Supplementary Information

Fungi form interkingdom microbial communities in the human primordial gut that develop with gestational age

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Supplemental Text

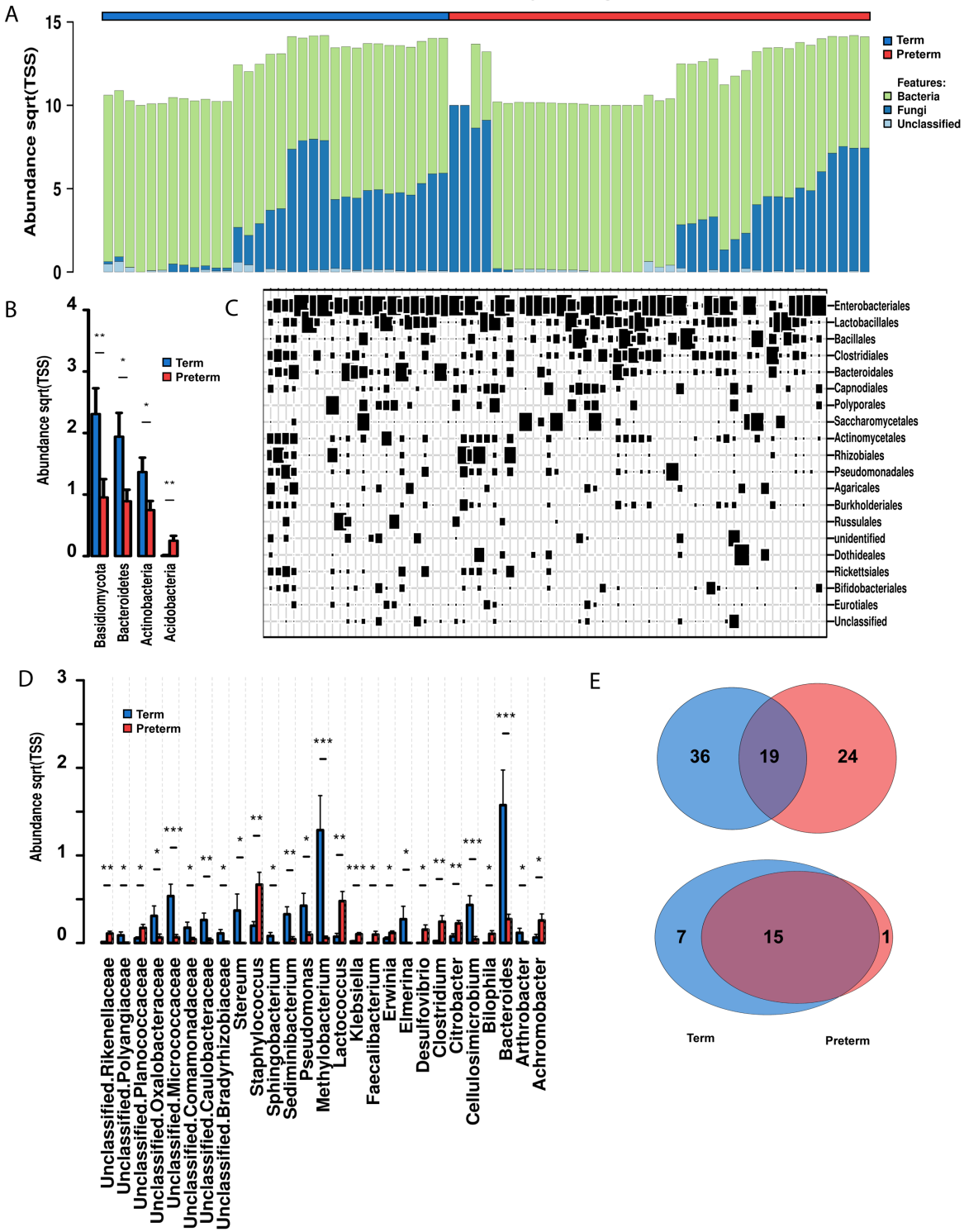
Bacterial colonization of the meconium

The bacterial colonization of first-pass meconium has been previously described[3,20,21]. Unlike previous work, both preterm and term-born newborns were equally likely to contain bacterial DNA on 16S rRNA sequencing at a read depth of 3000x (90% <33 weeks' and 94% >33 weeks' gestation, χ^2 p =0.5397). Gestational age also did not alter the alpha diversity of meconium samples. The most marked alteration in the relative composition of bacteria at the order level was in Bacteroidia (1.3% < 33 weeks' and 10.6% > 33 weeks' gestation) and Alphaproteobacteria (0.08% <33 weeks and 8.3% >33 weeks' gestation). In general, with increasing gestational age the relative abundance of rarer bacterial orders increased, with the exception of order Bacilli which decreased from 26.9% in preterm infants to 17.6% in those born at term. Gestational age significantly altered the bacterial community structure (PERMANOVA of unweighted UniFrac distances with 999 permutations, *f*-statistic 4.65, p =0.001).

