Electronic Supplementary Information

Human Milk Metals and Metalloids Shape Infant Microbiota

Eduard Flores Ventura,*[a] Manuel Bernabeu,[a] Belén Callejón-Leblic,[b] Raúl Cabrera-Rubio,[a] Laxmi Yeruva,[c] Javier Estañ-Capell,[d] Cecilia Martínez-Costa,[d] Tamara García-Barrera,[b] and María Carmen Collado*[a]

[a.] Institute of Agrochemistry and Food Technology- Spanish National Research Council (IATA-CSIC), Valencia, Spain
[b.] Research Centre of Natural Resources, Health and the Environment (RENSMA), Department of Chemistry, Faculty of Experimental Sciences, University of Huelva, Campus El Carmen, Fuerzas Armadas Ave., 21007, Huelva, Spain
[c.] Microbiome and Metabolism Research Unit, USDA-ARS, SEA, Little Rock, AR, USA; Arkansas Children's Nutrition Center, Little Rock, AR, USA.
[d.] Department of Pediatrics, University of Valencia, INCLIVA Biomedical Research Institute, Avenida Blasco Ibáñez 15-17, 46010 Valencia, Spain

* Corresponding author: MCC, Institute of Agrochemistry and Food Technology (IATA-CSIC), Department of Biotechnology, Unit of Lactic Acid Bacteria and Probiotics, Av. Agustin Escardino 7, 46980 Paterna, Valencia, Spain; Phone +34 96900022; e-mail: mcolam@iata.csic.es
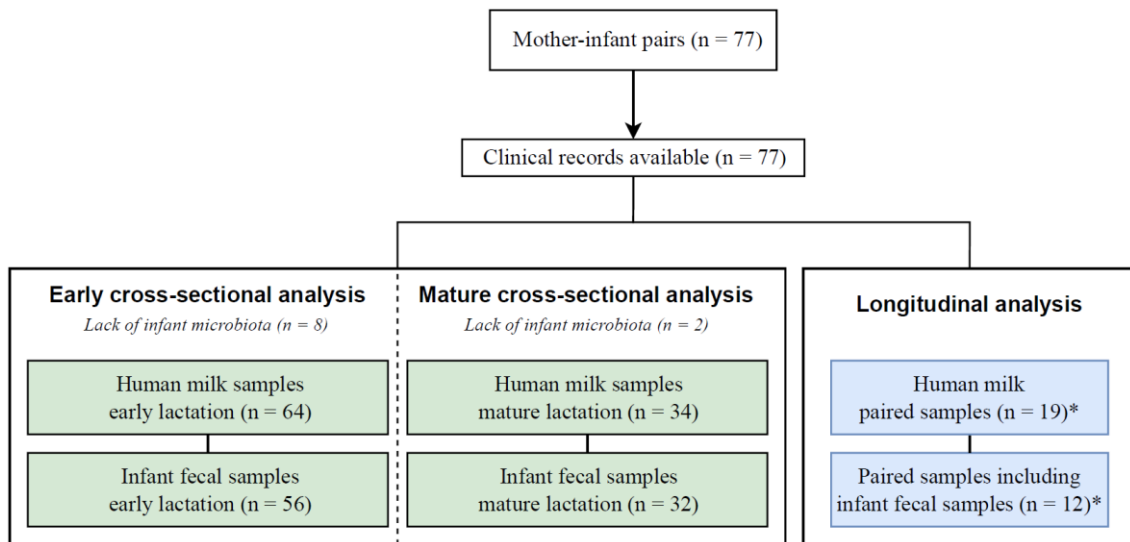
Fig. S1. Flow diagram of mother-infant pairs inclusion.

 *Mother infant samples available at two different times points: the early lactation stage (between 7 and 15 days postpartum) and the mature lactation stage (between 30 and 60 days postpartum). 71 human milk samples were analysed for lead (Pb), aluminium (Al), arsenic (As), and cadmium (Cd).

