1 **Dietary Epicatechin Is Available to Breastfed Infants through Human Breast**
2 **Milk in the Form of Host and Microbial Metabolites**

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ABSTRACT

Polyphenols play an important role in human health. To address their accessibility to a breastfed infant, we planned to evaluate whether breast milk (BM) (colostrum, transitional, and mature) epicatechin metabolites could be related to the dietary habits of mothers. The polyphenol consumption of breastfeeding mothers was estimated using a food frequency questionnaire and 24 h recalls. Solid-phase extraction−ultra performance liquid chromatography−tandem mass spectrometry (SPE−UPLC−MS/MS) was applied for direct epicatechin metabolite analysis. Their bioavailability in BM as a result of dietary ingestion was confirmed in a preliminary experiment with a single dose of dark chocolate. Several host and microbial phase II metabolites of epicatechin were detected in BM among free-living lactating mothers. Interestingly, a modest correlation between dihydroxyvalerolactone sulfate and the intake of cocoa products was observed. Although a very low percentage of dietary polyphenols is excreted in BM, they are definitely in the diet of breastfed infants. Therefore, evaluation of their role in infant health could be further promoted.

INTRODUCTION

Human breast milk (BM) is the optimal food for newborns and infants because it supplies nutrients and biologically active elements that are essential for their development and health.[1] Along with its main nutrients, it also supplies minor compounds, such as vitamins and micronutrients, which play important roles in the development and health of infants.[2] Despite the accumulated knowledge in the field of dietary polyphenols,[3,4] currently, little is known about their bioavailability in human BM of lactating mothers and their accessibility to breastfed infants. Accordingly, the effect of the consumption of dietary polyphenols through BM on the health of infants has not yet been widely studied. Thus far, only a few studies have addressed the question of the excretion of dietary polyphenols in human BM under controlled dietary ingestion,[5−7] and even fewer studies have

addressed their presence in the BM of lactating mothers under free-living conditions.[8,9] However, all of them measured the enzymatically hydrolyzed fraction of corresponding polyphenols, without taking into account the nature of the metabolites available in BM. Recently, the importance of investigating the metabolites of dietary polyphenols was highlighted.[3] To establish the health effects of polyphenol intake by infants, it is essential to have information on their disposition in BM, which is related to the previous dietary intake of a lactating woman. With the intention of assessing the delivery of polyphenols of a dietary origin to infants through breastfeeding, we planned to estimate whether the presence of epicatechin metabolites in BM could be related to the dietary habits of lactating mothers. Cocoa products are among the richest sources of polyphenols in our diets.[10] One of the most well-accepted and widely consumed cocoa products in the world is chocolate. In relation to the objectives of the present study, we consider dark chocolate (DCh) to be an appropriate food element to provide close to real-life ingestion of one of the most representative dietary polyphenols: flavan-3-ols. In addition, the bioavailability of flavanols, especially epicatechin, is one of the most well-understood among dietary polyphenols[11] and has also been extensively studied by our group.[12,13] Therefore, flavanol-rich DCh could be a good dietary choice to approximate the bioavailability and excretion of epicatechin in BM. In addition, a cocoa component in foods is easy to track in the dietary records of free-living subjects as a result of its relatively integral consumption and because its presence in food can be easily recognized and recalled by subjects. Therefore, we anticipate that the presence in BM of epicatechin metabolites might reflect dietary epicatechin consumption of either a total or specifically epicatechin-rich food (e.g., cocoa) origin by lactating women.

MATERIALS AND METHODS

Study Design, Subjects, and Sampling. Preliminary Experiment on Epicatechin BM Bioavailability. Two healthy lactating mothers (aged 32 and 37 and weighing 68 and 63 kg, respectively) in the 6th month of the postpartum period kindly provided their BM (mature milk) at several time points over a 12 h period on a voluntary basis. Neither woman smoked or took medication. The acute ingestion of DCh was carried out in the morning, prior to any other food intake.

