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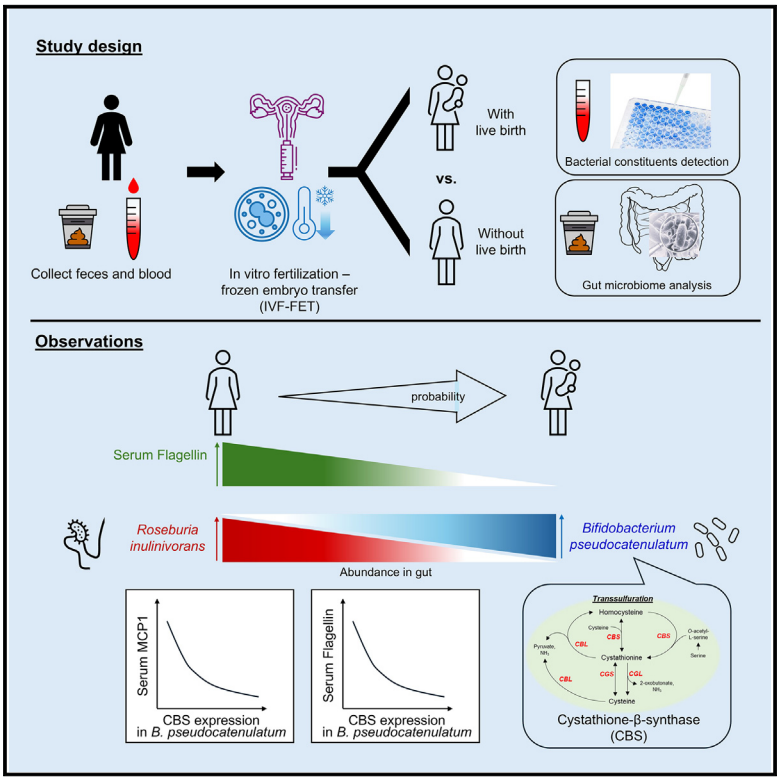
Flagellin in blood and *Bifidobacterium pseudocatenulatum* in gut are associated with live birth upon IVF-frozen embryo transfer

Graphical abstract



Highlights

- Higher serum flagellin levels in women without live birth after FET
- *R. inulinivorans* and *B. pseudocatenulatum* in gut predict live birth outcomes
- Negative correlation between bacterial CBS activity and serum flagellin and MCP1 levels

Authors

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In brief

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Flagellin in blood and *Bifidobacterium pseudocatenulatum* in gut are associated with live birth upon IVF-frozen embryo transfer

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SUMMARY

The role of gut microbiota in live birth attainment upon *in vitro* fertilization (IVF) remains unclear. We recruited 67 women, evaluated bacterial constituents in the serum, and analyzed the gut microbiota composition and functions prior to an IVF-frozen embryo transfer (FET). A higher serum flagellin level, residues from flagellated bacteria, was observed in women without live birth after FET. Twelve species showed significant differences in abundance between with and without live birth groups, of which *Roseburia inulinivorans* and *Bifidobacterium pseudocatenulatum* were the most important to predict live birth outcome. *R. inulinivorans* abundances were higher among women with high flagellin levels. The cystathionine β-synthase activity in *B. pseudocatenulatum*, which may play roles in gut integrity, was a critical factor in the negative correlation with serum flagellin and MCP1 levels. The presence of bacterial residues in the circulation may elicit systemic inflammation and decrease the chances of attaining live birth after FET.

INTRODUCTION

Infertility, a problem affecting around 17.5% of the adult population, is a major medical challenge globally.[1] The costs of treating infertility remain higher than the GDP per capita in many countries.[2] *In vitro* fertilization (IVF) is the most commonly employed assisted reproductive technology, and the global IVF market size has been growing since the birth of the first baby using this technique in 1978. Despite the advanced technology in recent decades that has improved the success rates of IVF, it only stays at around 45% among women aged less than 35 years and drops to less than 10% for those aged 40 years and older.[3,4] In particular, implantation failure of apparently good-quality embryos is a major obstacle, for which there is no good solution at present. Ongoing research is being conducted in an attempt to elucidate the factors that predict or modulate embryo implantation. Uncovering such factors would greatly help to alleviate the physiological and psychological stress of the patients.

