

RESEARCH ARTICLE

Intestinal *Enterococcus* abundance correlates inversely with excessive weight gain and increased plasma leptin in breastfed infants

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One sentence summary: The reported findings suggest that lack of seeding with *Enterococci* from breastmilk into the infant gut influences the unusual development of excessive weight gain in breastfed infants.

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ABSTRACT

Epidemiological evidence indicates that breastfeeding provides protection against development of overweight/obesity. Nonetheless, a small subgroup of infants undergo excessive weight gain during exclusive breastfeeding, a phenomenon that remains unexplained. Breast milk contains both gut-seeding microbes and substrates for microbial growth in the gut of infants, and a large body of evidence suggests a role for gut microbes in host metabolism. Based on the recently established SKOT III cohort, we investigated the role of the infant gut microbiota in excessive infant weight gain during breastfeeding, including 30 exclusively breastfed infants, 13 of which exhibited excessive weight gain and 17 controls which exhibited normal weight gain during infancy. Infants undergoing excessive weight gain during breastfeeding had a reduced abundance of gut *Enterococcus* as compared with that observed in the controls. Within the complete cohort, *Enterococcus* abundance correlated inversely with age/gender-adjusted body-weight, body-mass index and waist circumference, body fat and levels of plasma leptin. The reduced abundance of *Enterococcus* in infants with excessive weight gain was coupled to a lower content of *Enterococcus* in breast milk samples of their mothers than seen for mothers in the control group. Together, this suggests that lack of breast milk-derived gut-seeding *Enterococci* may contribute to excessive weight gain in breastfed infants.

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Keywords: excessive weight gain; breastfeeding; infant; gut microbiota; *Enterococcus*; leptin

INTRODUCTION

Epidemiological studies suggest that breastfeeding has a moderately protective effect against development of overweight/obesity (Itabashi et al. 2012; Victora et al. 2016; Azad et al. 2018). However, a small subgroup of infants (without genetic predisposition towards obesity) show unusually rapid weight gain during infancy despite being fully breastfed (Grunewald et al. 2014; Perrella and Geddes 2016; Saure et al. 2017; Larsson et al. 2019a), sometimes followed by weight catch-down after introduction of solid foods (Grunewald et al. 2014; Larsson et al. 2019a). This suggests that components in the breastmilk are contributing to the excessive weight gain (EWG) observed in this subset of infants. Indeed, hormones (insulin, ghrelin, adiponectin and leptin), cytokines (IL-6 and TNF- α) and macronutrients composition (e.g. protein content) in breastmilk have been suggested to impact infant adiposity although the literature on this is inconclusive (Fields, Schneider and Pavela 2016; Eriksen et al. 2018). Breast milk also contains human milk oligosaccharides (HMOs), which have been linked with infant growth and body composition (Alderete et al. 2015; Larsson et al. 2019b) and as a constituent of breast milk modulate the infant gut microbiota composition (Matsuki et al. 2016). Additionally, recent research has provided strong evidence that breast milk contains a variety of microbes (Hunt et al. 2011; Cabrera-Rubio et al. 2012; Boix-Amorós, Collado and Mira 2016; Moossavi et al. 2019), which reach the infant gut and affect its microbial population (Pannaraj et al. 2017). A range of studies have linked the infant gut microbiota to development of infant/childhood obesity (Kalliomaki et al. 2008; Dogra et al. 2015; Kozyrskyj et al. 2016; Korpela et al. 2017; Stanislowski et al. 2018). Although evidence of a causal relation between infant gut microbes and obesity is still lacking, studies in animals show that obesity phenotypes are transmissible via faecal microbiota transplants (Ridaura et al. 2013; Zhang et al. 2016), suggesting a contributing role of the gut microbiota to weight gain. However, very little is known about the influence of maternal milk and infant gut microbes on infant growth and body composition. Considering that breast milk is the sole dietary component for exclusively breastfed infants, and inversely associated with risk of obesity development, the fact that some infants show EWG during breastfeeding is intriguing. Motivated by the fact that breastmilk contains both gut-seeding microbes and selective substrates for microbial growth (e.g. HMOs) as well as by the growing evidence of a role for gut microbes in host metabolism, we set out to investigate associations between infant gut microbiota composition and EWG during breastfeeding in the recently established SKOT III cohort, which comprises breastfed infants showing either unusual EWG or normal weight gain (NWG) (control) during infancy (Larsson et al. 2018).

MATERIALS AND METHODS

Cohort subjects

The SKOT III cohort subjects, recruitment and inclusion criteria has been described previously (Larsson et al. 2018). The study protocol was approved by the Regional Ethical Committee of the Capital Region of Denmark in accordance with the Helsinki declaration (H-15008948) and registered at the Danish Data Protection Agency (2015-57-0117 and 2015-57-0116). Written informed