Table S1. Cohort characteristics

	Total population (**N=77≠**)
Maternal characteristics	
Gestational age (weeks)*	40 (39-41)
Pregestational BMI (kg/m^2)*^	22.55 (20.48-24.75)
Weight gain over pregnancy (Kg)*^	12 (10-15)
Antibiotic during pregnancy^ (nº cases)	
Yes	21
No	55
Not available	1
Antibiotics during labour (nº cases)	
Yes	33
No	44
Delivery mode (n)	
C-section	31
Vaginal	46
Infant characteristics	
Gender (n)	
Females	45
Males	32
Breastfeeding duration (months)*	6 (4.5-6)
Weight at birth (kg)*	3.28 (3-3.55)
Weight at 7 days (kg)*	3.29 (2.98-3.59)
Weight at 15 days (kg)*	3.5 (3.18-3.78)
Weight at 1 month (kg)*	4.05 (3.73-4.54)
BMI z-score at birth	-0.11(-0.62-0.6)

≠For some data, n=76 was provided as missing data from infant was missing.
*Values expressed as Median (Quartile 1 - Quartile 3).
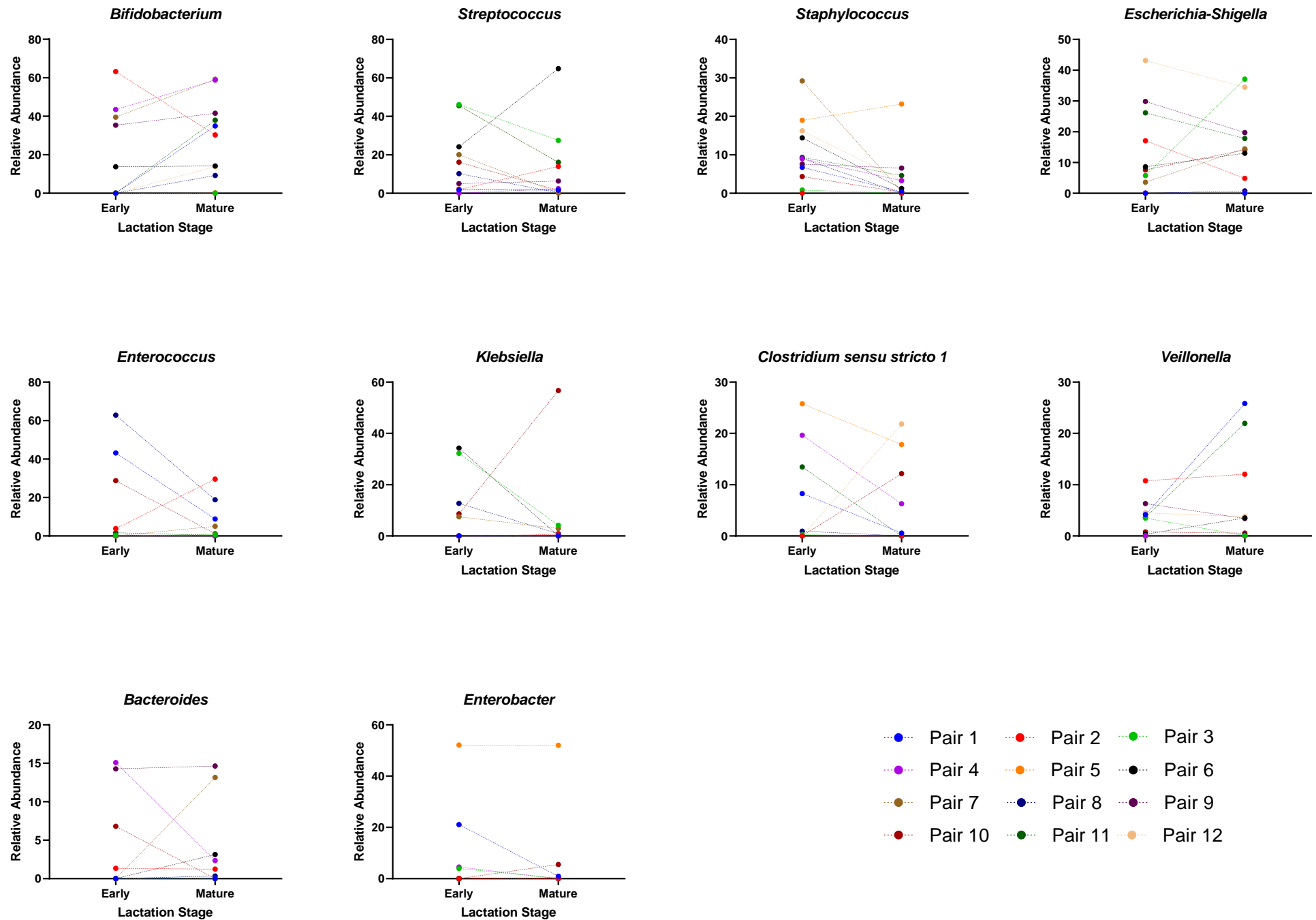^Variable with missing data in 1 mother.