The DCh consumed was from a domestic supply, and both volunteers reported on the amount (g) consumed and percentage of cocoa in the chocolate (according to the manufacturer) (Table S1 of the Supporting Information). During the intervention day, no other cocoa-containing products were consumed by either volunteer. The women reported their 24 h dietary habits on the day prior to the collection of BM samples. While total milk was collected from one breast using an electric pump over defined time periods after acute DCh ingestion, babies were nourished from the other breast on demand. The volume of collected BM was recorded. Free-Living Population Study. A total of 11 breastfeeding women (mean age of 33.2 years) under free-living conditions participated in the population study. They were recruited in their 30th week of pregnancy from the Barcelona Science Park and the San Cecilio University Hospital in Granada (Figure 1). Detailed information on the eligibility criteria is disclosed in the Supporting Information. All participants had read the study protocol, approved by the Ethics Committee of San Cecilio University Hospital in Granada and the Bioethics Committee of the University of Barcelona (IRB00003099), and had signed an informed consent form. The samples, metrics, and dietary data collection were performed in both recruiting centers. Manipulation of the samples, evaluation of dietary records, and analyses were performed by the Barcelona University group. A food frequency questionnaire (FFQ) was provided on the day of inclusion in the study (Figure 1). After a baby was born, three types of BM samples, colostrum (1−4 days postpartum), transitional (5−15 days postpartum), and mature (>30 days postpartum), and 24 h dietary recalls (24 h DR) completed on the previous day were collected from each volunteer. Up to 30−50 mL of BM was collected per sample in the morning or through the day using a manual or electric pump. However, in the course of the study, several dietary records and some BM samples were not available (Figure 1). All of the collected BM samples (from the preliminary experiment and population study) were labeled, immediately stored at −20 °C (in home freezers), and shortly afterward transferred in dry ice to the laboratory freezers, where they were stored at −80 °C until analysis.

Dietary Assessment of Polyphenol Intake. Food intake (g/ day) was evaluated using three 24 h DR. Dietary total and individual flavan-3-ol monomer (from here on flavan-3-ols will refer to monomers

97 only) intake was estimated using our food composition database on polyphenols.[14] This was based

98 on three United States Department of Agriculture (USDA) databases[15−17] and the Phenol- Explorer

99 database.[18] The FFQ, developed and validated in the Spanish population,[19] was applied on the day

100 of enrolment to evaluate food intake (g/day) over the previous 3 months by pregnant women in a free-

101 living population study.

102 **Determination of Epicatechins and Their Metabolites in BM Samples.** On the day of analysis, a

103 1 mL aliquot of the BM sample was thawed in an ice bath, acidified with 25 μL of phosphoric acid

104 (35%), sonicated for 15 min at room temperature, and afterward centrifuged at 15900g for 10 min at

105 4 °C. Through gentle aspiration, 0.8 mL of acidified aqueous phase of BM was collected and used

106 for solid-phase extraction (SPE). The concentrations of (−)-epicatechin (EpiCat), O-methyl-

107 epicatechin (MetEpiCat), and colon microbiotagenerated metabolites, such as 5-(3′ ,4′ -

108 dihydroxyphenyl)-γ-valerolactone (DHPV) and 5-(3′ -methoxy-4′ -hydroxyphenyl)-γ-

109 valerolactone (MHPV), along with their corresponding phase II conjugate sulfates (Sulf) and

110 glucuronides (Gluc) in BM, were analyzed according to the earlier published methodology,[20] with a

111 slightly modified elution step. Then, the retained epicatechin metabolites were eluted by applying 0.5

112 mL of basic methanol (0.1% ammonia) after 1 mL of acidified methanol (0.1% formic acid) to

113 optimize both glucuronide and sulfate extraction (>75% recovery; data not shown).[21] The

114 reconstituted-inmobile- phase samples were used for ultra performance liquid chromatography−

115 tandem mass spectrometry (UPLC−MS/MS) analysis. Ethyl gallate served as the internal standard

116 (IS). The previously established methodology was used for UPLC−MS/MS analysis of epicatechin

117 metabolites.[12,20] The epicatechin metabolites were identified according to the available standards

118 (EpiCat, Cat, MetEpiCat, EpiCat-Gluc-2, and EpiCat-Sulf-1) and as a result of the previously

119 described mass chromatographic behavior of compounds[12,20] (Table S2 of the Supporting