The microbiome has been increasingly studied in obstetrical care. In regard to its relevance to IVF, studies have been heavily focused on the vaginal and endometrial microbiota.[5,6] However, the use of vaginal probiotics to improve pregnancy outcomes shows limited evidence.[7,8] Low-grade inflammation is known to negatively impact the live birth rate in women undergoing IVF treatment.[9] Although it remains unclear how low-grade inflammation evolves, gut microbiota is suggested to be one of the contributors.[10] Gut bacteria are the major source of bacterial components in the circulation of individuals free of infection.[11,12] Unhealthy diet and disease conditions can disrupt the normal ecology of the gut microflora, leading to a state referred to as gut dysbiosis, which is often associated with the impairment of gut integrity resulting in the penetration of bacteria products.[10,13] The leakage of bacterial constituents, such as lipopolysaccharides, cell membrane components of gram(−) bacteria, and flagellins, structural proteins of flagella on flagellated bacteria, from the gut into the circulation can activate immune cells, resulting in production of proinflammatory molecules and consequently low-grade systemic inflammation.[10,14] Moreover, flagellins consist of conservative and variable regions, and these variable regions are species-depending and vary in immunogenicity.[15] Special diets, such as Mediterranean diet, that lower systemic inflammation have been shown to improve fecundability.[16]

We hypothesize that gut dysbiosis may affect the live birth rate in IVF treatment by modifying inflammatory status through the infiltration of bacterial constituents into the uterine environment.

Table 1. Comparison of demographic characteristics and clinical parameters between women with and without live birth after the index frozen embryo transfer cycle

Parameter	Women with live birth (N = 32)	Women without live birth (N = 35)	p value[a]
Age of women (years)	36 (34–38)	36 (35–38)	0.594
Body mass index (kg/m^2)	22.9 (20.2–24.4)	23.2 (21.6–26.1)	0.196
Smoking status			0.115
Non-smoker	32 (100.0%)	31 (88.6%)	
Ex-smoker	0 (0.0%)	4 (11.4%)	
Type of infertility			0.527
No infertility	1 (3.1%)	0 (0.0%)	
Primary	21 (65.6%)	21 (60.0%)	
Secondary	10 (31.3%)	14 (40.0%)	
Duration of infertility (years)	3 (3–5)	4 (3–5)	0.557
Cause of infertility			0.807
Tubal factor	6 (18.8%)	5 (14.3%)	
Endometriosis	2 (6.3%)	1 (2.9%)	
Male factor	9 (28.1%)	8 (22.9%)	
Unexplained	5 (15.6%)	9 (25.7%)	
Mixed causes	10 (31.3%)	12 (34.3%)	
Parity			0.755
0	27 (84.4%)	28 (80.0%)	
1	5 (15.6%)	7 (20.0%)	
Frozen embryo transfer protocol			0.551
Natural cycle	24 (75.0%)	28 (80.0%)	
Letrozole cycle	0 (0.0%)	1 (2.9%)	
Hormone replacement cycle	8 (25.0%)	6 (17.1%)	
Pre-implantation genetic testing			0.246
No	27 (84.4%)	33 (94.3%)	
Yes	5 (15.6%)	2 (5.7%)	
Stage of embryo transferred			0.159
Cleavage stage	5 (15.6%)	11 (31.4%)	
Blastocyst	27 (84.4%)	24 (68.6%)	

Data presented as number (percentage) or median (25th – 75th percentile).
[a]Mann-Whitney U test for continuous variables, Fisher's exact test for categorical variables.

This study aims to assess the relationship between gut microbiota and the live birth outcome of IVF treatment. We recruited women undergoing frozen embryo transfer (FET), a procedure in which a frozen embryo from a previous fresh IVF cycle is thawed and transferred back into the uterus, followed by analyzing bacterial constituents in their serum and gut microbiota samples that were collected prior to FET. Our results reveal that *Bifidobacterium* in the gut microbiota and lower serum flagellin levels are associated with live birth attainment after FET.