Fig. S2. Longitudinal trends in bacterial abundance at the genus level in infant faeces from the 7th day to the 2nd month postpartum.
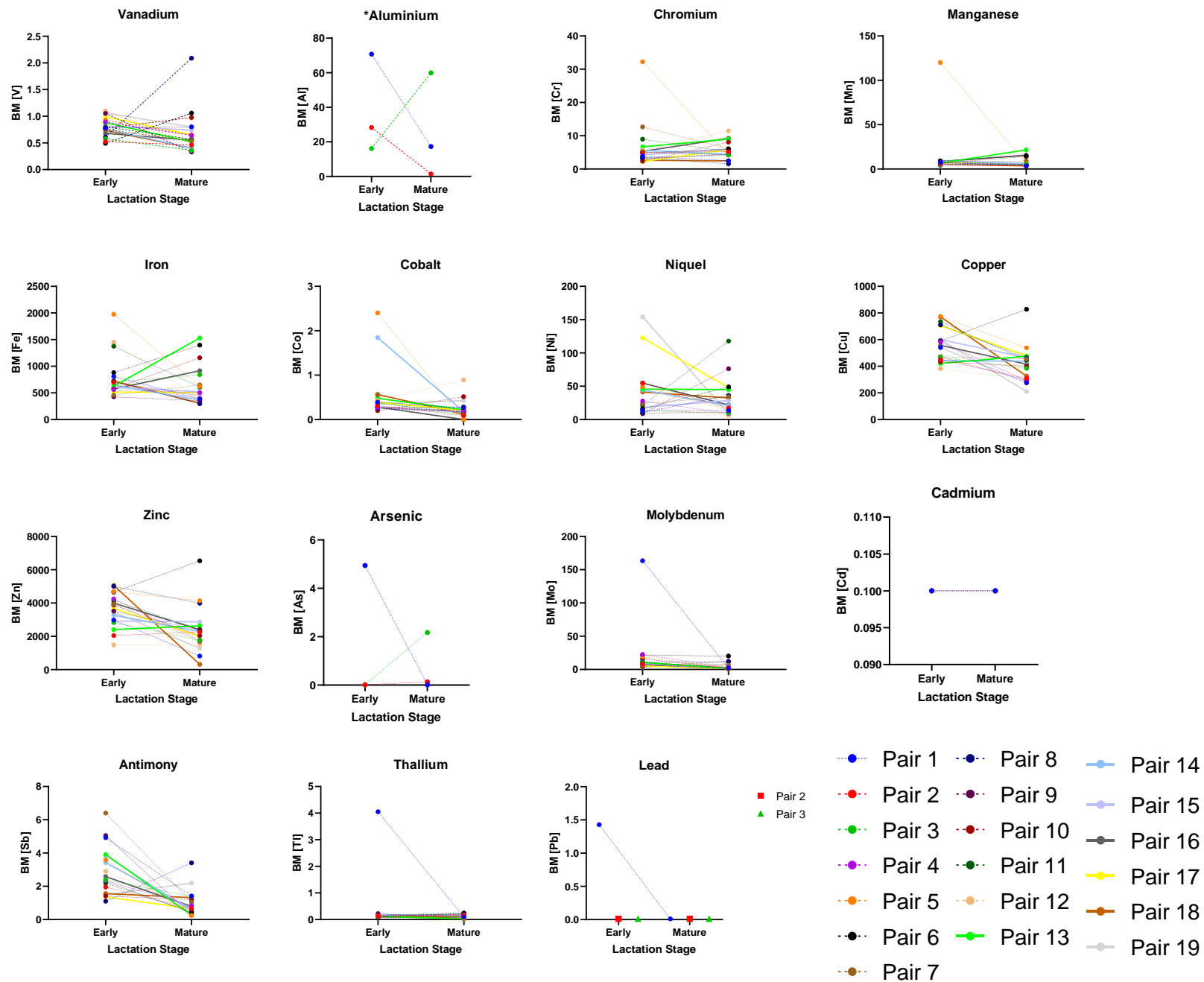Figure developed using GraphPad Prism 8 software.