We also performed LeFSe to identify bacterial taxa that could function as high-dimensional biomarkers of gestational age. The abundance of multiple bacterial genera identified preterm samples, including *Dermacoccus*, *Parabacterodies*, *Clostridium*, *Oribacterium*, *Anaerococcus*, *Citrobacter*, *Enterobacter* and *Erwinia*. The abundance of two taxa identified full term samples: family *Micrococcacea* and genus *Methylobacterium*.

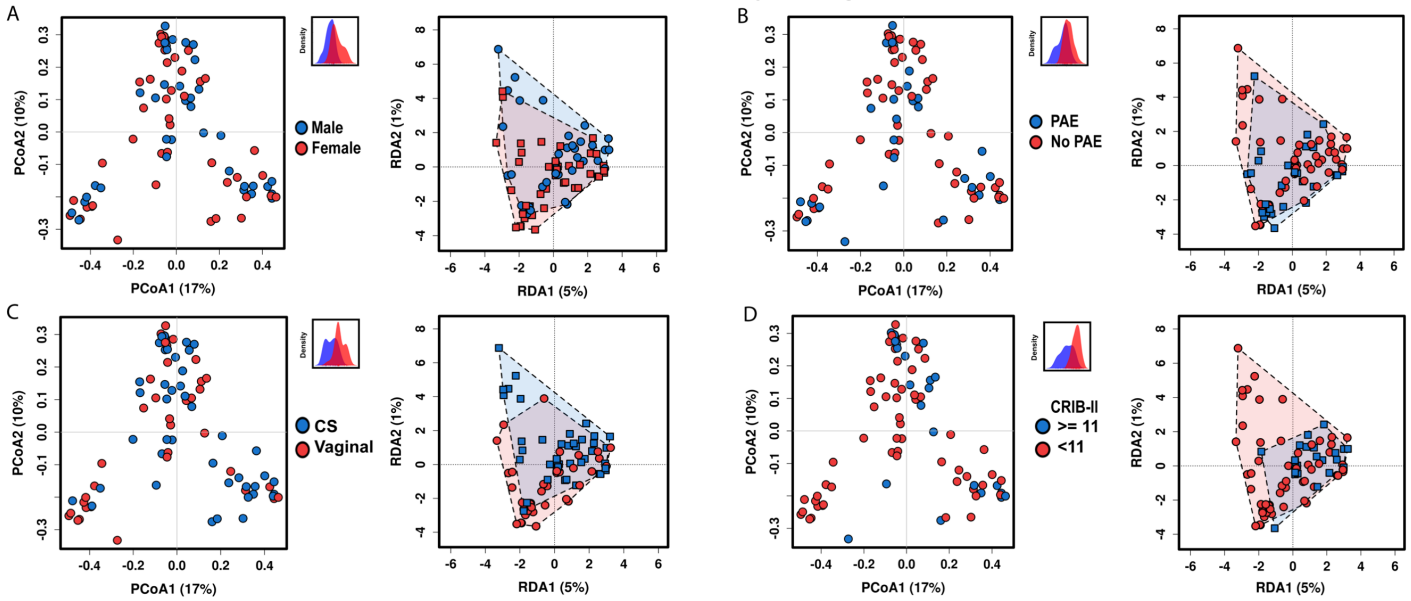
The alpha diversity was not significantly altered by any perinatal factor analyzed. As with fungi, mode of delivery was not associated with changes in bacterial community structure (PERMANOVA, *f*-statistic 1.192, p =0.19). After antibiotic exposure, the relative abundance of order Gammaproteobacteria increased (46.8% without perinatal antibiotics exposure and 64.7% with exposure), while the relative abundance of order Bacilli decreased (27.8% without exposure and 14.4% with exposure). However, overall bacterial community structures were not significantly altered by perinatal antibiotic exposure (PERMANOVA, *f*-statistic 1.47, p =0.052. Far less prominent changes in relative abundance were noted by host sex, and no differences were appreciated in bacterial community structure (PERMANOVA, *f*-statistic 1.18, p =0.179). Finally, illness severity as quantified by the CRIB-II score was also not associated with significant differences in bacterial community structure (PERMANOVA, *f*-statistic 1.49, p =0.051).