consent was obtained from the parents. After exclusion of six participants that did not meet the inclusion criteria or showed deviating growth patterns, the cohort consisted of 30 exclusively or fully breastfed (the latter defined as receiving human milk as a primary energy source, but allowed supplementation with water, minerals, vitamins and maximum one meal of formula per week) infants, with 13 exhibiting EWG (increment of ≥ 1 SDs in weight-for-age Z-score during the first five months postpartum and a weight-for-age Z-score > 2.00 at recruitment time) and 17 infants with NWG (weight-for-age Z-score between -1.00 and $+1.00$ SDs at first examination). Inclusion criteria applicable for both groups were exclusively or fully breastfed for at least four months postpartum. At first visit all infant were required to be within 5.0–6.5 months of age and have breastfeeding as the primary energy source, allowing maximum two meals per day of solid foods. At second visit infants were required to be 9 months ± 2 weeks. One infant (from the EWG group) dropped out before the nine months visit. In the present study one infant faecal sample at five months (NWG group) was excluded due to oral maternal antibiotics at time of sampling and one infant faecal sample was excluded at nine months (NWG group) due to oral infant antibiotics within seven days prior to sampling. Further, two faecal samples at five months (one from each group) and two faecal samples at nine months (one from each group) failed during sequencing, thus no data were available for these. An overview of all samples and data can be found in Table S1, Supporting Information.

Parental questionnaires

Information on age, gender, birth mode, presence of siblings, breastfeeding practices and introduction of solid foods were collected from parental questionnaires as described previously (Larsson et al. 2018).

Anthropometrics and body composition

Measurement of infant and mother body-weight, height and infant waist circumference were performed as described previously (Larsson et al. 2018), and age/gender specific Z-scores calculated using the software WHO Anthro (World Health Organisation 2011). Infant body fat mass was estimated by subtracting body weight with fat-free mass, measured using a tetrapolar Bioelectrical Impedance Analyzer Quantum III (RJL Systems, Clinton Township, Detroit, MI, USA). Fat mass % and fat-mass index were calculated as described previously (Larsson et al. 2018).

Blood samples

Venous blood samples were collected at five and nine months of age into EDTA tubes and kept on ice until plasma separation. Plasma leptin (DLP00) and adiponectin (DRP300) concentrations were measured by ELISA (R&D Systems, Inc. Minneapolis, MN, USA).

Faeces and milk samples and DNA extraction

Faecal samples were collected from all participants at 5–6 months and 9 months of age and stored at -80°C until DNA extraction from 200 mg faeces in random order using the DNeasy

PowerLyzer PowerSoil kit (Qiagen, 12855–100) with the following modification: bead beating was performed at 30 cycles/s for 10 min (Retsch MM 300 mixer mill) and the initial centrifugation steps were performed at 10 000 x *g* for 3 min, as recommended for clay matter. Breast milk samples were also collected from mothers at the same time points as the faecal sampling from infants. The mothers were instructed to collect 10 mL milk from the start of the breastfeeding session (foremilk) and to collect 10 mL at the end of the same breastfeeding session (hindmilk), both expressed from the same breast. All milk samples were collected in participants home into disposable tubes using a manual breast pump (Type Harmony™, Medela AG, Baar, Switzerland) and stored at –20°C until transportation (on ice) to the university where the samples were stored at –80 °C until DNA extraction. Prior to DNA extraction approximately 2 mL milk was centrifuged at 20 000 x *g* for 2 min. in micro-centrifuge tubes, after which the upper fat layer was removed using a pipette tip and the supernatant discarded by inverting the tube. The pellets were then re-suspended in 750 µL bead solution (from the DNeasy PowerLyzer PowerSoil kit) and transferred to a new tube. Total DNA was extracted in random order from these suspensions using the same protocol as for faecal samples. A total of 10 blank DNA extraction controls (one for each batch of DNA extraction) were included following all the same steps as in the procedure for the milk samples. DNA concentrations in both faeces and milk were measured by Qubit® dsDNA HS assay (Invitrogen™, Q32851).

PCR and 16S rRNA gene amplicon sequencing

PCR amplification and Ion Torrent sequencing was performed on DNA extracted from faecal material, as described previously (Laursen, Dalgaard and Bahl 2017). Briefly, the V3-region of the 16S rRNA gene was amplified using 1–5 ng community DNA as template, 0.2 µL Phusion High-Fidelity DNA polymerase (Fisher Scientific, F-553 L), 4 µL HF-buffer, 0.4 µL dNTP (10 mM of each base), 1 µM forward primer (PBU 5'-A-adaptor-TCAG-barcode-CCTACGGGAGGCAGCAG-3') and 1 µM reverse primer (PBR 5'-trP1-adaptor-ATTACCGCGGCTGCTGG-3') in 20 µL total reaction volume. Both primers (TAG Copenhagen A/S) were linked to sequencing adaptors and the forward primer additionally contained a unique 10 bp barcode (Ion Xpress™ Barcode Adapters) for each sample. The PCR program consisted of initial denaturation for 30 s at 98°C, followed by 24 cycles of 98°C for 15 s and 72°C for 30 s and finally 72°C for 5 min to allow final extension before cooling to 4°C. No template controls were included for each PCR run, all resulting in less than 0.05 ng/µL. A mock community (HM-276D, obtained through BEI Resources, NIAID, NIH as part of the Human Microbiome Project) consisting of 200 000 16S rRNA genes embedded into the genomes of 20 bacterial species was used as template in triplicates for each intended sequencing run to control for inter and intra sequencing run consistency. The PCR products were purified by use of HighPrep™ PCR Magnetic Beads (MAGBIO®, AC-60 005) with a 96-well magnet stand (MAGBIO®, MyMag 96), according to the manufacturers recommendations. DNA quantity was measured using Qubit® dsDNA HS assay (Invitrogen™, Q32851) and samples (including triplicates of mock communities) were pooled to obtain equimolar libraries and sequenced on two separate chips using the Ion OneTouch™ and Ion PGM systems with a 318-Chip v2 incorporating the Hi-Q chemistry in 200 bp runs. Sequence data has been deposited at NCBI's Sequence Read Archive under BioProject number PRJNA553872.

