significant difference

at 12–24 h. This implies that the difference in excretion of hippuric acid between CM and CW decreased over time and was less affected by milk as its metabolism progressed. Urinary excretion of hippuric acid has been associated with the microbial degradation of many phenolic compounds such as hydroxycinnamic acids (29) or flavanoids present in foods such as black or green tea (29, 30), wine powder (26), and sorghum bran (31), and it is also common to the metabolism of other dietary components, which makes it difficult to pinpoint its origin.

With regard to hydroxycinnamic acids, caffeic acid and its methylated derivative (ferulic acid) arising from its subsequent liver metabolism were excreted to a lesser extent after CM than after CW. This behavior was observed in all collected urine fractions, representing 50% (mean value) and 80% (mean value) of diminution for caffeic acid and ferulic acid, respectively (Figure 1). As could be observed from Table 1 and Figure 1, the increment in concentration found in the last fraction (12–24 h) for both caffeic and ferulic acids suggested that a longer period of urine collection was required for their complete excretion. Previously, Rios et al. (11) studied the urinary excretion of phenolic acids after acute consumption of 80 g of chocolate with bread and water. Similar to our results, they observed a significant increase of ferulic acid excretion at 9 h after consumption. According to the flavanol microbial degradation pathway partially elucidated by several authors (10, 12, 13) and the present results, it could be proposed that these compounds could come from several routes of metabolism, which could include dehydrogenation of 3,4-dihydroxyphenylpropionic acid as well as degradation from other phenolic derivatives present in cocoa powder, such as chlorogenic acid or *N*-phenylpropenoyl-L-amino acids (11). *N*-Phenylpropenoyl-L-amino acids are hydroxycinnamic acid amides of aspartic acid, tyrosine, and 3-hydroxytyrosine (3). In fact, Rios et al. (11) proposed that the urinary excretion of ferulic acid after consumption of chocolate was associated with the clovamide [(-)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine] content of cocoa foods.

Whereas a higher global urinary excretion of phenolic acids was observed with consumed CW, vanillic acid showed an excretion pattern contrary to those of other metabolites. This compound was excreted in higher significant amounts after consumption of CM at 0–6 and 6–12 h periods when compared with CW. This result could mean that liposoluble vanillin, when ingested with CM, could be absorbed more easily and rapidly than after its intake with CW. Vanillin, a flavoring product added in cocoa preparations (10, 11), could be oxidized to vanillic acid by aldehyde oxidase of the liver (32), although it could also arise from the methylation of protocatechuic acid.

As far as we know, little information is available concerning studies about the effect of milk in the production of phenolic acids by colonic microbiota after polyphenol-rich food consumption. Recently, Serafini et al. (33) studied the effect of milk on the plasma antioxidant capacity and on the plasma levels of ferulic and caffeic acids after the consumption of 200 g of blueberries with 200 mL of water or with 200 mL of whole milk in human volunteers. They observed enhanced plasmatic concentration of caffeic and ferulic acids when blueberries were consumed with water, whereas no increase in plasma antioxidant capacity and reduced plasmatic levels of these metabolites were observed when consumed with milk (33). There is some controversy about the effect of milk on the plasma antioxidant capacity as Lotito et al. (34) have reviewed in 2006. It has been suggested that in whole milk, with a higher fat content than skimmed milk, polypeptide chains are located around the fat globule membrane, which promotes their linkage with phenolic compounds (33, 35) and probably causes the decrease of accessibility by the colonic

microbiota and therefore their degradation. Also, recently the same behavior was observed with yogurt. Roowi et al. (36) determined the excretion of 9 phenolic acids after the consumption of orange juice with and without natural fat yogurt, 5 of which were associated with orange juice consumption (3-hydroxyphenylacetic acid, 3-hydroxyphenylhydracrylic acid, dihydroferulic acid, 3-methoxy-4-hydroxyphenylhydracrylic acid, and 3-hydroxyhippuric). The 24 h excretion of these 5 phenolic acids diminished from 62 ± 18 to 9.3 ± 4.4 μmol when orange juice was ingested with yogurt.