120 Information). Calibration curves were constructed in the range of 5−2000 μg/L with available

121 standards in aqueous extracts of BM and subjected to the same procedure as the samples. As a result

122 of the fact that standards for phase II metabolites were not available, the concentrations of all sulfated,

123 glucuronidated, and methylated metabolites were approximated using curves of in-house synthesized

124 EpiCat-Sulf-1, EpiCat-Gluc-2, and MetEpiCat, respectively, and the results were expressed as their

125 equivalents.[20] These compounds were synthesized and characterized as previously published.[22] The

126 purity of purchased standards [(−)-epicatechin, (+)-catechin, and ethyl gallate from Sigma-Aldrich

127 (St. Louis, MO)] was of analytical grade, and the purity of synthesized standards was ≥98, ≥98, and

128 ≥96%, respectively, as reported in our previous studies applying this methodology.[12,20]

129 **Statistical Analysis.** Dietary intake of polyphenols is shown as the mean and standard deviation

130 (SD). The contribution of each food to total dietary polyphenols and flavan-3-ol intake, in total and

131 specifically that of cocoa origin, and the contribution of flavan-3-ol to the polyphenol intake were

132 calculated as a percentage. The Friedman test was used to compare the consumption of polyphenols

133 among the three phases of lactation. We used Spearman correlations to assess whether flavan-3-ol

134 and epicatechin intakes were correlated to DHPVSulf- 2. SPSS statistical analysis system, version

135 18.0 (SPSS), was used, and the significance level for the performed statistical analyses was <0.05.

136 **RESULTS AND DISCUSSION**

137 **Epicatechin Metabolites in BM after Acute DCh Ingestion**. In accordance with the habitual dietary

138 consumption evaluated by 24 h DR collected on pre-intervention day, DCh was the principal dietary

139 source of flavan-3-ols (78− 92%) and epicatechin (92−96%) consumed during the intervention day

140 by both volunteers (Table S1 of the Supporting Information). In both lactating women, the acute DCh

141 intervention was estimated to provide an intake of around 80 mg of DCh-derived flavan-3-ol

142 monomers, of which about 48% corresponded to epicatechin (Table S1 of the Supporting

143 Information). Therefore, the DCh epicatechin content was estimated to be within the ranges reported

144 for other commercially available DChs.[23]

145 The DCh intake challenge led to the direct identification of two groups of epicatechin metabolites in

146 the BM samples of both volunteers collected over 12 h after ingestion: (i) host metabolites of

147 epicatechin, EpiCat-Sulf-1 and EpiCat-Sulf-2, EpiCat-Gluc-4, and Met-EpiCat-Sulf-3, and (ii)

148 microbial metabolites, DHPV-Sulf-2, DHPV-Gluc-1 and DHPV-Gluc-2, MHPV-Sulf-1 and MHPV-

149 Sulf-2, and MHPV-Gluc-1.12,20 The selected chromatograms of the detected epicatechin

150 metabolites in the BM samples are represented in Figure S1 of the Supporting Information.

151   BM is a special biological compartment linked closely to plasma, but on the other hand, similar to

152 urine, it can accumulate excreted compounds.24 Although we did not have corresponding standards,

153 it is very likely that the main detected host metabolites correspond to the major metabolites of

154 cocoaderived epicatechin recently described in postprandial plasma and urine:25,26 EpiCat-3′ -O-

155 Gluc (as EpiCat-Gluc-4), EpiCat- 3′ -O-Sulf (as EpiCat-Sulf-2), and 3′ -Methyl-EpiCat-5-O-Sulf

156 (as Met-EpiCat-Sulf-3). In general, their levels in the BM samples were quite low, even at maximum

157 concentration rates (nmol/L) (Figure S1 of the Supporting Information and panels A and B of Figure

158 2), being close to the plasmatic postprandial levels detected after intake of 2 times higher dose of

159 DCh.26 This was also in agreement with the BM ranges of other polyphenols previously reported in

160 dietary intervention volunteers6−8 and in some populations of free-living lactating mothers.5,9 Other,

161 less abundant metabolites could not be detected as a result of their very low, several times lower than

162 detected, metabolite26 concentrations. The changes in the BM concentrations of the detected host

163 metabolites were very similar in both volunteers (Figure 2B).