Bioinformatics

Raw sequencing reads were de-multiplexed according to barcode and trimmed to remove barcodes and 16S rRNA gene primers, maintaining only reads containing both forward and reverse primers and discarding reads below 125 bp or above 180 bp using the CLC Genomic Workbench v8.5 software (CLCbio, Qiagen, Aarhus, DK). ASV (Amplicon Sequence Variant) analysis was performed in R (R Development Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>) using the DADA2 pipeline v1.8 (Callahan et al. 2016). Briefly, the primer and adaptor filtered reads were quality filtered (*filterAndTrim* function) using maximum number of expected error of 1 (*maxEE* = 1) and no allowed ambiguous bases (*maxN* = 0). Errors were learned (*learnErrors* function) using *nbases* = 10¹⁰, reads dereplicated (*derepFastq* function) and denoised (*dada* function). Chimeric sequences were removed (*removeBimeraDenovo* function) and the resulting ASVs were assigned taxonomy (*assignTaxonomy* and *addSpecies* functions) by using the RDP 16S rRNA database (Wang et al. 2007). The output ASV table was converted into .biom format within the QIIME v1.9 (Caporaso et al. 2010) environment. In QIIME, low abundant ASVs were filtered (*filter_otus_from_otu_table.py/filter_faste.py*, *min_count_fraction* 0.005%), resulting in 307 ASVs. A phylogenetic tree was constructed (*make_phylogeny.py*, *FastTree*, default settings) based on alignment of all remaining ASV sequences (*align_seqs.py*, *filter_alignment.py*, default settings), including an archaea (*Methanosarcina*) full length 16S rRNA gene sequence which was included as outgroup for rooting, re-rooted to the outgroup and subsequently pruned, using Dendroscope v3.5.7 (Huson and Scornavacca 2012). The *core_diversity_analysis.py* script was run to sum relative abundances of ASVs to higher taxonomical levels (to genus, family, order, class and phylum) and to estimate alpha diversity (Shannon index, Observed ASVs), beta diversity (weighted/unweighted UniFrac and Bray-Curtis), with a sampling depth of 20 000 sequences per sample.

Quantitative PCR

Absolute abundance of *Enterococcus* was estimated with qPCR, using genus specific primers (Rinttila et al. 2004). The mastermix contained 6 µL PCR-grade water, 1 µL forward primer (Ent1, 5'-CCCTTATTGTTAGTTGCCATCATT-3', final conc. 0.5 µM), 1 µL reverse primer (Ent2, 5'-ACTCGTTGTACTTCCCATTGT-3', final conc. 0.5 µM), 10 µL SYBR Green I Master 2X (LightCycler® 480 SYBR Green I Master, Roche, 0 488 735 2001) and 2 µL template DNA, in a 20 µL total volume. Template DNA was either 10-fold serial dilutions of 1 ng/µL DNA extracted from a pure culture of *E. faecalis* DSM 20478^T or community DNA (1–5 ng/µL) extracted from faecal or milk samples, run in triplicates on the qPCR instrument (LightCycler® 480 Instrument II, Roche, 05015243001) using the following program: 5 min pre-incubation at 95°C, followed by 45 cycles with 10 s at 95°C, 15 s at 50°C and 15 s at 72°C. For the milk sample runs, 10 blank DNA extraction controls were run as negative controls for the DNA extraction procedure and 2 no template controls as negative controls for the qPCR. A melting curve analysis was subsequently performed with 5 min at 95°C, 1 min at 65°C and continuous temperature increase (ramp rate 0.11°C/s) until 98°C. Data were analyzed with the LightCycler® 480 Software (v 1.5) and *Enterococcus* abundance in each sample was quantified based on standard curves of pure culture DNA from *E. faecalis* DSM 20478^T.

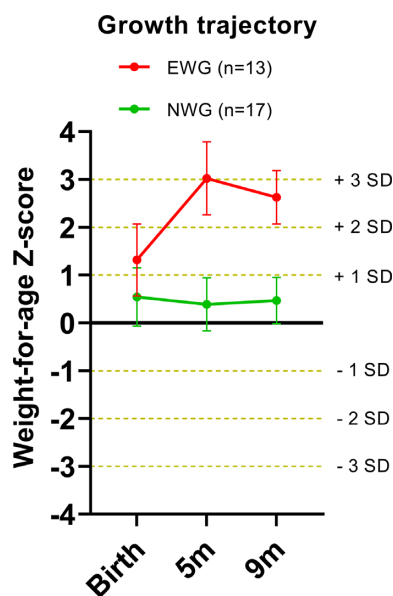


Figure 1. Growth trajectories of infants in the SKOT III cohort. Dashed yellow lines indicate standard deviations from the median growth trajectory (black line) of the reference population based on the WHO child growth standards (Espejo 2007). Dots and error-bars indicate mean \pm sd.

