

Supporting information to research article:

Dietary epicatechin is available to breastfed infants through human breast milk in the form of host and microbial metabolites.

Olha Khymenets^{1*}, Montserrat Rabassa^{1*}, María Rodríguez Palmero²,
Montserrat Rivero-Urgell², Mireia Urpi-Sarda¹, Sara Tulipani^{1,3}, Pilar Brandi⁴,
Cristina Campoy^{4,5}, Celestino Santos-Buelga⁶, Cristina Andres-Lacueva¹.

¹Nutrition and Food Science Department, Biomarkers and Nutrimetabolomics Lab., XaRTA, INSA, Faculty of Pharmacy and Food Sciences, University of Barcelona, Barcelona, Spain.

²Laboratorios Ordesa S.L., Parc Científic de Barcelona, Barcelona, Spain.

³Biomedical Research Institute (IBIMA), Service of Endocrinology and Nutrition, Hospital Complex Virgen de la Victoria, Malaga, Spain.

⁴Excellence Centre for Paediatric Research, Biomedical Research Centre, Health Sciences Technological Park, Hospital Universitario San Cecilio, Granada, Spain.

⁵Department of Paediatrics, School of Medicine, University of Granada, Granada, Spain

⁶GIP-USAL, Faculty of Pharmacy, University of Salamanca, Salamanca, Spain.

*These authors contributed equally to the manuscript.

Correspondence: Dr. Cristina Andres-Lacueva, Tel: +34 934034840; Fax: +34 934035931; E-mail: candres@ub.edu

Eligibility criteria for study population:

Inclusion criteria:

- Healthy women, aged between 30 and 40 years.
- Pregnant women, with gestation over 30 weeks.
- Intention to breastfeed throughout the study period.
- The consumption of products with added probiotics (Actimel, bio yogurts, bio cheese, etc.) should not exceed more than 2 per week.

If the contact with the mother was made before childbirth, only those who met the following requirements were allowed to participate in the study:

- Childbirth term (> 36 weeks gestation), without major complications.
- Infants should be exclusively breastfed, i.e. receive only breast milk for feeding.
- Infants had to be healthy.

Exclusion criteria:

- Mother's pathological background: neurological diseases, metabolic diseases, type I diabetes, chronic gastrointestinal diseases, ToRCH complex infections.
- Background disease during pregnancy: gestational diabetes, chronic disease (hypothyroidism), risk of abortion, hypertension, pre-eclampsia.

After childbirth, the following exclusion criteria were established:

- Antibiotic consumption during the study period.
- The consumption of bio products during the study period should not exceed more than 2 per week.
- Breastfeeding abandonment.
- The introduction of complementary feeding during the study.

Table S1 (Supporting Information): DCh flavan-3-ols^a and epicatechin ingestion by both volunteers, participating in the preliminary experiment on epicatechin BM bioavailability, calculated on the base of their 24 hr DRs

	Total dietary flavan-3-ols (mg/day) ^a	DCh intervention (g (% cocoa))	DCh flavan-3-ols			
			Total flavan-3-ols (mg/day) ^a	% of total dietary flavan-3-ols ^a	EpiCat (mg/day)	% of total dietary EpiCat
Volunteer 1						
DCh intervention within habitual diet ^b	87.19	40 (85%)	80.08	91.84	38.59	91.84
Volunteer 2						
DCh intervention within habitual diet ^c	102.38	50 (75%)	80.08	78.22	38.59	96.86

DCh – dark chocolate; 24 hr DR – 24 h dietary records; EpiCat – epicatechin; Vol – volunteer.

^a- flavan-3-ols refer only to monomers and are expressed as aglycone equivalents (mg/d) and were calculated as the sum of catechin, catechin-3-gallate, epicatechin, epicatechin-3-gallate, epigallocatechin, epigallocatechin-3-gallate and galocatechin.

^b - Volunteer 1 on the morning of the day before the intervention had consumed the same type and amount of DCh as for the intervention as a part of her habitual diet. In this case, the evaluation of the day before's habitual consumption (24 h DR) included the DCh intervention.

^c- estimated by adding DCh intervention to the habitual diet evaluated the day before using 24 hr DR.

