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

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Supporting Information

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.

KEYWORDS: human breast milk, dietary polyphenols, cocoa, epicatechin host and microbiota metabolites, breastfeeding

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. 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. Despite the accumulated knowledge in the field of dietary polyphenols, 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 and even fewer studies have addressed their presence in the BM of lactating mothers under free-living conditions. 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.

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. 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\(^\text{11}\) and has also been extensively studied by our group.\(^\text{12,13}\) 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 only) intake was estimated using our food composition database on polyphenols.\(^\text{14}\) This was based on three United States Department of Agriculture (USDA) databases\(^\text{15-17}\) and the Phenol-Explorer database.\(^\text{16}\) The FFQ, developed and validated in the Spanish population,\(^\text{19}\) was applied on the day of enrolment to evaluate food intake (g/day) over the previous 3 months by pregnant women in a free-living population study.

**Determination of Epicatechins and Their Metabolites in BM Samples.** On the day of analysis, a 1 mL aliquot of the BM sample was thawed in an ice bath, acidified with 25 μL of phosphoric acid (35%), sonicated for 15 min at room temperature, and afterward centrifuged at 15900g for 10 min at 4 °C. Through gentle aspiration, 0.8 mL of acidified aqueous phase of BM was collected and used for solid-phase extraction (SPE). The concentrations of (+)-epicatechin (EpiCat), O-methyl-epicatechin (MetEpiCat), and colo. microbiota-generated metabolites, such as S-(5′,4′-dihydroxyphenyl)-γ-valerolactone (DHPV) and S-(3′-methoxy-4′-hydroxyphenyl)-γ-valerolactone (MHPV), along with their corresponding phase II conjugates sulfates (Sulf) and glucuronides (Gluc) in BM, were analyzed according to the earlier published methodology,\(^\text{20}\) with a slightly modified elution step. Then, the retained epicatechin metabolites were eluted by applying 0.5 mL of basic methanol (0.1% ammonia) after 1 mL of acidified methanol (0.1% formic acid) to optimize both glucuronide and sulfate extraction (>75% recovery; data not shown).\(^\text{21}\) The reconstituted-in-mobile-phase samples were used for ultra performance liquid chromatography—tandem mass spectrometry (UPLC—MS/MS) analysis. Ethyl gallate served as the internal standard (IS). The previously established methodology was used for UPLC—MS/MS analysis of epicatechin metabolites.\(^\text{22}\)

The epicatechin metabolites were identified according to the available standards (EpiCat, Cat, MetEpiCat, EpiCat-Gluc-2, and EpiCat-Sulf-1) and as a result of the previously described mass chromatographic behavior of compounds.\(^\text{22,20}\) (Table S2 of the Supporting Information). Calibration curves were constructed in the range of 5–2000 μg/L with available standards in aqueous extracts of BM and subjected to the same procedure as the samples. As a result of the fact that standards for phase II metabolites were not available, the concentrations of all sulfated, glucuronidated, and methylated metabolites were approximated using curves of in-house synthesized EpiCat-Sulf-1, EpiCat-Gluc-2, and MetEpiCat, respectively, and the results were expressed as their equivalents.\(^\text{20}\) These compounds were synthesized and characterized as previously published.\(^\text{22}\) The purity of purchased standards (−)-epicatechin, (+)-catechin, and ethyl gallate from Sigma-Aldrich (St. Louis, MO) was of analytical grade, and the purity of synthesized standards was ≥98%, ≥98%, and ≥96%, respectively, as reported in our previous studies applying this methodology.\(^\text{22,20}\)

**Statistical Analysis.** Dietary intake of polyphenols is shown as the mean and standard deviation (SD). The contribution of each food to total dietary polyphenols and flavan-3-ol intake, in total and specifically that of cocoa origin, and the contribution of flavan-3-ol to the three phases of lactation were calculated as a percentage. The Friedman test was used to compare the consumption of polyphenols among the three phases of lactation. We used Spearman correlations to assess whether flavan-3-ol and epicatechin intakes were correlated to DHPV-Sulf-2. SPSS statistical analysis system, version 18.0 (SPSS), was used, and the significance level for the performed statistical analyses was <0.05.
RESULTS AND DISCUSSION