Statistics

Normal distribution of data was evaluated by Shapiro-Wilk test using the GraphPad Prism software (v.8.1, GraphPad Software Inc., La Jolla, CA). Parametric and non-parametric tests (T-tests, Mann-Whitney U tests, Fisher's exact test, Spearman's Rank correlations) were performed with the GraphPad Prism software or R software. For testing of differential abundance taxa (at ASV and genus levels) between EWG and NWG groups, a cut-off of 25% prevalence across all samples was used. When appropriate, raw P-value were adjusted for multiple testing by the Benjamini-Hochberg False Discovery Rate approach (Benjamini and Hochberg 1995), using a $q < 0.15$ as a cutoff. ADO-NIS and PERMDISP test's (compare.categories.py, permutations = 999) of weighted/unweighted UniFrac distances or Bray-Curtis dissimilarities was performed in QIIME v1.9 (Caporaso et al. 2010).

RESULTS

Cohort characteristics

The SKOT III cohort (Larsson et al. 2018) consist of 30 full-term breastfed infants, 13 of which exhibited EWG and 17 of which were controls that exhibited NWG during infancy (Fig. 1). Both groups were examined at approximately five and nine months of age, however, one infant dropped out before the nine month visit and for the present study a few samples/data points were excluded in the further analysis due to either use of oral/maternal antibiotics ($n = 2$) or no sequencing reads obtained ($n = 4$) in the 16S rRNA amplicon sequence analysis of faecal material, ending up with 27 and 26 infants with gut microbiota data at 5 and 9 months, respectively (Table 1 and Table S1, Supporting Information). The two groups did not differ with respect to age, gender distribution, caesarean section prevalence or maternal BMI (Table 1). However, as expected the EWG group had markedly and significantly higher BMI-for-age, weight-for-age and height-for-age Z-scores, higher waist

circumference as well higher fat mass, fat mass % and fat mass index than the NWG group at both five and nine months of age (Table 1). At five months, all infants in each group were breastfed, none received formula, and only limited amount of complementary foods had been introduced. At nine months, most infants were still partially breastfed and the two groups did not differ significantly in breastfeeding practices (Table 1). The age at introduction of complementary foods in the form of vegetables, fruits/berries and porridge as reported by the parents did not differ between the groups (Fig. S1A-C, Supporting Information). However, compared to the NWG group, infants in the EWG group were significantly earlier exposed to meat, fish and poultry food products ($P = 0.0005$, Fig. S1D, Supporting Information).

Infants with EWG show reduced intestinal *Enterococcus* abundance

16S rRNA amplicon sequencing of faecal material from the infants at both 5 and 9 months of age revealed that while alpha diversity measures (Observed ASVs, Shannon index) significantly increased over time, they did not differ between the two groups at 5 months. However, a higher number of observed ASVs were present in EWG than in NWG at 9 months (Fig. S2, Supporting Information), possibly due to the earlier introduction of meats, fish, and poultry food products in the EWG group (Fig. S1D, Supporting Information). We found a significant association between these food groups and the number of observed ASVs (Fig. S2C, Supporting Information), which is in line with our observations from the SKOT I and II cohorts (Laursen et al. 2016). Analysis of weighted UniFrac distances showed that the gut microbiota was significantly affected by age ($r^2 = 0.16$, $P < 0.001$, Adonis, Fig. 2A), characterized by a marked reduction of *Bifidobacterium* and increase in Clostridiales genera (*Faecalibacterium*, *Anaerostipes*, *Blautia*, *Roseburia*, *Lachnospiraceae* and *Ruminococcus2*), *Veillonella* and *Escherichia* occurring between five months and nine months of age. Weighted/unweighted UniFrac distance and weighted/unweighted Bray-Curtis dissimilarity measures at either five or nine months revealed that differences between microbiota communities were not significantly explained by exact age at sampling, gender, breastfeeding, age of introduction to selected food products, caesarean section prevalence and presence of siblings or maternal obesity (Table S2 and S3, Supporting Information). Additionally, no apparent clustering of the EWG and NWG groups was observed at each of the sampling points ($P > 0.05$, Adonis, weighted UniFrac, Fig. 2B and C; Table S2 and S3, Supporting Information). Likewise, the genus level microbiota composition was similar between the two groups at each time point (Fig. 2B and C; Table S4 and S5, Supporting Information). Nevertheless, infants with EWG during the first five months of life were found to have a markedly reduced relative abundance of *Enterococcus* ($P = 0.008$, $q = 0.147$) at five months of age (Fig. 2D, left panel and Table S4, Supporting Information), which was however, no longer apparent at nine months of age (Fig. 2E, left panel). The reduction in *Enterococcus* colonization was confirmed by specific qPCR, which showed a more than 10-fold lower median of absolute abundances in the EWG group at 5 months of age (Fig. 2D, centre panel), but no difference at 9 months of age (Fig. 2E, right panel). The three most abundant/prevalent ASVs within the *Enterococcus* genus were 100% identical to *E. faecalis* (ASV16), *E. faecium* group (ASV13) and *E. casseliflavus* group (ASV69) species, respectively (Table S6, Supporting Information). Of these, particularly ASV16 and ASV69

Table 1. Characteristics of the SKOT III cohort infants with gut microbiota data.