Fig. S3. Longitudinal trends in metal and metalloid concentrations at the ng g^{-1} or ng ml^{-1} level in human milk samples from the 7th day to the 2nd month postpartum. Figure developed using GraphPad Prism 8 software. Out of 98 human milk samples, 71 were analysed for lead (Pb), aluminium (Al), arsenic (As), and cadmium (Cd), with 3 longitudinal pairs available. The limits of detection (LODs) for each element were as follows: Pb (0.01), Al (1.41), As (0.01), and Cd (0.1). When concentrations were below the LOD, they were reported accordingly.
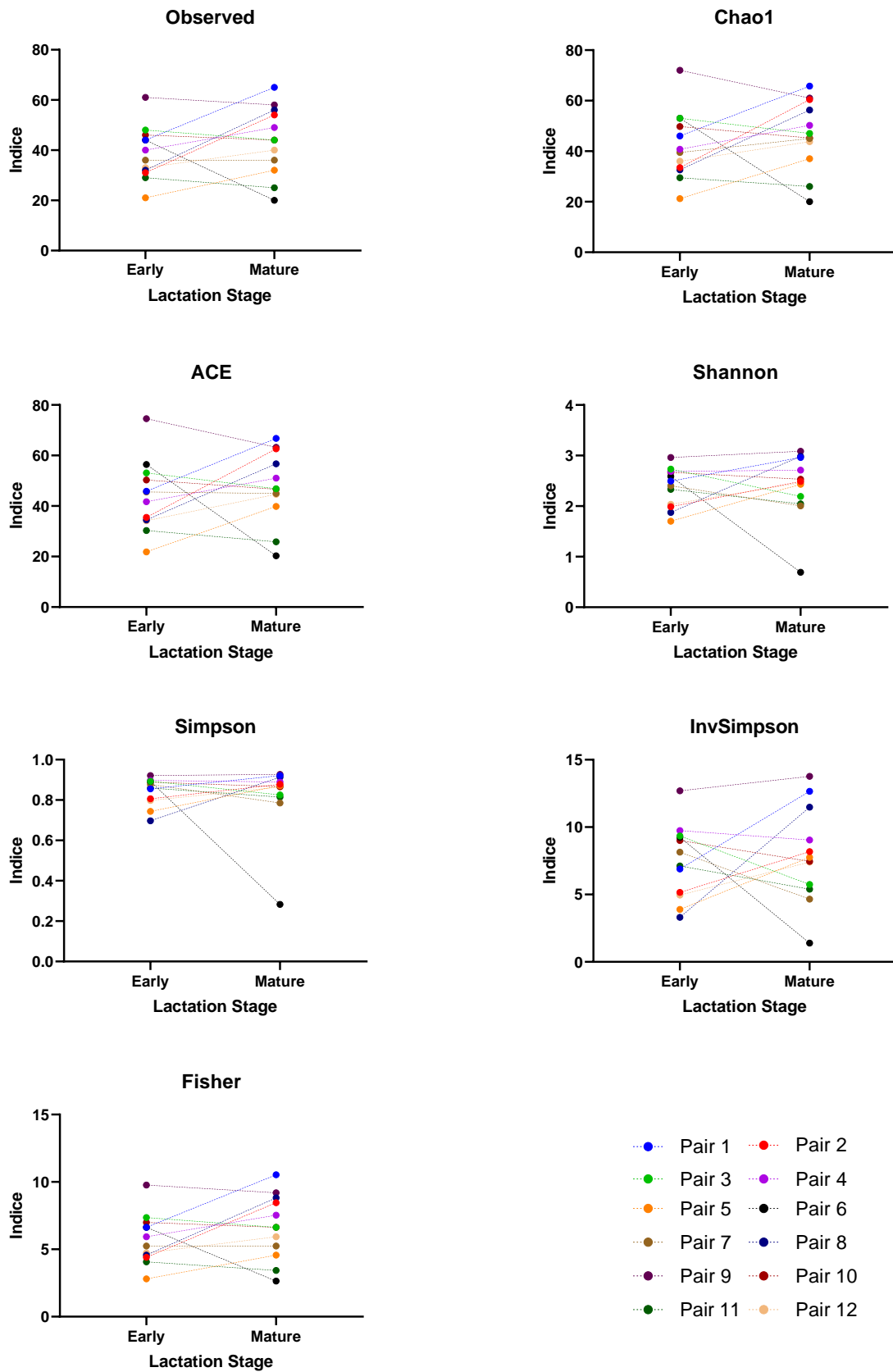
Fig. S4. Longitudinal trends in alpha diversity indices in infant faecal samples from the 7th day to the 2nd month postpartum.
Figure developed using GraphPad Prism 8 software.