Epicatechin Metabolites in BM after Acute DCh Ingestion. In accordance with the habitual dietary consumption evaluated by 24 h DR collected on pre-intervention day, DCh was the principal dietary source of flavan-3-ols (78–92%) and epicatechin (92–96%) consumed during the intervention day by both volunteers (Table S1 of the Supporting Information). In both lactating women, the acute DCh intervention was estimated to provide an intake of around 80 mg of DCh-derived flavan-3-ol monomers, of which about 48% corresponded to epicatechin (Table S1 of the Supporting Information). Therefore, the DCh epicatechin content was estimated to be within the ranges reported for other commercially available DChs.23

The DCh intake challenge led to the direct identification of two groups of epicatechin metabolites in the BM samples of both volunteers collected over 12 h after ingestion: (i) host metabolites of epicatechin, EpiCat-Sulf-1 and EpiCat-Sulf-2, EpiCat-Gluc-4, and Met-EpiCat-Sulf-3, and (ii) microbial metabolites, DHPV-Sulf-2, DHPV-Gluc-1 and DHPV-Gluc-2, MHPV-Sulf-1 and MHPV-Sulf-2, and MHPV-Gluc-1.12,20 The selected chromatograms of the detected epicatechin metabolites in the BM samples are represented in Figure S1 of the Supporting Information.

BM is a special biological compartment linked closely to plasma, but on the other hand, similar to urine, it can accumulate excreted compounds.24 Although we did not have corresponding standards, it is very likely that the main detected host metabolites correspond to the major metabolites of cocoa-derived epicatechin recently described in postprandial plasma and urine:25,26 EpiCat-3′-O-Gluc (as EpiCat-Gluc-4), EpiCat-3′-O-Sulf (as EpiCat-Sulf-2), and 3′-Methyl-EpiCat-S-O-Sulf (as Met-EpiCat-Sulf-3). In general, their levels in the BM samples were quite low, even at maximum concentration rates (nmol/L) (Figure S1 of the Supporting Information and panels A and B of Figure 2), being close to the plasmatic postprandial levels detected after intake of 2 times higher dose of DCh.26 This was also in agreement with the BM ranges of other polyphenols previously reported in dietary intervention volunteers6–8 and in some populations of free-living lactating mothers.5,9 Other, less abundant metabolites could not be detected as a result of their very low, several times lower than detected, metabolite26 concentrations. The changes in the BM concentrations of the detected host metabolites were very similar in both volunteers (Figure 2B).

Continuously collected samples from volunteer 2 were used to evaluate the BM excretion kinetics of detected host epicatechin metabolites over 12 post-intervention hours (Figure 2A). The peaks of maximum concentration for each metabolite were defined by visual inspection of excretion graphs. Thus, the highest levels of EpiCat-Sulfs and EpiCat-Gluc metabolites were reached in BM 4 h after DCh intake and after 6 h for Met-EpiCat-Sulf in both sample sets (Figure 2B).

Dietary epicatechin was rapidly absorbed in the small intestine, and its metabolites were quickly eliminated from plasma within 6–8 h and excreted in urine.26 Our data showed
that the main epicatechin host metabolites were still present in the BM at detectable levels 12 h after DCh ingestion (panels A and B of Figure 2). According to the dietary recalls, no other potent source of flavanols other than DCh was consumed on the intervention day by either volunteer (Table S1 of the Supporting Information). Therefore, this accumulation of DCh-derived host epicatechin metabolites in the BM resembled that previously reported for urine, where they were mostly excreted between the 5th and 10th postprandial hour and were still present at lower concentrations after 10 h.\(^{26}\) Unfortunately, we did not collect either blood or urine samples from the two volunteers within the present study. However, the relation between the plasmatic, urinary, and BM kinetics of epicatechin metabolites should be further studied, because it will indicate in detail the bioavailability, excretion, and accumulation of dietary polyphenols in BM.