	5 months of age (n = 27)			9 months of age (n = 26)		
	NWG (n = 15)	EWG (n = 12)	P-value	NWG (n = 15)	EWG (n = 11)	P-value
Age (mean months ± sd)	5.91 ± 0.32	5.61 ± 0.53	0.08 ¹	9.08 ± 0.20	9.03 ± 0.30	0.58 ¹
Gender (boys/girls, %)	40.0/60.0	58.3/41.7	0.45 ²	40.0/60.0	54.5/45.5	0.69
Birth mode (C-section/vaginal, %) ^a	6.7/92.3	27.3/72.7	0.28 ²	13.3/86.7	36.4/63.6	0.35
Maternal BMI (mean BMI ± sd)	22.51 ± 2.30	25.86 ± 6.70	0.12 ³	22.92 ± 2.14	26.04 ± 6.08	0.13
Siblings (yes/no, %)	46.7/53.3	63.6/36.4	0.45 ²	53.3/46.7	63.6/36.4	0.70
Breastfed (%)	100.0	100.0	–	93.3	90.9	0.99
No longer breastfed (%)	0.0	0.0		6.7	9.1	
Weight-for-age (mean Z-score ± sd)	0.31 ± 0.14	3.11 ± 0.21	<0.0001¹	0.44 ± 0.13	2.67 ± 0.17	<0.0001¹
BMI-for-age (mean Z-score ± sd)	–0.20 ± 0.21	2.64 ± 0.25	<0.0001¹	0.13 ± 0.22	2.18 ± 0.25	<0.0001¹
Height-for-age (mean Z-score ± sd)	0.81 ± 0.19	1.90 ± 0.20	0.0006¹	0.63 ± 0.20	1.84 ± 0.18	0.0002¹
Waist circumference (mean cm ± sd)	42.1 ± 1.50	50.4 ± 2.25	<0.0001¹	43.4 ± 1.72	50.2 ± 1.79	<0.0001¹
Fat mass (mean kg ± sd) ^b	2.07 ± 0.31	3.65 ± 0.54	<0.0001¹	2.79 ± 0.27	4.23 ± 0.64	<0.0001¹
Fat mass percent (mean % ± sd) ^b	27.41 ± 2.99	35.35 ± 3.34	<0.0001¹	30.84 ± 2.16	36.05 ± 3.67	0.0002¹
Fat mass index (mean kg/m ² ± sd) ^b	4.46 ± 0.68	7.38 ± 0.98	<0.0001¹	5.32 ± 0.59	7.43 ± 1.03	<0.0001¹

^aData on was missing from one individual; ^bData missing on one individual at 5 months and one individual at 9 months.

¹Unpaired T-test; ²Fishers exact test; ³Unpaired T-test with Welch's correctoion; P-values indicating significant differences are given in bold text.

had reduced relative abundance in the EWG group (Fig. 1D, right panel), however no ASVs were significantly different from the NWG group after FDR correction (Table S7 and S8, Supporting Information).

Enterococcus abundance correlate with anthropometric measures, body fat and plasma levels of adipocyte-derived hormones

Significant negative correlations were found between relative abundance of *Enterococcus* at five months of age and BMI-for-age Z-score ($\rho = -0.63$, $P = 0.0004$), waist circumference ($\rho = -0.54$, $P = 0.004$), weight-for-age Z-score ($\rho = -0.63$, $P = 0.0004$), but not height-for-age Z-score ($\rho = -0.21$, $P = 0.29$) across all cohort individuals at five months (Fig. 3A). The correlations with *Enterococcus* abundance measured at 5 months were still present for anthropometric measures assessed at 9 months of age (Fig. S3A–D, Supporting Information). Additionally, significant negative correlations were found between relative abundance of *Enterococcus* at five months of age and fat mass percentage as well as fat mass index at both five and nine months of age (Fig. 3B and Fig. S3E–F, Supporting Information). Finally, we found that relative abundances of *Enterococcus* at five months of age correlated negatively with plasma leptin at both five months ($\rho = -0.67$, $P = 0.002$) and nine months ($\rho = -0.54$, $P = 0.012$), but positively with plasma adiponectin at both five months ($\rho = 0.68$, $P = 0.001$) and nine months ($\rho = 0.56$, $P = 0.009$) (Fig. 2C; Fig. S3G–H, Supporting Information). Within the NWG group (but not within the EWG group), significant correlations were also observed between relative abundance of *Enterococcus* at five months and BMI-for age Z-score, FMI, leptin and adiponectin at five months of age (Table S9, Supporting Information). Furthermore, absolute abundances of this genus at five months correlated with anthropometric measures, body fat mass as well as plasma leptin/adiponectin at five and/or nine months of age (Fig. S4, Supporting Information). However, the relative and absolute *Enterococcus* abundance at nine months of age did not correlate with anthropometric measures or plasma leptin/adiponectin (data not shown), suggesting that the potential impact of *Enterococcus* abundance is restricted to early infancy.

Breastmilk of mothers to EWG infants contain lower absolute abundance of *Enterococcus*

Since breastmilk was the primary diet of infants in our cohort at age five months and breastmilk has been indicated to be a major source of gut-seeding microbes (Pannaraj et al. 2017), we investigated whether the reduced *Enterococcus* abundance observed in the EWG group could arise from lack of these bacteria in maternal breastmilk. We found that both foremilk and hindmilk of mothers in the EWG group contained significantly lower absolute amounts of *Enterococcus* than that of mothers in the NWG group at infant age of five months, but not at nine months (Fig. 4), mirroring our observations in the gut. Thus, poor seeding of *Enterococcus* from breastmilk in the EWG group might explain the reduced abundance of *Enterococcus* species in the gut of these infants.