In conclusion, among the 15 studied compounds, the excretion of 9 phenolic acids was significantly affected by the matrix in which the cocoa powder was dissolved (water or milk). According to the results, these 9 phenolic acids can be distributed in two groups: (1) positively affected by milk intake, vanillic acid and phenylacetic acid; and (2) negatively affected by milk intake, (2a) 3,4-dihydroxyphenylacetic acid, protocatechuic acid, 4-hydroxybenzoic acid, 4-hydroxyhippuric acid, and hippuric acid, and (2b) caffeic and ferulic acids probably related to the metabolism of hydroxycinnamic acid amides present in cocoa products as well as to other compounds. These results showed that milk had a partial effect on the phenolic acid excretion profile after the consumption of cocoa powder.

ABBREVIATIONS USED

PAD, photodiode array detector; MRM, multiple reaction monitoring; CM, cocoa with milk; CW, cocoa with water.

SAFETY

Guidelines for work with organic solvents and acids were respected. Universal precautions for the handling of chemicals and fluids were applied.

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LITERATURE CITED

- (1) Corti, R.; Flammer, A. J.; Hollenberg, N. K.; Luscher, T. F. Cocoa and cardiovascular health. *Circulation* **2009**, *119*, 1433–1441.
- (2) Cooper, K. A.; Donovan, J. L.; Waterhouse, A. L.; Williamson, G. Cocoa and health: a decade of research. *Br. J. Nutr.* **2008**, *99*, 1–11.
- (3) Tomas-Barberan, F. A.; Cienfuegos-Jovellanos, E.; Marin, A.; Muguerza, B.; Gil-Izquierdo, A.; Cerda, B.; Zafrilla, P.; Morillas, J.; Mulero, J.; Ibarra, A.; Pasamar, M. A.; Ramon, D.; Espin, J. C. A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. *J. Agric. Food Chem.* **2007**, *55*, 3926–3935.
- (4) Rusconi, M.; Conti, A. *Theobroma cacao* L., the food of the gods: a scientific approach beyond myths and claims. *Pharmacol. Res.* **2010**, *61*, 5–13.
- (5) Gu, L.; House, S. E.; Wu, X.; Ou, B.; Prior, R. L. Procyanidin and catechin contents and antioxidant capacity of cocoa and chocolate products. *J. Agric. Food Chem.* **2006**, *54*, 4057–4061.
- (6) Williamson, G.; Manach, C. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am. J. Clin. Nutr.* **2005**, *81*, 243S–255S.
- (7) Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727–747.
- (8) Urpi-Sarda, M.; Monagas, M.; Khan, N.; Lamuela-Raventos, R. M.; Santos-Buelga, C.; Sacanella, E.; Castell, M.; Permanyer, J.; Andres-Lacueva, C. Epicatechin, procyanidins, and phenolic microbial metabolites after cocoa intake in humans and rats. *Anal. Bioanal. Chem.* **2009**, *394*, 1545–1556.