164   Continuously collected samples from volunteer 2 were used to evaluate the BM excretion kinetics

165 of detected host epicatechin metabolites over 12 post-intervention hours (Figure 2A). The peaks of

166 maximum concentration for each metabolite were defined by visual inspection of excretion graphs.

167 Thus, the highest levels of EpiCat-Sulfs and EpiCat- Gluc metabolites were reached in BM 4 h after

168 DCh intake and after 6 h for Met-EpiCat-Sulf in both sample sets (Figure 2B). Dietary epicatechin

169 was rapidly absorbed in the small intestine, and its metabolites were quickly eliminated from plasma

170 within 6−8 h and excreted in urine.26 Our data showed that the main epicatechin host metabolites

171 were still present in the BM at detectable levels 12 h after DCh ingestion (panels A and B of Figure

172 2). According to the dietary recalls, no other potent source of flavanols other than DCh was consumed

173 on the intervention day by either volunteer (Table S1 of the Supporting Information). Therefore, this

174 accumulation of DChderived host epicatechin metabolites in the BM resembled that previously

175 reported for urine, where they were mostly excreted between the 5th and 10th postprandial hour and

176   were still present at lower concentrations after 10 h.26 Unfortunately, we did not collect either blood

177   or urine samples from the two volunteers within the present study. However, the relation between the

178   plasmatic, urinary, and BM kinetics of epicatechin metabolites should be further studied, because it

179   will indicate in detail the bioavailability, excretion, and accumulation of dietary polyphenols in BM.

180   In addition to host metabolites, the only microbial-derived metabolite of epicatechin, DHPV-Sulf-2,

181   was detected in the set of BM samples of volunteer 2 (Table S3 of the Supporting Information). In

182   contrast, other microbial metabolites were also detected in all of the BM samples of volunteer 1:

183   DHPV-Sulf-1, DHPV-Gluc-2, MHPV-Sulf-1, MHPV-Sulf-2, and MHPV-Gluc- 1, along with the

184   most pronounced DHPH-Sulf-2 metabolite (Figure S1 and Table S3 of the Supporting Information).

185   All of them, except DHPV-Sulf-1, were increasing in concentration toward the end of the BM sample

186   collection period (12 h). Unfortunately, there are no reported data on the urine or plasmatic kinetics

187   of microbial ring-fusion metabolites of cocoaderived epicatechin to be compared to our BM results.

188   According to recently reported data on tea epicatechins,27 first-stage microbial metabolites

189   (valerolactones) appeared in plasmatic circulation with a 2−4 h lag time and at a maximum

190   concentration in 4−8 h, exceeding those of host epicatechin metabolites by 1 order of magnitude.

191   They remained in circulation for about 20 h after single-dose consumption of black tea. On the basis

192   of these pharmacokinetic data, we assume that the BM kinetics of the microbial metabolites could

193   not be evaluated within the present study as a result of the relatively short time for postprandial

194   sample collection. The late appearance of the ring-fusion metabolites in BM is due to the fact that

195   they are generated through microbiota catabolic activities and, as result, are absorbed in the large

196   intestine.28 We assume that, in contrast to volunteer 2, who did not consume any cocoa products

197   prior to the intervention, epicatechin microbial metabolites were detected in the preintervention (0 h)

198   and early (2−8 h) postprandial samples of volunteer 1 most likely as a result of the consumption of

199   DCh from the previous day within her habitual diet (data not shown). In general, our data on microbial

200   epicatechin metabolites are also in line with those previously reported, where the main microbial

201   metabolite in 24 h urine after longterm cocoa powder consumption was DHPV-Sulf-2, along with the

202   main host metabolites EpiCat-Gluc-4, EpiCat-Sulf-2, and Met-EpiCat-Sulf-2.12 In support of our

203   findings, in the study with tea polyphenols, it was observed that sulfated conjugates of valerolactones

204 were almost twice more represented in plasma than glucuronidated forms.[27] Thus, the authors

205 suggested that sulfation was preferred over glucuronidation at the site of valerolactone metabolism.[27]