DISCUSSION

Although breastfeeding is linked to protection against development of overweight/obesity (Itabashi et al. 2012; Victora et al. 2016; Azad et al. 2018), unusual, EWG during exclusive breastfeeding is observed in a small subgroup of individuals (Grunewald et al. 2014; Perrella and Geddes 2016; Saure et al. 2017; Larsson et al. 2019a). The explanation for this phenomenon remains unknown. We recently established the SKOT III cohort, which includes 30 exclusively breastfed infants, of which 13 exhibited EWG and 17 underwent NWG during the first five months of life (Larsson et al. 2018). In spite of the relatively low sample size, this cohort provides a unique opportunity for investigation of the potential causes of EWG which is only rarely observed in exclusively breastfed infants. As reported in previous studies (Grunewald et al. 2014; Larsson et al. 2019a), the EWG infants exhibited a marked growth/adiposity catch-down during the complementary feeding period (Larsson et al. 2018), suggesting an important role of breast milk constituents. However, with the exception of milk-leptin concentrations, which were lower in the EWG group, breast milk concentrations of macronutrients, bioactive proteins and hormones or the amount of breast milk ingested did not differ between the two groups (Larsson et al. 2018). Additionally, we have recently reported that a limited number of HMOs in the maternal breast milk differed

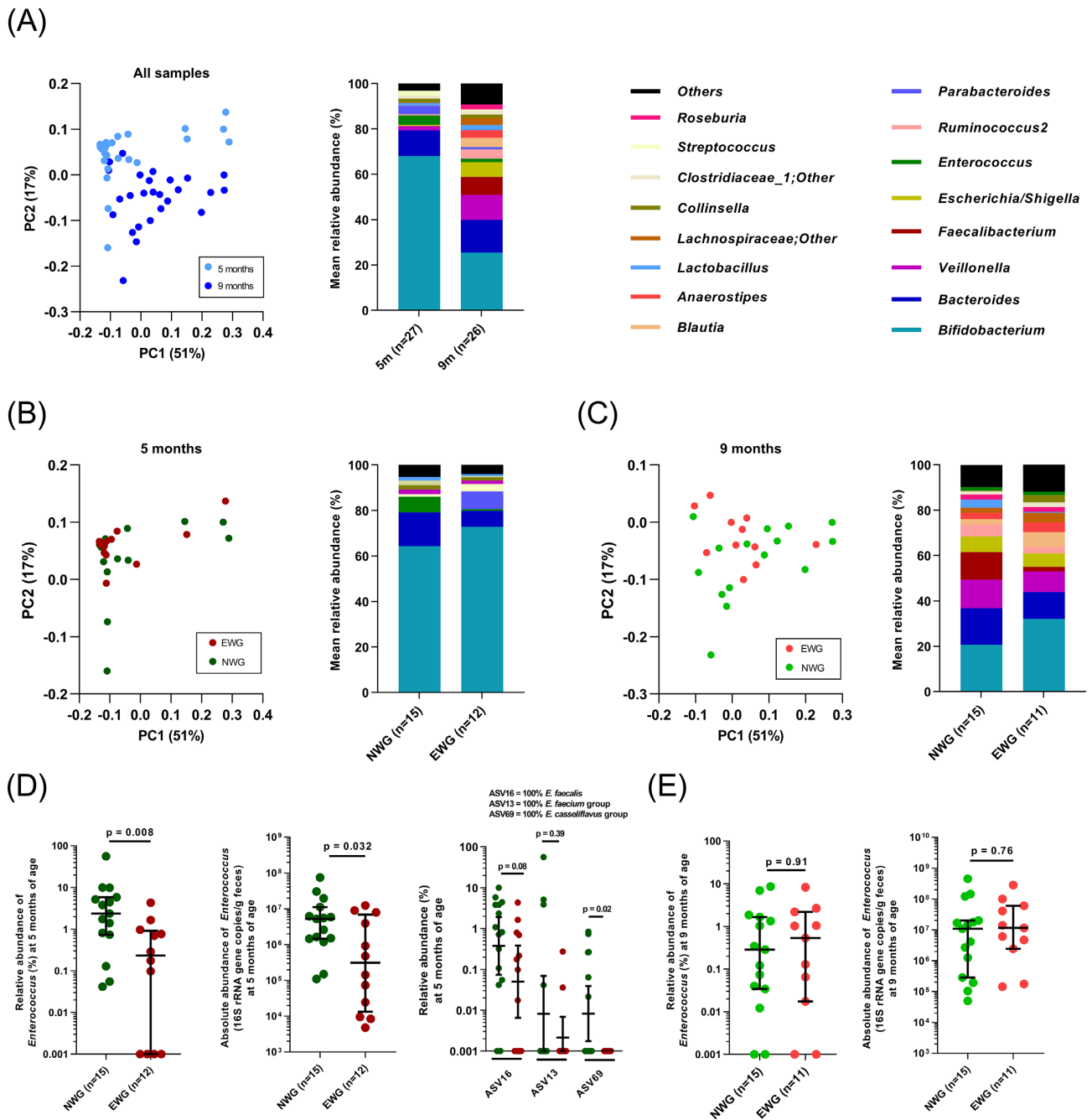


Figure 2. *Enterococcus* gut abundance during early infancy is lower in breastfed infants with excessive weight gain. (A-C) PCoA plots of weighted UniFrac distances based on all detected ASVs and bar plots of average relative abundances of genera derived from faecal samples of the SKOT III cohort infants; (A) all samples ($n = 53$) coloured according to age, (B) five months samples ($n = 27$) coloured according to group and (C) 9 months samples ($n = 26$) coloured