Table S2. Infant gut microbiota alpha-diversity indices

	Early lactation stage (n = 56)	Mature lactation stage (n =32)	p-values*
Observed	33.5 (27.8-44)	42 (30.5-54.5)	0.036
Chao1	37.63 (30.38-49.15)	45.1 (35.83-61)	0.057
ACE	41.33 (32.03-50.33)	45.81 (35.96-62.22)	0.06
Shannon	2.17 (1.87-2.45)	2.19 (1.88-2.53)	0.65
Simpson	0.82 (0.75-0.86)	0.83 (0.77-0.88)	0.66
InvSimpson	5.67 (4.01-7.51)	5.86 (4.27-8.26)	0.64
Fisher	4.82 (4.03-6.64)	6.28 (4.32-8.54)	0.042

Median (Quartile 1-Quartile 3) of alpha diversity indices calculated for 88 infant stool samples. *p-values under 0.05, show significant differences between unpaired early and mature samples as determined by a Mann-Whitney U test.

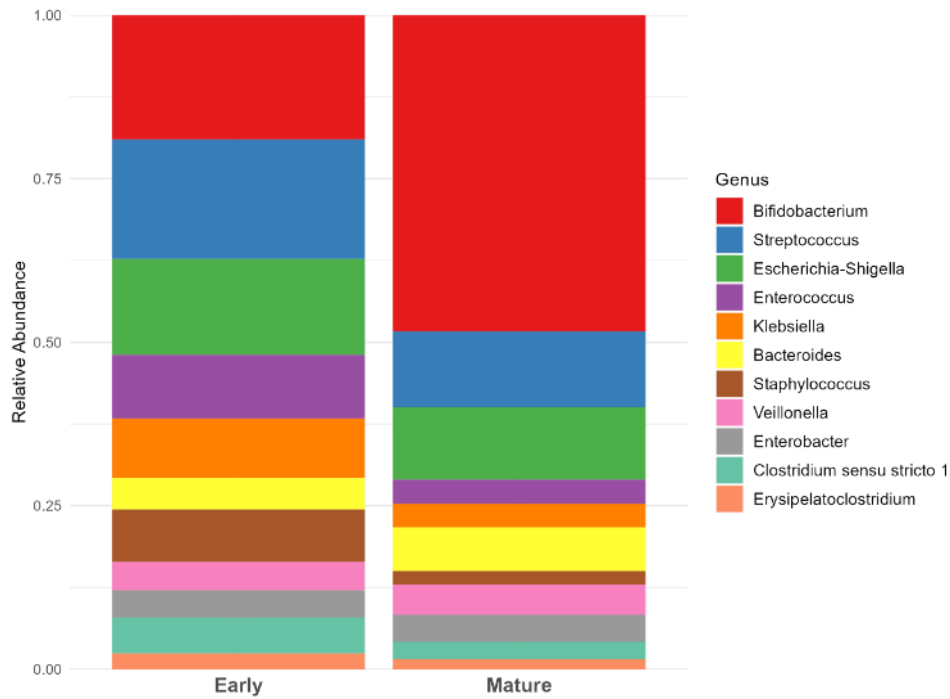**Fig. S5.** Infant gut microbiota at genus level. Data is shown as relative abundances.
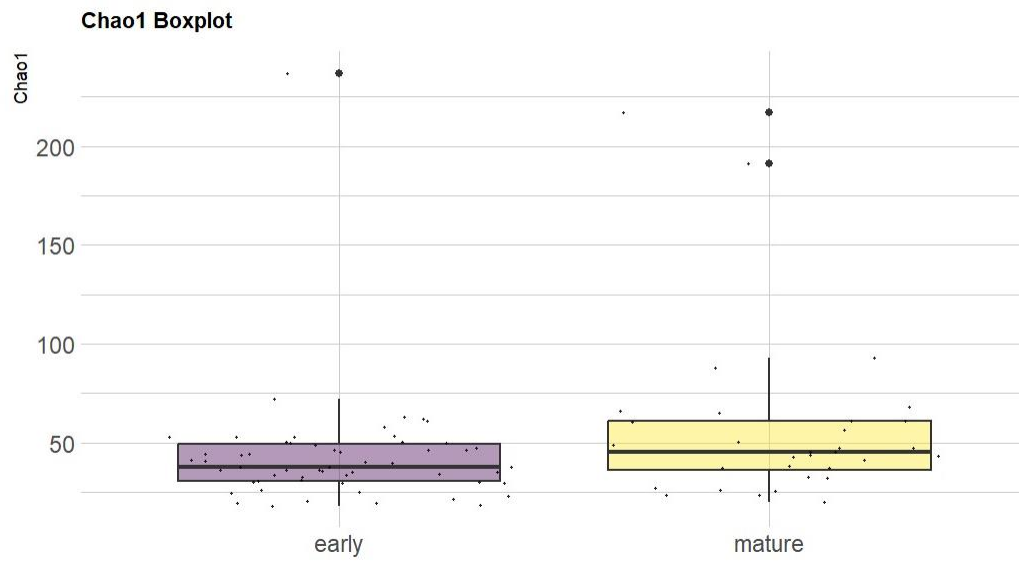
Chao1 Boxplot