Table S2 (Supporting Information): Mass spectrometric and chromatographic characteristics of epicatechin metabolites along with the reference compound used for UPLC-MS/MS^a determination (isomer numeration is according to earlier published methodology^{1, 2})

Compound	Q1	Q3	CE (V)	RT (min)
Flavan-3-ol monomers				
EpiCat-Gluc-1&2, -3 and -4	465	289	-25	0.48; 0.64 and 0.82
Cat and EpiCat	289	245	-25	0.75 and 0.91
EpiCat-Sulf-1 and -2	369	289	-25	0.72 and 0.87
Met-EpiCat-Gluc-1, -2 and -3	479	303	-30	0.6; 0.69 and 0.84
Met-EpiCat-Sulf-1, -2 and -3	383	303	-25	0.89; 0.97 and 1.08
3'- and 4'-Met-EpiCat	303	137	-25	1.13 and 1.23
Hydroxyphenylvalerolactones				
DHPV-Gluc-1 and -2	383	207	-25	0.83 and 0.9
DHPV-Sulf-1 and -2	287	207	-25	0.71 and 0.92
MHPV-Gluc-1	397	221	-25	0.93
MHPV-Sulf-1 and -2	301	221	-25	0.99 and 1.08
References				
Ethyl gallate (I.S.)	197	169	-25	1.16

Cat – catechin; CE – collision energy; DHPV - 5-(3',4'-dihydroxyphenyl)-γ-valerolactone; EpiCat – epicatechin; Gluc – glucuronidate; I.S. – internal standard; Met-EpiCat – methyl-O-epicatechin; MHPV - 5-(3'-methoxy,4'-hydroxyphenyl)-γ-valerolactone; Q – quadrupole; Sulf – sulphate; ^a - analysis performed in negative ionization mode.

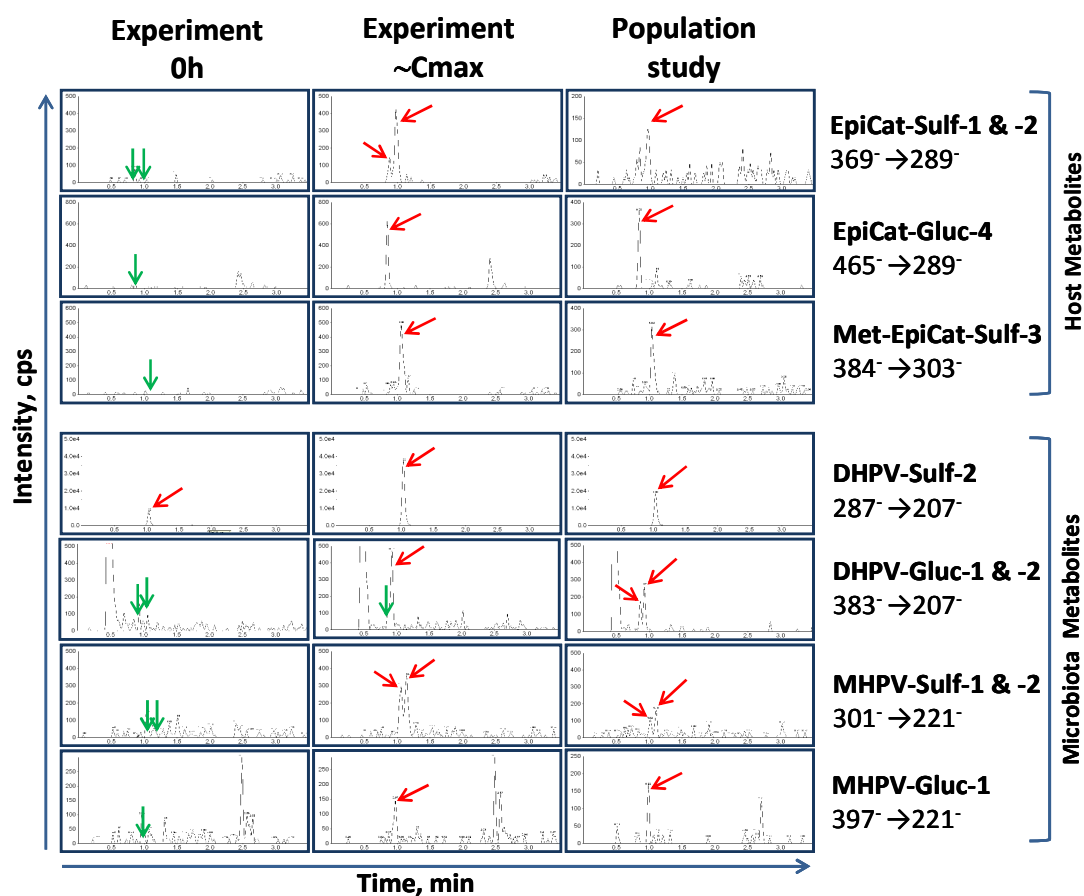


Figure S1 (Supporting Information): Selected chromatograms of identified epicatechin metabolites in pre- and post-intervention (close to maximum concentration (Cmax) of each compound) samples from bioavailability experiment and identified compounds in selected samples from population study. Red arrow marks point to detected compounds; green arrow marks point to the time where the compound peak is expected to elute.