according to group. NWG, normal weight gain, EWG, excessive weight gain. (D-E), Scatterplots of relative and absolute abundances of the *Enterococcus* genus or ASVs stratified according to group at D) 5 months and E) 9 months of age. Lines and error bars indicate median \pm IQR. Limit of detection = relative abundance of 0.001%. Statistical significance was evaluated by Mann-Whitney U test.

in concentration between the NWG and EWG groups (Larsson *et al.* 2019b), however, these were not associated with *Enterococcus* abundance in milk or infant faeces (data not shown). Nevertheless, the present study we revealed a reduced abundance of *Enterococcus* in the EWG infants, which correlated with anthropometric measures, body fat and levels of plasma adipocyte-derived hormones. We also report the first evidence of reduced abundance of *Enterococcus* in breast milk samples from mothers

of EWG infants, suggesting that the reduced *Enterococcus* abundance in the intestine results from poor breast milk-seeding of *Enterococcus* spp. into the gut. The same *Enterococcus* species as found by us (*E. faecalis* and *E. faecium*) have previously been isolated from breast milk and infant faeces of mother-infants pairs (Albesharat *et al.* 2011; Kozak *et al.* 2015), supporting the milk-to-gut seeding with *Enterococci* proposed here. In addition, *Enterococcus* is one of just a few gut-related genera from which species

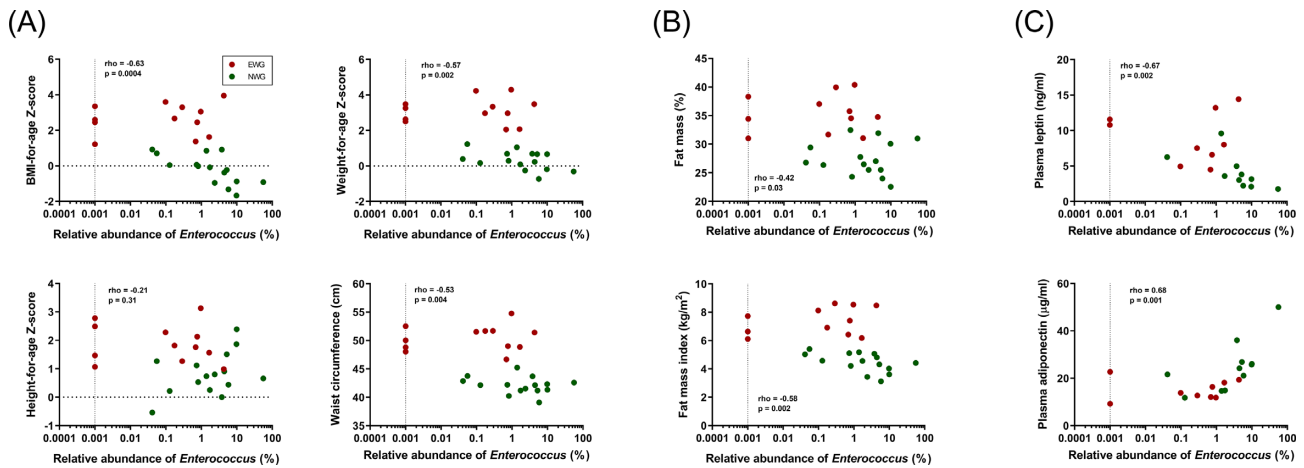


Figure 3. *Enterococcus* abundance correlates with anthropometrics, body fat and adipocyte-derived hormones. Spearman's Rank correlations between relative abundance of *Enterococcus* and (A), anthropometric measures (EWG, n = 12; NWG, n = 15), (B) fat mass %/index (EWG, n = 11; NWG, n = 15) and (C), plasma levels of adipocyte-derived hormones (EWG, n = 9; NWG, n = 10). Vertically dashed lines indicate limit of detection. Horizontally dashed lines indicate the Z-score reference value.