Supplementary Figure 1



Supplemental Fig 1. Fungal and bacterial communities increase in complexity with advancing gestational age at birth.
A Superkingdom (interkingdom) relative abundance. Bacteria are displayed in green and fungi are displayed in blue.
B Distribution of key taxa at the phylum level. Sqrt(TSS), square root total sum normalization (Hellinger transformation).
C Twenty most abundant interkingdom taxa at the order level.
D Distribution of key taxa at the genus level.
E Core interkingdom OTUs and unique phyla between preterm and term-born infants.
Data are median ± IQR (n=71). Preterm samples are displayed in red and term-born samples in blue.
For both (**A, D**) ANOVA, Bonferroni, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

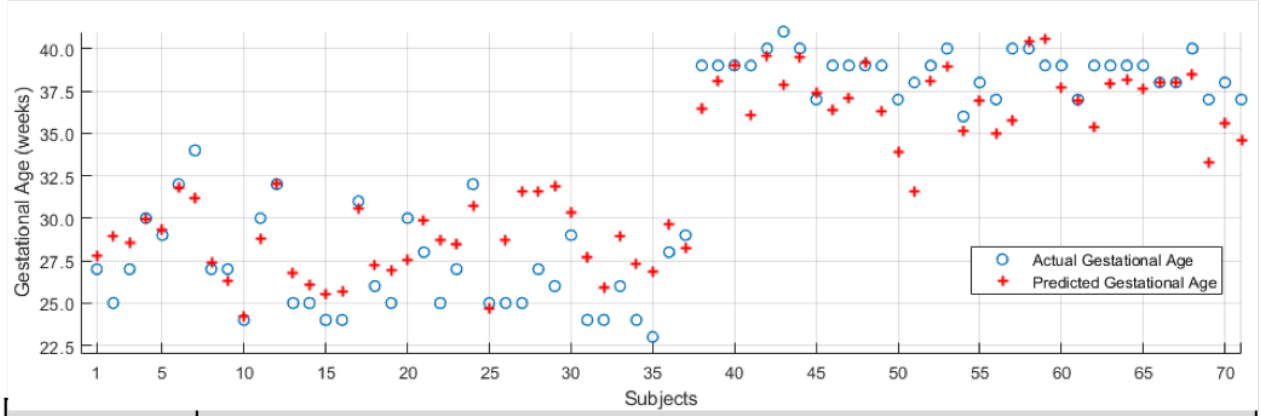
Supplementary Figure 2



Supplemental Fig. 2. Interkingdom community structure is not determined by mode of delivery, prenatal antibiotic exposure, host sex or illness severity.
A Principal coordinate analysis (PCoA) of Bray-Curtis dissimilarity matrices showing host sex does not significantly alter community structure, PERMANOVA R^2 =0.00977 p =0.749. Redundancy analysis (RDA), variance =4.21 f =0.90 p =0.710. The subset displays a discriminant analysis of principal components.
B Prenatal antibiotic exposure (PAE) does not significantly alter community structure, PCoA of Bray-Curtis dissimilarity matrices PERMANOVA R^2 =0.0101 p =0.699. RDA variance =4.24 f =0.91 p =0.691.
C Mode of delivery does not significantly alter community structure, PCoA of Bray-Curtis dissimilarity matrices PERMANOVA R^2 =0.0216 p =0.0526. RDA variance =5.34 f =1.14 p =0.169.
D Clinical illness severity does not significantly alter community structure, PCoA of Bray-Curtis dissimilarity matrices PERMANOVA R^2 =0.00718 p =0.964. RDA variance =3.16 f =0.68 p =0.997. CRIB-II, Critical Risk Index for Babies II.
Gestational age (data not shown), RDA variance =17.14 f =3.67 p =0.001.
For all analyses n = 71.