RESULTS

Serum flagellin level in women with and without attainment of live birth after FET

The demographic and clinical characteristics of the participants with and without attainment of live birth in the index FET cycle are shown in Table 1. There was no significant difference in all the listed parameters (Table 1). Various cytokines that commonly indicate systemic inflammation and bacterial components in serum samples were measured, and no significant difference in the serum levels of C-reactive protein (CRP), interferon-γ (IFN-γ), and monocyte attractant protein-1 (MCP1) between women with and without live birth after FET was observed, except serum amyloid A (SAA) level (Table 2, Figure S1). The group with live birth showed a higher serum SAA level (Table 2, Figure S1). By contrast, serum flagellin concentration was significantly lower in the group with live birth than those without, whereas no difference in serum LPS concentration was observed (Table 2, Figure S1A). To examine whether the increase in serum flagellin level was owing to alterations in the specific antibodies, we measured anti-flagellin immunoglobin G (IgG) and immunoglobin A (IgA) antibodies in the serum. Regardless of the source of flagellins used as the antigens for antibody measurement, there was no difference in either anti-flagellin IgG or IgA between the two groups (Table 2, Figure S1A). On the other hand, the analysis using a univariate binary logistic regression model demonstrated that although both serum flagellin and SAA levels were able to predict pregnancy, only serum flagellin in the prediction of live birth reached statistical significance (Table 3). The odds ratios (OR) indicated that a 10-fold increase in serum flagellin level was associated with an 82.9% decrease in the odds of a woman attaining live birth upon FET, while a 10-fold increase in serum amyloid A level was 3.9-fold more likely to have a successful pregnancy (Table 3, Figure S1B). Receiver-operating characteristic curve analysis for prediction of live birth by serum flagellin concentration revealed an area under the curve of 0.729 (95% CI 0.605–0.851, $p < 0.001$) (Figure S2). Furthermore, the best cut-off of serum flagellin concentration at 0.6 ng/mL based on the Youden's index gave a sensitivity of 71.9% and a specificity of 68.6% in predicting successful live birth.

R. inulinivorans and *B. pseudocatenulatum* as the most important species to predict live birth outcome

The differences in gut microbiota between the participants with and without live birth after FET were then examined. The two groups displayed the same degree of diversity in gut bacteria, as indicated by the Shannon index (Figure 1A). When we compared the composition of the gut microbiota as intra-sample diversity (β-diversity), there was a significantly distinctive pattern between subjects with and without live birth (PERMANOVA, $p < 0.05$, Figure 1A). The linear discriminant analysis effect size (LEfSe) revealed that *Roseburia inulinivorans* and *Bifidobacterium pseudocatenulatum* were the species with the most significant differences in abundance between the two groups,

Table 2. Comparison of various bacterial constituent and cytokines levels in serum between women with and without live birth after the index frozen embryo transfer cycle

Parameter	Women with live birth (*N* = 32)	Women without live birth (*N* = 35)	*p* value[a]
Flagellin (ng/mL)	0.40 (0.16–0.605)	0.71 (0.45–1.02)	<0.001[b]
Lipopolysaccharides (LPS, EU/ml)	10.46 (7.79–13.95)	9.12 (7.13–11.45)	0.120
Interleukin-1β (IL-1β, pg/ml)	ND[c]	ND[c]	N/A
Interferon-γ (IFNγ, pg/ml)	24.57 (0–78.71)	28.06 (0–77.80)	0.682
Monocyte chemoattractant protein-1 (MCP1, pg/ml)	121.61 (90.48–154.23)	131.99 (97.89–188.82)	0.283
C-reaction protein (CRP, μg/ml)	0.72 (0.23–2.03)	1.21 (0.31–2.24)	0.441
Serum amyloid A (SAA, μg/ml)	1.77 (0.87–2.70)	0.96 (0.45–1.94)	0.039[b]
Anti-flagellin IgA (*S*. typhimurium) (R.U.)	133.68 (86.26–192.66)	135.36 (101.85–201.71)	0.743
Anti-flagellin IgG (*S*. typhimurium) (R.U.)	144.52 (104.30–234.60)	124.82 (95.74–207.94)	0.598
Anti-flagellin IgA (*B*. *subtilis*) (R.U.)	89.88 (77.68–121.25)	90.2 (67.07–133.92)	0.715
Anti-flagellin IgG (*B*. *subtilis*) (R.U.)	219.00 (136.45–380.18)	211.19 (162.11–361.77)	0.762