In addition to host metabolites, the only microbial-derived metabolite of epicatechin, DHPV-Sulf-2, was detected in the set of BM samples of volunteer 2 (Table S3 of the Supporting Information). In contrast, other microbial metabolites were also detected in all of the BM samples of volunteer 1: DHPV-Sulf-1, DHPV-Gluc-2, MHPV-Sulf-1, MHPV-Sulf-2, and MHPV-Gluc-1, along with the most pronounced DHPH-Sulf-2 metabolite (Figure S1 and Table S3 of the Supporting Information). All of them, except DHPV-Sulf-1, were increasing in concentration toward the end of the BM sample collection period (12 h). Unfortunately, there are no reported data on the urine or plasmatic kinetics of microbial ring-fusion metabolites of cocoa-derived epicatechin to be compared to our BM results. According to recently reported data on tea epicatechins,\(^{27}\) first-stage microbial metabolites (valerolactones) appeared in the plasmatic circulation with a 2–4 h lag time and at a maximum concentration in 4–8 h, exceeding those of host epicatechin metabolites by 1 order of magnitude. They remained in circulation for about 20 h after single-dose consumption of black tea. On the basis of these pharmacokinetic data, we assume that the BM kinetics of the microbial metabolites could not be evaluated within the present study as a result of the relatively short time for postprandial sample collection.

The late appearance of the ring-fusion metabolites in BM is due to the fact that they are generated through microbiota catabolic activities and, as result, are absorbed in the large intestine.\(^{25}\) We assume that, in contrast to volunteer 2, who did not consume any cocoa products prior to the intervention, epicatechin microbial metabolites were detected in the pre-intervention (0 h) and early (2–8 h) postprandial samples of volunteer 1 most likely as a result of the consumption of DCh from the previous day within her habitual diet (data not shown). In general, our data on microbial epicatechin metabolites are also in line with those previously reported, where the main microbial metabolite in 24 h urine after long-term cocoa powder consumption was DHPV-Sulf-2, along with the main host metabolites EpiCat-Gluc-4, EpiCat-Sulf-2, and Met-EpiCat-Sulf-2.\(^{24}\) In support of our findings, in the study with tea polyphenols, it was observed that sulfated conjugates of valerolactones were almost twice more represented in plasma than glucuronidated forms.\(^{25}\) Thus, the authors suggested that sulfation was preferred over glucuronidation at the site of valerolactone metabolism.\(^{25}\)

The 2 h fractionated samples from volunteer 2 showed approximately 12 h cumulative excretion of the detected host metabolites in BM. It was not possible to conduct analysis for volunteer 1, because the collection of samples was interrupted as a result of lactating circumstances. Thus, the combined 12 h excretion of the host metabolites in BM, expressed in catechin equivalents, was estimated to account for about 0.01% (assuming equal excretion in both breasts) of the chocolate-ingested epicatechin (Table S3 of the Supporting Information). Considering just non-colonic metabolites, the bioavailability value during the first 12 postprandial hours observed in this study would provide a breastfed child with about 3.8 μg of catechin equivalent. This approximately corresponds to a cumulative dose of 0.772 μg/kg (for a baby weighing 5 kg) spread over 12 h,\(^{26}\) when the single dose of the lactating mother was estimated to be about 0.61 mg/kg (for a woman weighing 63 kg) as DCh-derived epicatechin. Thus, the dose for the child appears to be 3 orders of magnitude lower than that of the mother. Unfortunately, we cannot take into account the metabolites generated by colonic microbiota for mother and infant dose approximation, although their weight in relation to epicatechin metabolism and disposition is expected to be essential.\(^{27,28}\) On the other hand, other potential sources of epicatechin and valerolactone phase II metabolites, such as procyanidins,\(^{32}\) were not considered in our preliminary experiment. Estimation of the accessibility of dietary polyphenols to breastfed infants could be especially relevant in the light of recent reports accentuating their potential to modulate gut microbiota activities.\(^{30,31}\)