- (9) Holt, R. R.; Lazarus, S. A.; Sullards, M. C.; Zhu, Q. Y.; Schramm, D. D.; Hammerstone, J. F.; Fraga, C. G.; Schmitz, H. H.; Keen, C. L. Procyanidin dimer B2 [epicatechin-(4 β -8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa. *Am. J. Clin. Nutr.* **2002**, *76*, 798–804.
- (10) Urpi-Sarda, M.; Monagas, M.; Khan, N.; Llorach, R.; Lamuela-Raventos, R. M.; Jauregui, O.; Estruch, R.; Izquierdo-Pulido, M.; Andres-Lacueva, C. Targeted metabolic profiling of phenolics in urine and plasma after regular consumption of cocoa by liquid chromatography–tandem mass spectrometry. *J. Chromatogr., A* **2009**, *1216*, 7258–7267.
- (11) Rios, L. Y.; Gonthier, M. P.; Remesy, C.; Mila, I.; Lapiere, C.; Lazarus, S. A.; Williamson, G.; Scalbert, A. Chocolate intake increases urinary excretion of polyphenol-derived phenolic acids in healthy human subjects. *Am. J. Clin. Nutr.* **2003**, *77*, 912–918.
- (12) Gonthier, M. P.; Donovan, J. L.; Texier, O.; Felgines, C.; Remesy, C.; Scalbert, A. Metabolism of dietary procyanidins in rats. *Free Radical Biol. Med.* **2003**, *35*, 837–844.
- (13) Appeldoorn, M. M.; Vincken, J. P.; Aura, A. M.; Hollman, P. C.; Gruppen, H. Procyanidin dimers are metabolized by human microbiota with 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)- γ -valerolactone as the major metabolites. *J. Agric. Food Chem.* **2009**, *57*, 1084.
- (14) Monagas, M.; Khan, N.; Andres-Lacueva, C.; Urpi-Sarda, M.; Vazquez-Agell, M.; Lamuela-Raventos, R.; Estruch, R. Hydroxylated phenolic acids derived from microbial metabolism reduce lipopolysaccharide-stimulated cytokine secretion by human peripheral blood mononuclear cells. *Br. J. Nutr.* **2009**, *102*, 201–206.
- (15) Hole, A. S.; Grimmer, S.; Naterstad, K.; Jensen, M. R.; Paur, I.; Johansen, S. G.; Balstad, T. R.; Blomhoff, R.; Sahlstrom, S. Activation and inhibition of nuclear factor κ B activity by cereal extracts: role of dietary phenolic acids. *J. Agric. Food Chem.* **2009**, *57*, 9481–9488.
- (16) Wikoff, W. R.; Anfora, A. T.; Liu, J.; Schultz, P. G.; Lesley, S. A.; Peters, E. C.; Siuzdak, G. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 3698–3703.
- (17) Jacobs, D. M.; Deltimple, N.; van Velzen, E.; van Dorsten, F. A.; Bingham, M.; Vaughan, E. E.; van Duynhoven, J. (1)H NMR metabolite profiling of feces as a tool to assess the impact of nutrition on the human microbiome. *NMR Biomed.* **2008**, *21*, 615–626.
- (18) Selma, M. V.; Espin, J. C.; Tomas-Barberan, F. A. Interaction between phenolics and gut microbiota: role in human health. *J. Agric. Food Chem.* **2009**, *57*, 6485–6501.
- (19) Schroeter, H.; Holt, R. R.; Orozco, T. J.; Schmitz, H. H.; Keen, C. L. Nutrition: milk and absorption of dietary flavanols. *Nature* **2003**, *426*, 787–788.
- (20) Serafini, M.; Bugianesi, R.; Maiani, G.; Valtuena, S.; De, S. S.; Crozier, A. Plasma antioxidants from chocolate. *Nature* **2003**, *424*, 1013.
- (21) Roura, E.; Andres-Lacueva, C.; Estruch, R.; Lourdes Mata, B. M.; Izquierdo-Pulido, M.; Lamuela-Raventos, R. M. The effects of milk as a food matrix for polyphenols on the excretion profile of cocoa (–)-epicatechin metabolites in healthy human subjects. *Br. J. Nutr.* **2008**, *100*, 846–851.
- (22) Serafini, M.; Ghiselli, A.; Ferro-Luzzi, A. In vivo antioxidant effect of green and black tea in man. *Eur. J. Clin. Nutr.* **1996**, *50*, 28–32.