Fig.S6. Boxplot showing differences between Chao1 indices during early and mature lactation stage in infant faecal microbiota.
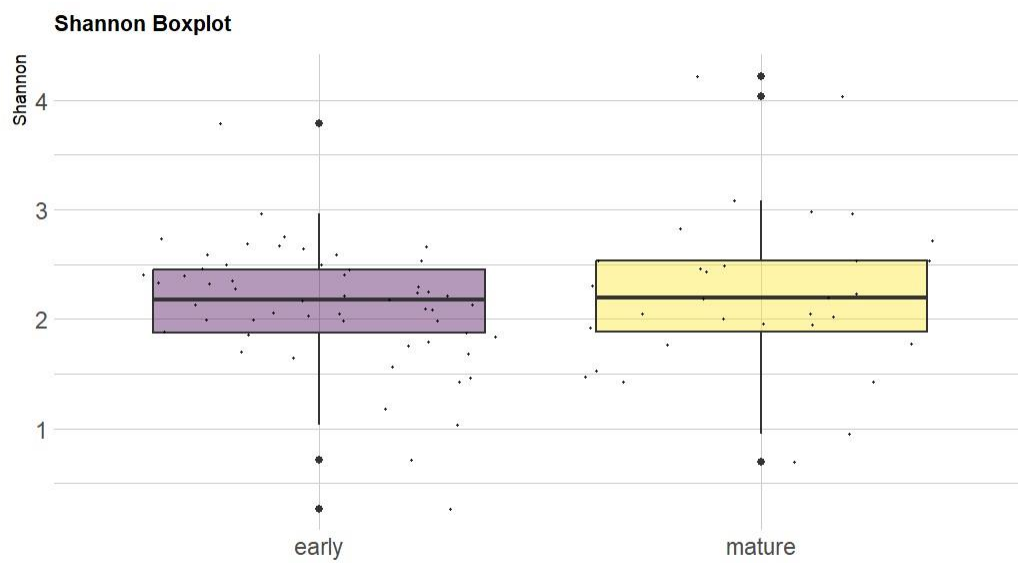
Shannon Boxplot

Fig. S7. Boxplot showing differences between Shannon indices during early and mature lactation stage in infant faecal microbiota.