Supplementary Figure 3

a



b

Model 1	Predicted			
	Category	Term	Preterm	Accuracy (%)
Actual	Term	31	6	Specificity = 83.8
	Preterm	2	32	Sensitivity = 94.1
	Predictive Value (%)	NPV = 93.9	PPV = 84.2	Overall = 88.7

c

Actual / Predicted	Term	Preterm	Accuracies
Term	29.0	5.0	Specificity = 85.3%
Preterm	5.1	31.9	Sensitivity = 86.2%
	Negative Predicted Value = 85.1%	Positive Predicted Value = 86.5%	Accuracy = 85.8%

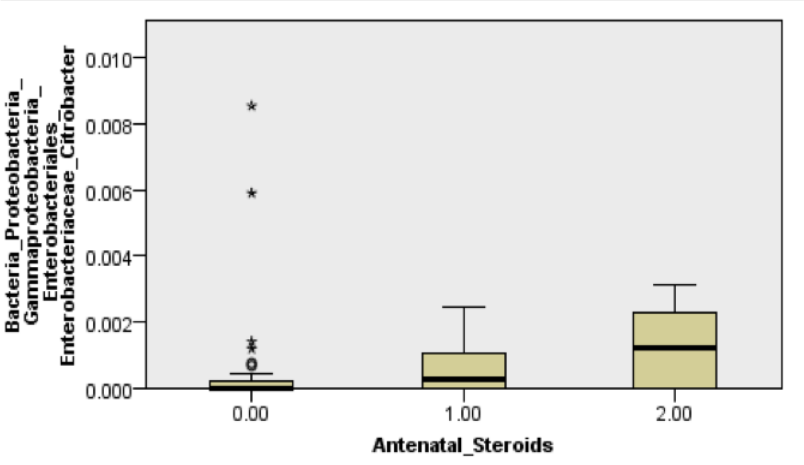
d

Algorithm	AUC with 96% CI (5-fold cross-validation results)
Decision Trees	0.70 (0.58, 0.82)
Logistic Regression	0.44 (0.31, 0.57)
Support Vector Machines	0.65 (0.52, 0.78)
K-nearest neighbors	0.56 (0.43, 0.69)

Supplemental Fig. 3. Machine learning models to predict preterm versus term gestational age using microbial taxa. **A** Utilizing 51 fungal and 209 bacterial taxa that were identified at least twice in the dataset, we utilized LASSO with 5-fold cross validation to identify significant predictors, leading to the identification of 9 fungal and 34 bacterial taxa as predictors of gestational age yielding an R^2 of 0.85 and an adjusted R^2 of 0.62.
B Confusion matrix from a support vector machine model utilizing these key microbial taxa.
C Confusion matrix from the final genera-level random forest machine learning model.
D Comparison of other machine learning models with less accuracy than random forest.