Data presented as median (25th – 75th percentile), R.U., Relative unit. See also Figure S1.
[a]Mann-Whitney U test.
[b]Statistically significant ($p < 0.05$).
[c]Detectable only in 6 women with live birth and 6 women without live birth.

where *R. inulinivorans* was enriched in the group without live birth and *B. pseudocatenulatum* in the group with live birth (Figure 1B). By applying the same analysis as for flagellin, we found that the best cut-off of *B. pseudocatenulatum* abundance at 0.371% gave a sensitivity of 50% and a specificity of 87.1% in predicting successful live birth, and the best cut-off of *R. inulinivorans* abundance at 0.597% gave a sensitivity of 45.2% and 93.3% in predicting the absence of live birth attainment.

We then evaluated the importance of these species and serum flagellin level in predicting successful live birth with random forest classification and repeated cross-validation due to the small sample size (Figure 1C). The abundance of *R. inulinivorans* and *B. pseudocatenulatum*, along with serum flagellin levels, consistently ranked as the most important factors to predict the outcome of live birth (Figure 1C). These two bacterial species were present in more than 70% of subjects in this cohort (Figure 1C). The predictive performance of the combination of *R. inulinivorans* abundance and serum flagellin level was better than each alone, and the addition of *B. pseudocatenulatum* abundance to the combination enhanced the predictive performance further (Figure 1D). Taken together, serum flagellin level and the abundances of *R. inulinivorans* and *B. pseudocatenulatum* are the three most important factors for predicting live birth outcomes of FET.

Table 3. Odd ratios of serum flagellin and serum amyloid A levels to predict different outcomes of FET

Predictor[a]	Outcomes	Odd ratio	Confidence interval (95%)	*p* value
Flagellin (ng/mL)	Pregnancy[b]	0.290	0.076–0.892	0.045[c]
	Live birth	0.171	0.040–0.553	0.007[c]
Serum amyloid A (SAA) (μg/mL)	Pregnancy	3.900	1.171–14.939	0.034[c]
	Live birth	3.174	0.976–11.910	0.067

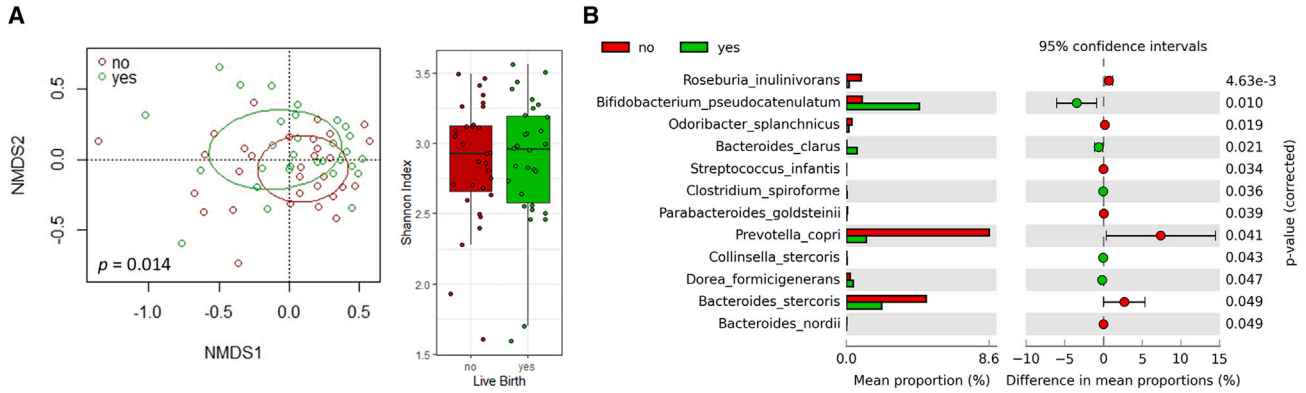
See also Figures S1 and S2.
[a]Log_{10}-transformed.
[b]Detection on the initial pregnancy test.
[c]Statistically significant.