Table S3. Correlations between infant gut microbiota and alpha-diversity indices and Pb, Al and As

		*	Pb (n = 11)	Al (n = 59)	As (n = 32)
Infant gut microbiota	*Bifidobacterium*	rho	-0.32722	-0.08823	-0.16843
		p-values	0.3561	0.551	0.382439
	Streptococcus	rho	0.030488	-0.06568	-0.04631
		p-values	0.9334	0.6574	0.811233
	Staphylococcus	rho	0.347567	0.043969	0.347783
		p-values	0.3251	0.7667	0.065129
	Escherichia-Shigella	rho	0.05033	0.098837	-0.0376
		p-values	0.8902	0.5039	0.8465
	Enterococcus	rho	0.204467	-0.05303	-0.06481
		p-values	0.571	0.7204	0.7384
	Gemella	rho	0.352313	0.15081	0.197038
		p-values	0.3181	0.3062	0.3056
	Klebsiella	rho	-0.51853	0.1822	0.006535
		p-values	0.1246	0.2152	0.9732
	Lacticaseibacillus	rho	NA	-0.00673	0.003363
		p-values	NA	0.9638	0.9862
	Clostridium sensu stricto 1	rho	-0.12963	0.019403	-0.17902
		p-values	0.7211	0.8959	0.3528
	Veillonella	rho	0.154137	0.07621	-0.2223
		p-values	0.6707	0.6067	0.2464
	Bacteroides	rho	-0.02251	-0.07989	-0.23477
		p-values	0.9508	0.5893	0.2202
	Enterobacter	rho	0.288525	-0.02647	-0.11875
		p-values	0.4188	0.8582	0.5395
	Acinetobacter	rho	NA	0.7257	0.039597
		p-values	NA	0.051975	0.8384
	Ruminococcus_gnavus_group	rho	-0.07502	-0.0545	-0.28059
		p-values	0.8368	0.7129	0.1404
	Erysipelatoclostridium	rho	-0.22628	-0.13595	0
		p-values	0.5296	0.3569	1
	Sediminibacterium	rho	NA	NA	NA
		p-values	NA	NA	NA
Diversity indices	Shannon	rho	-0.63416	0.071219	-0.06207
		p-values	0.04894	0.6305	0.7485
	Chao1	rho	0.134969	-0.08575	0.022677
		p-values	0.7101	0.5623	0.907
	Fisher	rho	-0.01829	-0.08686	0.016778
		p-values	0.96	0.5572	0.9312
	ACE	rho	-0.01829	-0.13082	0.065517
		p-values	0.96	0.3755	0.7349
	Simpson	rho	-0.7744	0.046032	-0.0468
		p-values	0.008543	0.756	0.8093
	InvSimpson	rho	-0.72562	0.055477	-0.01724
		p-values	0.01753	0.708	0.9298

*Rho and p-values represent the outputs of Spearman correlations. The available sample sizes for Pb, Al, and As were 11, 59, and 32, respectively. All metal concentrations correspond to early lactation stage, between 7th and 15th day post-partum. NA: not available, correlation and p-values were not computable.

Table S4. Comparison of Metal and Metalloid Concentrations with Literature Values.