**Epi catechin in BM of Free-Living Lactating Women.** Among the 11 women participating in our population study, FFQs were collected from 9 volunteers. All three 24 h DRs were available only for 8 volunteers, whereas the second and third 24 h DRs (corresponding to transition and mature milk collections) were available for 10 volunteers; for one of them, it was repeated twice for transition, and for another, it was repeated twice for mature milk collection (Figure 1 and Table S4 of the Supporting Information). From the 33 samples planned to be 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).\(^{32}\) 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

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

Using the previously tested DCh intervention methodology,
we were able to detect various epicatechin metabolites in BM samples collected from free-living lactating
mothers (Table S4 of the Supporting Information). The main
phase II host metabolites reported in the preliminary study
(EpiCat-Gluc-4, EpiCat-Sulf-2, and Met-EpiCat-Sulf-3) were
sporadically identified in some of the samples of the population
study. On the other hand, the most pronounced metabolite,
DHPV-Sulf-2, which belongs to the colonic microbial
epicatechin metabolites, was detected in practically all of the
samples (Table S4 of the Supporting Information), whereas
other microbial metabolites were only detected in some of
them.

On the whole, the levels of detected host metabolites were
low, only up to several tens of nanomoles per liter (Table S4
of the Supporting Information). A recent study with free-living
lactating mothers reported a higher concentration of epicatechin in BM (63.7–828.5 nmol/L).33 On the one hand,
our methodology differs from that applied in the American
study, in which total enzymatically hydrolyzed epicatechin was
analyzed. In addition, the collection of BM samples differs in
the methodology. On the other hand, the dietary habits during
lactation might be different among countries, providing distinct
dietary uptake of flavan-3-ols. Unfortunately, no data on the
dietary habits of American breastfeeding mothers were
collected within the study7 to compare to our population.

Samples collected during the population study corresponded
to three different stages of lactation: colostrum, transition, and
mature milk (Figure 1). Changes in the concentration of the
detected metabolites over these periods could not be examined
because of their sporadic detection in a limited number of samples. For the same reason, no correlation studies could be
performed between the epicatechin metabolites detected in BM
and the dietary data of volunteers, with the exception of
DHPV-Sulf-2. The levels of DHPV-Sulf-2 detected in practi-
cally all of the BM samples were correlated with the dietary
epicatechin and flavan-3-ol consumption reported by both the
FFQ (data not shown) and 24 h DR (Table 1), but no relation
was observed. After consideration of the heterogeneity in the
types of cocoa product and in the percentage of cocoa flavan-3-
ol consumption (Table S4 of the Supporting Information) for
the three periods of lactation and the physiological specificity of
the colostrum secretion is taken into account,19 the colostrum
samples (n = 3) were withdrawn from the analysis. As a result,
the analysis performed on the transition and 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, valer-
olactones 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 microbial-
derived 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

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 Mothersa

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<th>bivariate Spearman correlations [r (p value)]</th>
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<tr>
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<td>24 h DR versus DHPV-Sulf2 (n = 24)b</td>
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<tr>
<td>cocoa food flavan-3-ols</td>
<td>0.201 (0.347)</td>
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<tr>
<td>EpiCat</td>
<td>0.273 (0.197)</td>
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<tr>
<td>total diet flavan-3-ols</td>
<td>0.064 (0.767)</td>
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<tr>
<td>EpiCat</td>
<td>0.079 (0.715)</td>
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aEpiCat, epicatechin; DHPV, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone; 24 h DR, 24 h dietary recall; M, mature (BM); Sulf, sulfate; and Tr,
transition (BM). Samples (BM corresponding to their dietary data) are considered as independent measurements. "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 gallatechin.

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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**

1. **Supporting Information**  
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), cocoa-derived 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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**Author Contributions**  
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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, S-(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, S-(3′-methoxy,4′-hydroxyphenyl)-γ-valerolactone; Sulf, sulfate

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