- (23) Mullen, W.; Borges, G.; Donovan, J. L.; Edwards, C. A.; Serafini, M.; Lean, M. E.; Crozier, A. Milk decreases urinary excretion but not plasma pharmacokinetics of cocoa flavan-3-ol metabolites in humans. *Am. J. Clin. Nutr.* **2009**, *89*, 1784–1791.
- (24) Andres-Lacueva, C.; Monagas, M.; Khan, N.; Izquierdo-Pulido, M.; Urpi-Sarda, M.; Permanyer, J.; Lamuela-Raventos, R. M. Flavanol and flavonol contents of cocoa powder products: influence of the manufacturing process. *J. Agric. Food Chem.* **2008**, *56*, 3111–3117.
- (25) Roura, E.; Andres-Lacueva, C.; Estruch, R.; Lamuela-Raventos, R. M. Total polyphenol intake estimated by a modified Folin–Ciocalteu assay of urine. *Clin. Chem.* **2006**, *52*, 749–752.
- (26) Gonthier, M. P.; Cheynier, V.; Donovan, J. L.; Manach, C.; Morand, C.; Mila, I.; Lapiere, C.; Remesy, C.; Scalbert, A. Microbial aromatic acid metabolites formed in the gut account for a major fraction of the polyphenols excreted in urine of rats fed red wine polyphenols. *J. Nutr.* **2003**, *133*, 461–467.
- (27) Granvogl, M.; Bugan, S.; Schieberle, P. Formation of amines and aldehydes from parent amino acids during thermal processing of cocoa and model systems: new insights into pathways of the strecker reaction. *J. Agric. Food Chem.* **2006**, *54*, 1730–1739.
- (28) Panoutsopoulos, G. I. Metabolism of 2-phenylethylamine to phenylacetic acid, via the intermediate phenylacetaldehyde, by freshly prepared and cryopreserved guinea pig liver slices. *In Vivo* **2004**, *18*, 779–786.
- (29) Olthof, M. R.; Hollman, P. C.; Buijsman, M. N.; van Amelsvoort, J. M.; Katan, M. B. Chlorogenic acid, quercetin-3-rutinoside and black tea phenols are extensively metabolized in humans. *J. Nutr.* **2003**, *133*, 1806–1814.
- (30) Mulder, T. P.; Rietveld, A. G.; van Amelsvoort, J. M. Consumption of both black tea and green tea results in an increase in the excretion of hippuric acid into urine. *Am. J. Clin. Nutr.* **2005**, *81*, 256S–260S.
- (31) Gu, L.; House, S. E.; Rooney, L.; Prior, R. L. Sorghum bran in the diet dose dependently increased the excretion of catechins and microbial-derived phenolic acids in female rats. *J. Agric. Food Chem.* **2007**, *55*, 5326–5334.
- (32) Panoutsopoulos, G. I.; Beedham, C. Enzymatic oxidation of vanillin, isovanillin and protocatechuic aldehyde with freshly prepared guinea pig liver slices. *Cell Physiol. Biochem.* **2005**, *15*, 89–98.
- (33) Serafini, M.; Testa, M. F.; Villaño, D.; Pecorari, M.; van Wieren, K.; Azzini, E.; Brambilla, A.; Maiani, G. Antioxidant activity of blueberry fruit is impaired by association with milk. *Free Radical Biol. Med.* **2009**, *46*, 769–774.
- (34) Lotito, S. B.; Frei, B. Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: cause, consequence, or epiphenomenon? *Free Radical Biol. Med.* **2006**, *41*, 1727–1746.
- (35) Patton, S.; Keenan, T. W. The milk fat globule membrane. *Biochim. Biophys. Acta* **1975**, *415*, 273–309.
- (36) Roowi, S.; Mullen, W.; Edwards, C. A.; Crozier, A. Yoghurt impacts on the excretion of phenolic acids derived from colonic breakdown of orange juice flavanones in humans. *Mol. Nutr. Food Res.* **2009**, *53* (Suppl. 1), S68–S75.

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