206 The 2 h fractionated samples from volunteer 2 showed approximated 12 h cumulative excretion of

207 the detected host metabolites in BM. It was not possible to conduct analysis for volunteer 1, because

208 the collection of samples was interrupted as a result of lactating circumstances. Thus, the combined

209 12 h excretion of the host metabolites in BM, expressed in catechin equivalents, was estimated to

210 account for about 0.01% (assuming equal excretion in both breasts) of the chocolateingested

211 epicatechin (Table S3 of the Supporting Information). Considering just non-colonic metabolites, the

212 bioavailability value during the first 12 postprandial hours observed in this study would provide a

213 breastfed child with about 3.8 μg of catechin equivalent. This approximately corresponds to a

214 cumulative dose of 0.772 μg/kg (for a baby weighing 5 kg) spread over 12 h,[29] when the single dose

215 of the lactating mother was estimated to be about 0.61 mg/kg (for a woman weighing 63 kg) as DCh-

216 derived epicatechin. Thus, the dose for the child appears to be 3 orders of magnitude lower than that

217 of the mother. Unfortunately, we cannot take into account the metabolites generated by colonic

218 microbiota for mother and infant dose approximation, although their weight in relation to epicatechin

219 metabolism and disposition is expected to be essential.[27,28] On the other hand, other potential

220 sources of epicatechin and valerolactone phase II metabolites, such as procyanidins,[12] were not

221 considered in our preliminary experiment. Estimation of the accessibility of dietary polyphenols to

222 breastfed infants could be especially relevant in the light of recent reports accentuating their potential

223 to modulate gut microbiota activities.[30,31]

Epicatechin in BM of Free-Living Lactating Women.

225 Among the 11 women participating in our population study, FFQs were collected from 9 volunteers.

226 All three 24 h DRs were available only for 8 volunteers, whereas the second and third 24 h DRs

227 (corresponding to transition and mature milk collections) were available for 10 volunteers; for one of

228 them, it was repeated twice for transition, and for another, it was repeated twice for mature milk

229 collection (Figure 1 and Table S4 of the Supporting Information). From the 33 samples planned to be

230 collected for epicatechin metabolite analysis, 4 colostrum samples and 1 mature milk sample from

different volunteers and all three BM samples from one volunteer were not available as a result of lactating circumstances. In total, 10 volunteers participated in the study, providing 24 BM samples matched to corresponding dietary records (Figure 1 and Table S4 of the Supporting Information). The total polyphenol consumption in our population was 1104.97 ± 465.97 mg/day according to the FFQs, which was higher than that assessed by the 24 h DRs, where the average consumption corresponded to about 751.58 mg/day (Table S5 of the Supporting Information). According to the FFQs and average 24 h DRs, cocoa polyphenols made a moderate contribution to the total dietary polyphenol consumption in our free-living population, 8 and 9%, respectively (Table S5 of the Supporting Information), which was in line with recent data (6.7%) on the south European region.[32] In general, daily flavan- 3-ol consumption by free-living breastfeeding mothers was in the range of 1.8−47.5 mg/day (Table S4 of the Supporting Information), on average 18.29 ± 11.83 mg/day, which was relatively lower than that recently reported for habitual consumption among Spanish adult males (∼24 mg/day)[32] and females (∼26 mg/day).[33] However, the impact of cocoa products on the provision of a relatively small percentage of dietary flavan-3-ols (∼4% of total polyphenols according to the FFQ and average 24 h DRs) was noticeable: 44% (according to the FFQs) and in the range of 13−54% during the three stages of lactation (according to the 24 h DRs) (Table S5 of the Supporting Information). According to the FFQs, DCh was the principal source of flavan-3-ols, providing 23% of the total flavan-3-ol monomer consumption, followed by cocoa (14%), green tea (9%), apples (8%), cocoa products (7%), black tea (5%), red grapes (4%), and white grapes (2%) (data not shown). Thus, cocoa provided on average more than 30% of the flavan-3-ols and about 40% of the epicatechin consumed within their habitual diet by breastfeeding mothers (Table S5 of the Supporting Information), which was much higher than the 6.7% reported recently for the southern region of Europe.[32] Among the dietary records matched to BM samples, there were 6 participants who declared no consumption of cocoa products over the 24 h prior to sample collection (Table S4 of the Supporting Information). Overall, the consumption of both total polyphenols and flavan-3-ols, assessed by 24 h DR, did not differ significantly ($p = 0.88$) among the three lactating periods. Both global dietary and specifically cocoa origin daily intakes of flavan-3-ols and epicatechin correlated highly (minimum $r > 0.75$ at $p < 0.025$) between the FFQs and the average data from the parallel 24