Table S3 (Supporting Information): Brief summary of excretion kinetics for epicatechin metabolites detected in BM after acute DCh intake by lactating mothers

Detected metabolites	C_{\max} , nmol/L		T_{\max} , h	Cumulative excretion (12 h)*, nmol	% of consumed EpiCat**
	Volunteer 1	Volunteer 2			
EpiCat-Sulf-1	ND	15.70	6	1.256	0.0009
EpiCat-Sulf-2	20.84	25.51	4-5	1.588	0.0012
EpiCat-Gluc-4	38.45	37.08	4-5	1.771	0.0013
Met-EpiCat-Sulf-3	25.42	26.07	5-8	1.720	0.0013
Total host metabolites				6,335	0,0048
DHPV-Sulf-1	19.44	ND	NA	NA	NA
DHPV-Sulf-2	2097.29	254.20	NA	NA	NA
DHPV-Gluc-2	59.66	ND	NA	NA	NA
MHPV-Sulf-1	25.03	ND	NA	NA	NA
MHPV-Sulf-2	29.47	ND	NA	NA	NA
MHPV-Gluc-1	17.75	ND	NA	NA	NA

BM – breast milk; C_{\max} – maximum concentration; DCh – dark chocolate; DHPV – 5-(3',4'-dihydroxyphenyl)- γ -valerolactone; EpiCat – epicatechin; Gluc – glucuronidate; Met-EpiCat – methyl-epicatechin; MHPV – 5-(3'-methoxy,4'--hydroxyphenyl)- γ -valerolactone; NA – not available; ND – not detected; Sulf – sulphate; T_{\max} – time when maximum concentration was reached. ^a – calculated for Volunteer 2, one breast only on the basis of the detected epicatechin metabolites; ^b – calculated for Volunteer 2 for one breast only and according to EpiCat consumed with DCh.

Table S4 (Supporting Information): Cocoa-derived and total flavan-3-ol (monomer) consumption by free-living lactating mothers according to collected 24 hr DRs and epicatechin metabolites detected in corresponding BM samples

Vol_ID	sample ID	BM type	cocoa-derived flavan-3-ols (mg/d)		total dietary flavan-3-ols (mg/d)		host EpiCat metabolites detected in BM (nmol/L)			microbiota-derived EpiCat metabolites detected in BM (nmol/L)					
			EpiCat	Flavan-3-ols ^a	EpiCat	Flavan-3-ols ^a	EpiCat-Gluc-4	EpiCat-Sulf-2	MetEpiCat-Sulf-3	DHPV-Gluc-1	DHPV-Gluc-2	DHPV-Sulf-2	MHPV-Gluc-1	MHPV-Sulf-1	MHPV-Sulf-2
1	O_02	Tr	0.31	0.51	1.23	2.35	n.d.	n.d.	n.d.	n.d.	n.d.	57.07	n.d.	n.d.	n.d.
	O_03	M	0.00	0.00	0.93	7.35	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	O_04	C	3.12	9.22	3.14	15.34	n.d.	n.d.	n.d.	n.d.	n.d.	510.88	n.d.	n.d.	n.d.
	O_05	Tr	3.12	9.22	3.12	9.22	n.d.	n.d.	n.d.	n.d.	n.d.	175.24	n.d.	14.94	n.d.
	O_06	M	3.12	9.22	3.15	15.63	n.d.	13.53	n.d.	n.d.	19.04	415.46	n.d.	19.63	n.d.
3	O_07	Tr	0.00	0.00	6.94	9.54	n.d.	n.d.	13.23	n.d.	n.d.	34.24	n.d.	n.d.	n.d.
	O_08	M	5.62	16.60	6.57	18.48	n.d.	n.d.	n.d.	n.d.	n.d.	58.39	n.d.	n.d.	n.d.
	O_09	M	5.62	16.60	6.00	17.63	n.d.	n.d.	n.d.	n.d.	n.d.	86.95	n.d.	n.d.	16.89
4	O_12	Tr	6.28	12.18	29.67	45.36	n.d.	n.d.	n.d.	n.d.	n.d.	713.35	n.d.	18.68	20.10
	O_13	M	3.13	9.38	10.27	22.33	n.d.	n.d.	n.d.	n.d.	n.d.	320.94	n.d.	n.d.	17.25
5	O_15	Tr	6.04	13.07	7.91	17.12	n.d.	13.43	17.19	n.d.	n.d.	216.17	n.d.	n.d.	n.d.
	O_16	M	3.12	9.22	3.15	9.31	n.d.	14.49	15.62	n.d.	n.d.	204.68	n.d.	16.50	17.26
6	O_18	Tr	5.83	7.69	6.51	20.13	36.37	n.d.	n.d.	n.d.	39.16	1909.84	n.d.	26.26	n.d.
7	O_20	Tr	11.24	33.20	11.26	33.23	32.64	n.d.	23.74	n.d.	n.d.	83.05	n.d.	n.d.	n.d.
	O_21	Tr	11.24	33.20	19.71	47.52	n.d.	n.d.	n.d.	n.d.	n.d.	109.41	n.d.	n.d.	n.d.
	O_22	M	0.00	0.00	7.92	11.44	n.d.	n.d.	n.d.	n.d.	n.d.	19.53	n.d.	n.d.	n.d.
8	O_23	C	7.86	10.28	14.94	19.40	15.39	n.d.	15.79	n.d.	n.d.	164.53	n.d.	n.d.	n.d.
	O_24	Tr	1.46	1.92	16.82	29.32	n.d.	n.d.	n.d.	n.d.	n.d.	23.78	n.d.	n.d.	n.d.
	O_25	M	2.84	8.50	4.43	24.47	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9	O_26	Tr	2.92	3.84	3.86	5.71	n.d.	ND	n.d.	n.d.	22.38	340.71	0.00	n.d.	n.d.
	O_27	M	0.00	0.00	14.24	18.93	n.d.	ND	n.d.	25.58	39.25	434.82	24.28	n.d.	n.d.
10	O_28	C	0.00	0.00	7.51	10.92	25.51	n.d.	23.67	n.d.	36.58	780.94	n.d.	n.d.	20.20
	O_29	Tr	5.83	7.69	20.53	26.47	n.d.	n.d.	n.d.	n.d.	n.d.	54.06	n.d.	n.d.	n.d.
	O_30	M	0.00	0.00	0.92	1.84	n.d.	n.d.	13.91	n.d.	n.d.	80.82	n.d.	n.d.	n.d.