Study	Year	samples	Location	As	Al	Cd	Pb	Zn	Mn	Cu	Fe	Ni
MAMI cohort	2024	98*	Valencia, Spain	0.92-12.52	13.5-67.18	0.3	0.285-0.35	1520.7-4172.65	4.91-8.79	302.52-651.75	391.6-1031.73	13.68-47.23
Hasballah and Beheary (1)	2016	10	Damietta, Egypt	NA	NA	1.504±1.371	1.675±2.426	2.311±5.36	0.254±0.42	0.076±0.15	2.655±4.16	0.014±0.04
Dillon et al (2)	1974	29	7 US cities	NA	NA	NA	0.006-0.058	NA	NA	NA	NA	NA
Lamm et al (3)	1971	14	Connecticut, US	NA	NA	NA	0.0-0.07	NA	NA	NA	NA	NA
Souad et al (4)	2006	396	Marrakech, Morocco	NA	36.3 (3.1–117.4)	NA	36.3 ± 26.1	NA	NA	NA	NA	NA
Al-Saleh et al (5)	2003	171	Riyadh, Saudi Arabia	NA	NA	1.011 (<LOD-11.672)	16.397 (<DL-354.901)	NA	NA	NA	NA	NA
Al-Saleh et al (5)	2003	201	Al-Ehssa, Saudi Arabia	NA	NA	1.483 (<LOD-9.224)	26.889 (<LOD-489.832)	NA	NA	NA	NA	NA
Turan et al (6)	2001	30	Ankara, Turkey	NA	NA	2.8 (1.2-9)	14.6 (8.8-35.4)	12900 (4500-27100)	43.2 (12.5-70.7)	278 (179-454)	3500 (2100-11000)	27.8 (14.9-47.9)
Leotsinidis et al (7)	2005	95	Patras, Greece	NA	NA	0.127 (<LOD-0.487)	<LOD (0.94)	2969 (864-6550)	2.56 (0.17-9.89)	408 (120-614)	369 (96-1544)	NA
Namihira et al (8)	1986	35	Mexico City, Mexico	NA	NA	NA	61.6	NA	NA	NA	NA	NA
Toxicological guidance value: (9,10)				BMDL0.5: 3.0 µg/kg bw per day (2.0-7.0 µg/kg bw per day (for inorganic arsenic), NOAEL of 30 mg/kg	2 mg/kg bw (expressed as Al), 30 mg/kg bw per day	PTMI 25 µg/kg bw (2010)	Children: 0.03 to 9 µg/kg bw/d (mean), 0.2 to 8.2 µg/kg bw/d (90th to 97.5th percentile)	PMTDI 0.3-1 mg/kg bw (1982)	NA	PMTDI 0.05 - 0.5 mg/kg bw (1982)	PMTDI 0.8 mg/kg bw (iron from all sources except for iron oxides used as colouring agent, supplemental iron taken during pregnancy and lactation, and supplemental iron for specific clinical requirements)	TDI of 13 µg/kg bw

All concentrations are in ng/ml. 71 human milk samples analysed for Pb, Al, As, and Cd. Values shown are medians and standard deviations. NA; not available, Benchmark Dose Lower Confidence Limit, BMDL0; No Observed Adverse Effect Level, NOAEL; Provisional Tolerable Monthly Intake, PTMI; tolerable daily intake, TDI. See references below.

List of references table S4:

1. Hasballah AF, Beheary MS. Detection of Heavy Metals in Breast Milk and Drinking Water in Damietta Governorate, Egypt. Asian Journal of Biology 2016;1–7.

2. Dillon HK, Wilson DJ, Schaffner W. Lead concentrations in human milk. Am J Dis Child 1974;128:491–2.

3. Lamm S, Cole B, Glynn K, Ullmann W. Lead Content of Milks Fed to Infants — 1971–1972. New England Journal of Medicine Massachusetts Medical Society; 1973;289:574–5.

4. Souad C, Farida Z, Nadra L, François B, Bougle D, Azeddine S. Trace element level in infant hair and diet, and in the local environment of the Moroccan city of Marrakech. Science of The Total Environment 2006;370:337–42.

5. Al-Saleh I, Shinwari N, Mashhour A. Heavy metal concentrations in the breast milk of Saudi women. Biol Trace Elem Res 2003;96:21–37.

6. Turan S, Saygi Ş, Kiliç Z, Acar O. Determination of Heavy Metal Contents in Human Colostrum Samples by Electrothermal Atomic Absorption Spectrophotometry. Journal of Tropical Pediatrics 2001;47:81–5.

7. Leotsinidis M, Alexopoulos A, Kostopoulou-Farri E. Toxic and essential trace elements in human milk from Greek lactating women: association with dietary habits and other factors. Chemosphere 2005;61:238–47.

8. Namihira D, Saldivar L, Pustilnik N, Carreón GJ, Salinas ME. Lead in human blood and milk from nursing women living near a smelter in Mexico City. Journal of Toxicology and Environmental Health Taylor & Francis; 1993;38:225–32.

9. Codex Alimentarius Commision. WORKING DOCUMENT FOR INFORMATION AND USE IN DISCUSSIONS RELATED TO CONTAMINANTS AND TOXINS IN THE GSCTFF. Japan and The Netherlands; 2023. Report No.: CF/16 INF/1.

10. World Health Organization, Food and Agriculture Organization of the United Nations, Joint FAO/WHO Expert Committee on Food Additives. Meeting (73rd : 2010 : Geneva S. Evaluation of certain food additives and contaminants: seventy-third [73rd] report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 2011 [cited 2024 Aug 12]; Available from: https://iris.who.int/handle/10665/44515