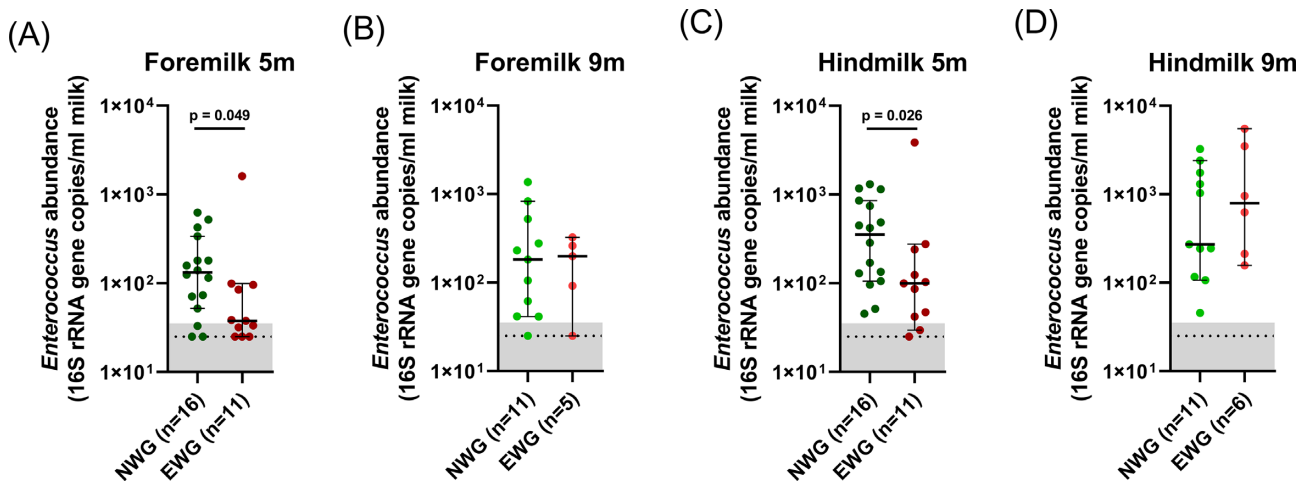


Figure 4. *Enterococcus* absolute abundance in breast milk of mothers to infants with excessive compared to normal weight gain is reduced at infant age five months. Scatterplots of absolute abundances of *Enterococcus* in (A and B) foremilk and (C and D) hindmilk at infant age five and nine months. Line and error bars indicate median ± 95% CI. Dots are colored according to group (green, normal weight gain; red, excessive weight gain). Dashed lines indicate the theoretical limit of detection and grey-shaded areas mark the range of signals obtained from 10 blank DNA extraction controls. Statistical significance was evaluated by Mann–Whitney U test.

have been isolated from maternal feces, maternal breast milk and infant feces (Jost et al. 2013), indicating vertical transmission. The existence of a so-called entero-mammary pathway for transmission of maternal gut microbes into the mammary gland has been suggested (Rodriguez 2014). The putative mechanism involves gut lumen sampling dendritic cells that carry live bacteria from the maternal gut into the mammary gland during pregnancy and/or lactation, which eventually ends up in the breast milk (Rodriguez 2014). DNA belonging to the same *Enterococcus* species has been found in pre-colostrum samples from pregnant woman and in their corresponding infants saliva after birth (Ruiz et al. 2019), suggesting that maternal-to-infant transmission of *Enterococcus* can occur directly via breast milk as opposed to retrograde inoculation via infant saliva or maternal skin/environmental contamination of breastmilk during lactation, which could also be potential sources of enterococci.

We did not record maternal diet in the present study. However, maternal diet during pregnancy (Lundgren et al. 2018) and lactation (Padilha et al. 2019) has been linked with abundance of *Enterococcus* in the infant gut and in maternal breast milk,

respectively. Lundgren et al., showed that intake of red and processed meat during pregnancy was positively associated with *Enterococcus* abundance in the gut of infants aged six weeks (Lundgren et al. 2018). Additionally, Padilha et al., found maternal intake of B vitamins thiamin, folate and riboflavin during lactation to be negatively associated with *Enterococcus* abundance in breast milk (Padilha et al. 2019).

In line with our observations, a number of studies in animals have linked serum leptin to intestinal microbiota, and specifically to enterococci. Conventionalization of germ-free mice led to significant increase in serum leptin levels (Bäckhed et al. 2004). In obese rodent models, supplementation with heat-killed *E. faecalis* FK-23 strain inhibited body weight gain, fat mass, epididymal fat pad weight, blood glucose and leptin concentrations (Motonaga et al. 2009) and fat accumulation in the liver (Kondoh et al. 2014). In broiler chickens, supplementation with *E. faecium* CGMCC 4847 in the diet led to a significant decrease in serum leptin as compared to controls (Zhao et al. 2013). Such studies suggest a causal impact of *Enterococci* on leptin and adiposity. However, studies in leptin receptor deficient mice imply that

leptin itself may regulate gut microbiota composition by affecting gut epithelial production of antimicrobial peptides (Rajala et al. 2014). The current study does not provide proof of causality between *Enterococcus* colonisation and excessive infant weight gain, but it suggests that infant gut microbes may play a role in host lipid metabolism and weight gain during breastfeeding in this group of infants. However, further studies are needed to clarify the direction of a putative causal association between weight gain and gut *Enterococci* in breastfed infants.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](https://academic.oup.com/femsec) online.

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AUTHORS CONTRIBUTIONS

KFM, MWL, CM and AL designed the SKOT III study and were in charge of data and sample collection. MWL and MVL performed initial data analysis of study participant characteristics. MFL, MIB and TRL conceived and designed the studies related to gut microbes. MFL and MIB performed DNA extraction, qPCR and 16S rRNA amplicon library preparation. MFR analyzed the 16S rRNA amplicon sequence data and interpreted it in the context of the study. MFL wrote the manuscript with substantial contribution from MIB and TRL. All authors read and approved the final manuscript.

Conflicts of Interests

The authors declare that they have no competing interests.

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