Supplementary Figure 4

a



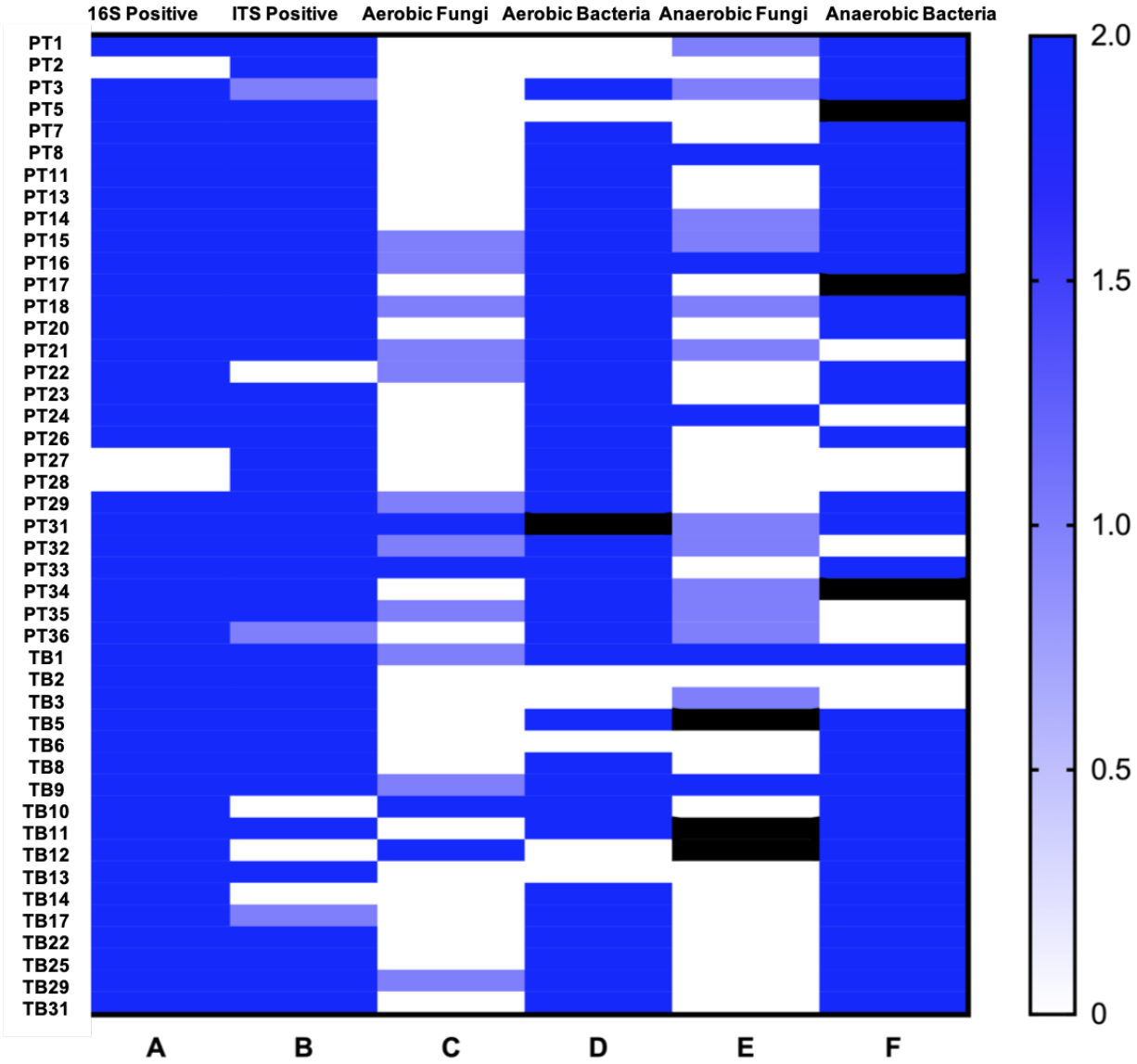
b

Analysis Level	Accuracy (95% CI)	Number of Features
Level 1: Kingdom level	57.63% [56.81%-58.44%]	7
Level 2: Phylum Level	68.22% [67.41%-69.02%]	27
Level 3: Class Level	74.27% [73.56%-74.98%]	53
Level 4: Order Level	79.04% [78.43%-79.66%]	87
Level 5: Family Level	84.22% [83.65%-84.79%]	167
Level 6: Genus Level	85.74% [85.23%-86.24%]	265

Supplemental Fig. 4. Machine learning models of interkingdom community importance.
A Box plot showing the alteration of 7 of the 10 most important bacterial taxa by the use of antenatal steroids as quantified by independent sample Kruskal-Wallis tests.
B Confusion matrix from a random forest classifier model for each phylogenic level.
Significant predictors are as follows:

Variable:	Importance (%)
Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, Rikenellaceae, undefined	4.49062131
Maternal Age	3.07380941
Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, Porphyromonadaceae, Parabacteroides	3.03080201
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Citrobacter	2.62700202
Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, Bacteroidaceae, Bacteroides	2.59241299
Bacteria, Proteobacteria, Deltaproteobacteria, Desulfovibrionales, Desulfovibrionaceae, Desulfovibri	2.55982523
Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, mitochondria, Other	2.51801403
Bacteria, Firmicutes, Bacilli, Lactobacillales, Streptococcaceae, Lactococcus	2.39478017
Maternal BMI	2.3822576
Bacteria, Firmicutes, Clostridia, Clostridiales, Ruminococcaceae, Oscillospira	2.24234257

Supplementary Figure 5



Supplemental Figure 5. Live fungi and bacteria are present by culture-based techniques.
A 16S rRNA (bacteria and archaea).
B ITS rDNA (fungi).
C Aerobic yeast-extract-peptone-dextrose (YPD) with chloramphenicol broth (aerobic fungi).
D Aerobic brain heart infusion (BHI) with fluconazole broth (aerobic bacteria).
E Anaerobic YPD with chloramphenicol broth (anaerobic fungi).
F Anaerobic BHI with fluconazole (anaerobic bacteria).
2 = strongly positive, 1 = weakly positive, 0 = undetected. Black cells represent quantity not sufficient.
(n = 46; PT, preterm n = 30; TB, term-born n = 17)