259   h DRs (data not shown), thereby ensuring the consistency of our dietary evaluations over time. Using

260   the previously tested DCh intervention experiment methodology, we were able to detect various

261   epicatechin metabolites in BM samples collected from free-living lactating mothers (Table S4 of the

262   Supporting Information). The main phase II host metabolites reported in the preliminary study

263   (EpiCat-Gluc-4, EpiCat-Sulf-2, and Met-EpiCat-Sulf-3) were sporadically identified in some of the

264   samples of the population study. On the other hand, the most pronounced metabolite, DHPV-Sulf-2,

265   which belongs to the colonic microbial epicatechin metabolites, was detected in practically all of the

266   samples (Table S4 of the Supporting Information), whereas other microbial metabolites were only

267   detected in some of them. On the whole, the levels of detected host metabolites were low, only up to

268   several tens of nanomoles per liter (Table S4 of the Supporting Information). A recent study with

269   free-living lactating mothers reported a higher concentration of epicatechin in BM (63.7−828.5

270   nmol/L).9 On the one hand, our methodology differs from that applied in the American study, in

271   which total enzymatically hydrolyzed epicatechin was analyzed. In addition, the collection of BM

272   samples differs in the methodology. On the other hand, the dietary habits during lactation might be

273   different among countries, providing distinct dietary uptake of flavan-3-ols. Unfortunately, no data

274   on the dietary habits of American breastfeeding mothers were collected within the study9 to compare

275   to our population. Samples collected during the population study corresponded to three different

276   stages of lactation: colostrum, transition, and mature milk (Figure 1). Changes in the concentration

277   of the detected metabolites over these periods could not be examined because of their sporadic

278   detection in a limited number of samples. For the same reason, no correlation studies could be

279   performed between the epicatechin metabolites detected in BM and the dietary data of volunteers,

280   with the exception of DHPV-Sulf-2. The levels of DHPV-Sulf-2 detected in practically all of the BM

281   samples were correlated with the dietary epicatechin and flavan-3-ol consumption reported by both

282   the FFQ (data not shown) and 24 h DR (Table 1), but no relation was observed. After consideration

283   of the heterogeneity in the types of cocoa product and in the percentage of cocoa flavan-3- ol

284   consumption (Table S4 of the Supporting Information) for the three periods of lactation and the

285   physiological specificity of the colostrum secretion is taken into account,1,34 the colostrum samples

286   (n = 3) were withdrawn from the analysis. As a result, the analysis performed on the transition and

287   mature sample sets, as independent ones, showed a moderate positive correlation ($r = 0.434$; $p = 0.049$) between 24 h consumption of cocoa epicatechin and levels of detected DHPV-Sulf-2 in these BM samples (Table 1). The fact that the epicatechin host metabolites were detected in BM only sporadically but colonic metabolites were detected more often was supported by the data on the BM kinetics of these metabolites observed in our preliminary bioavailability experiment. In our free-living population, dietary data were recorded 24 h prior to BM sampling, and therefore, the chance of detecting low-abundant host metabolites would be limited to the late, close-to-sampling epicatechin consumption as a result of their short circulation time. However, as a result of the specificity of the colonic metabolism, the appearance of microbial metabolites of epicatechin could be extended to a much longer period at more elevated levels than host metabolites,[12,27] thereby increasing the possibility of being monitored during an uncontrolled study. Therefore, valerolactones could be more readily detected in BM and, thus, used in relation to 24 h previous epicatechin consumption. Although our data showed that the concentration of the main microbial epicatechin metabolite DHPV-Sulf-2 in BM could be correlated with cocoa epicatechin intake within an uncontrolled habitual diet, we are aware of the small size of our population. Therefore, a larger study should be carried out to confirm this association. Our study demonstrates that dietary polyphenols, such as cocoa-derived epicatechin, are bioavailable to breastfed infants through maternal milk as a phase II host and colonic microbialderived metabolites. According to the approximated cumulative excretion data, only a very small amount of dietary-ingested epicatechin could be provided with BM within the first 12 postprandial hours as host epicatechin metabolites. However, it seems that microbial metabolites represent another important part of the BM-excreted epicatechin metabolites. Both host and first-stage microbial metabolites (valerolactones) could even be detected in BM samples collected from lactating mothers under non-controlled free-living conditions. However, as a result of its relatively elevated concentration and timely prolonged excretion rates compared to other epicatechin metabolites, only the principal first-stage microbial metabolite DHPV-Sulf-2 could be used in correlation analysis. Thus, its presence in BM was modestly correlated with dietary intake of cocoa epicatechin by breastfeeding mothers during transition and mature periods of lactation. Our