BM – breast milk; C – colostrum milk; DHPV – 5-(3',4'-dihydroxyphenyl)-γ-valerolactone; EpiCat – epicatechin; Gluc – glucuronidate; M – mature milk; Met-EpiCat – methyl-epicatechin; MHPV – 5-(3'-methoxy,4'--hydroxyphenyl)-γ-valerolactone; n.d.– not detected; Sulf – sulphate; Tr – transition milk.

^a – Total and cocoa flavan-3-ol monomers were expressed as aglycone equivalents (mg/d) and were calculated as the sum of catechin, catechin-3-gallate, epicatechin, epicatechin-3-gallate, epigallocatechin, epigallocatechin-3-gallate and gallocatechin. Cells marked in light grey correspond to no-cocoa consumption data and those marked in dark grey indicate epicatechin metabolites detected in BM.

Table S5 (Supporting Information): Dietary intake of flavan-3-ols (monomers) and epicatechin in total and specifically of cocoa origin by breastfeeding mothers in the population study based on FFQ and 24 h DR (data are presented as mean (SD) or in % as stated in the table)

dietary records	dietary polyphenols				dietary flavanols		cocoa-derived flavanols	
	total polyphenols (mg/day) ^b	cocoa polyphenols by food (%) ^c	flavan-3-ols (%) ^c	cocoa flavan-3-ols by food (%) ^c	flavan-3-ols (mg/day) ^d	EpiCat (mg/day)	flavan-3-ols (mg/day) ^d	EpiCat (mg/day)
FFQ (n=9)	1104.97 (465.97)	5% cocoa, 3% DCh	4.40	23% DCh. 14% cocoa. 7% chocolate products	48.59 (36.31)	19.39 (12.19)	18.59 (19.66)	8.25 (8.42)
24 hr DR colostrum (n=8)	874.98 (578.02)	3% chocolate products, 2% cocoa	8.57	10% chocolate products. 3% cocoa	74.99 (175.20)	11.88 (17.46)	10.05 (15.85)	5.06 (8.02)
24 hr DR transition (n=11 or 12 ^a)	594.39 (251.86)	10% cocoa, 3% MCh, 1% DCh	3.22	37% cocoa. 9% MCh. 6% chocolate products 3% DCh	19.16 (15.45)	7.94 (8.57)	8.35 (9.30)	3.56 (3.33)
24 hr DR mature (n=11 or 12 ^a)	785.36 (498.41)	5% cocoa, 2% MCh, 1% DCh	1.98	28 % cocoa. 11% MCh. 5% DCh	15.57 (8.61)	8.35 (6.13)	5.87 (5.85)	2.74 (2.51)

DCh – dark chocolate; EpiCat – epicatechin; FFQ – frequency food questionnaire; MCh – milk chocolate; 24 hr DR – 24-hour dietary records. ^a – some data/samples were collected twice from the same volunteer at different time points; ^b – dietary total polyphenols intake was calculated as the sum of flavonoids (anthocyanidins, flavonols, flavanones, flavones, flavanols [including flavan-3-ol monomers, theaflavins and proanthocyanidins] and isoflavones), phenolic acids, lignans, stilbenes and other polyphenols and was expressed as aglycone equivalents (mg/day); ^c – % of total dietary polyphenol; ^d – 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 gallic catechin.

References:

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