findings provide support for further potential research evaluating the impact of dietary polyphenols on the health of infants.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b01947. Eligibility criteria for the study population, DCh flavan-3- ol and epicatechin ingestion by volunteers in the bioavailability experiment (Table S1), mass spectrometric and chromatographic characteristics of the compounds used for the present UPLC−MS/MS analysis (Table S2), extracted chromatograms of identified epicatechin metabolites in BM samples (Figure S1), brief summary of the excretion kinetics for the epicatechin metabolites detected in BM after acute DCh intake by lactating mothers (Table S3), cocoaderived and total flavan-3-ol consumption by free-living lactating mothers and epicatechin metabolites detected in BM samples (Table S4), and dietary intake of flavan-3- ols (monomers) and epicatechin in total and specifically of cocoa origin by breastfeeding mothers in the population study (Table S5) (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

BM, breast milk; DCh, dark chocolate; DHPV, 5-(3′,4′- dihydroxyphenyl)-γ-valerolactone; FFQ, food frequency questionnaire; Gluc, glucuronide; 24 h DR, 24 h dietary recall; EpiCat, epicatechin; IS, internal standard; Met, methyl; MHPV, 5-(3′-methoxy,4′--hydroxyphenyl)-γ-valerolactone; Sulf, sulfate

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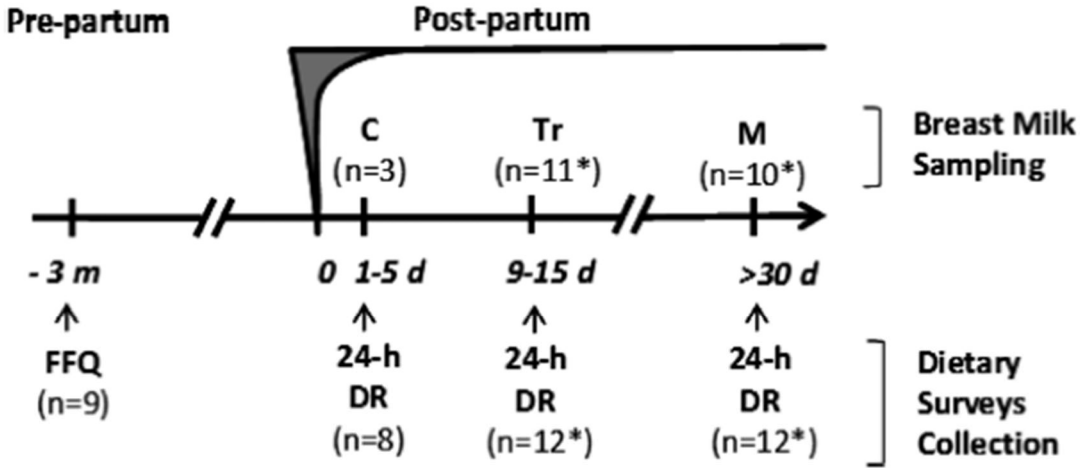
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FIGURES

Figure 1. Scheme of population study with 11 free-living lactating women. (∗) Some data/samples were collected twice from the same volunteer at different time points.
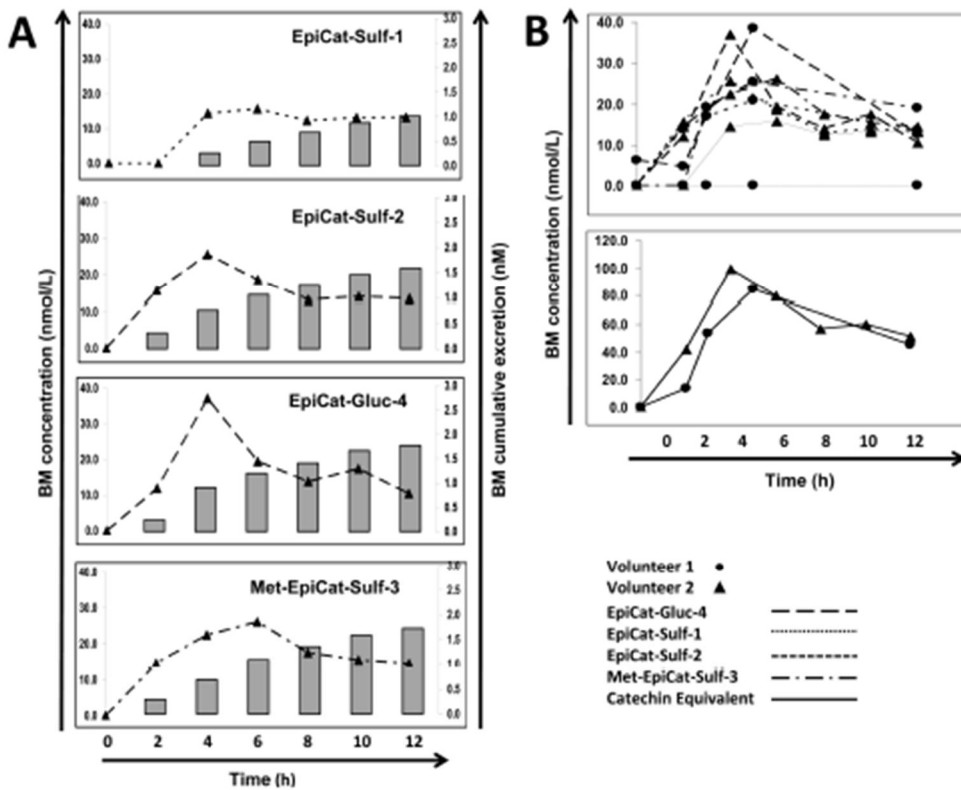
Figure 2. Postprandial (12 h) host epicatechin metabolites in BM: (A) metabolite concentrations (lines) versus their cumulative excretion (bars) in BM of volunteer 2 and (B) kinetics of detected metabolites in BM of both volunteers.

TABLES

Table 1. Bivariate Correlation between Dietary Intake of Flavan-3-ols and Levels of DHPV-Sulf-2, a Microbial Metabolite of Epicatechin, Detected in BM Samples of Free-Living Breastfeeding Mothers[a]

		bivariate Spearman correlations [r (p value)]	
		24 h DR versus DHPV-Sulf2 (n = 24)[b]	24 h DR (Tr + M) versus DHPV-Sulf2 (n = 21)[b]
cocoa food	flavan-3-ols[c]	0.201 (0.347)	0.332 (0.142)
	EpiCat	0.273 (0.197)	0.434 (0.049)
total diet	flavan-3-ols[c]	0.064 (0.767)	0.126 (0.586)
	EpiCat	0.079 (0.715)	0.140 (0.544)

[a]EpiCat, epicatechin; DHPV, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone; 24 h DR, 24 h dietary recall; M, mature (BM); Sulf, sulfate; and Tr, transition (BM). [b]Samples (BM corresponding to their dietary data) are considered as independent measurements. [c]Total dietary and cocoa flavan-3-ol monomers were expressed as aglycone equivalents (mg/day) and were calculated as the sum of catechin, catechin-3-gallate, epicatechin, epicatechin-3-gallate, epigallocatechin, epigallocatechin-3-gallate, and gallocatechin.

Table 1.