Abundance of *B. pseudocatenulatum* negatively related to serum flagellin level

R. inulinivorans is a flagellated species, so we evaluated whether serum flagellin level was directly associated with the abundance of flagellated microbiota in feces. The functional profile of the gut microbiota was generated from Gene Ontology (GO) functional enrichment analysis on the genes identified from the microbiome analysis. No difference in the abundances of the flagellum-related pathways was observed between the participants with and without live birth after FET (Figure 2A). Moreover, the abundance of flagellum-related GO terms derived from *R. inulinivorans* was not correlated with the serum flagellin level of the hosts (Table S1). Conversely, the abundance of *B. pseudocatenulatum* but not *R. inulinivorans* was significantly and negatively correlated with serum flagellum level (Figure 2B). Upon dividing the cohort into quartiles according to serum flagellin level, a higher abundance of *B. pseudocatenulatum* was observed at the 1st and 2nd quartiles (Figures 2B and 2C). The abundance of *R. inulinivorans* was notably elevated in the 4th quartile (Figures 2B and 2C). Taken together, the abundance of *B. pseudocatenulatum* is associated with the suppression of serum flagellin levels and live birth attainment after FET.

Association between bacterial cysteine synthesis via cystathionine and serum flagellin level

The GO terms from the functional profiles under the category of biological processes of the gut microbiota were subjected to correlation analysis with serum flagellin level (Table S2), and the GO terms contributed by bacteria in Figure 1B were further examined (Table S3). We separated these GO terms according to whether the contributing bacteria were enriched in the with or without live birth group; the common terms were excluded



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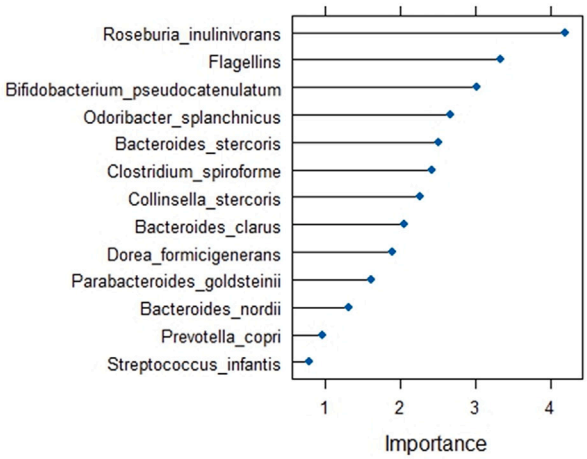

Species	Prevalence (%)
Roseburia inulinivorans	75.41
Bifidobacterium pseudocatenulatum	73.77
Odoribacter splanchnicus	59.02
Bacteroides stercoris	60.66
Clostridium spiroforme	29.51
Collinsella stercoris	73.77
Bacteroides clarus	27.87
Dorea formicigenerans	70.49
Parabacteroides goldsteinii	31.15
Bacteroides nordii	19.67
Prevotella copri	18.03
Streptococcus infantis	8.20

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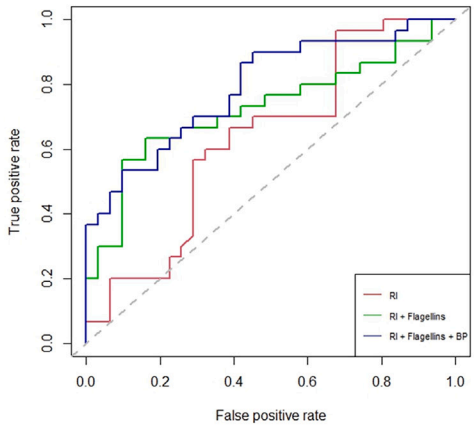


Classifiers	AUC
Roseburia inulinivorans (RI)	0.632
Flagellins	0.599
Bifidobacterium pseudocatenulatum (BP)	0.489
RI + Flagellins	0.725
BP + Flagellins	0.689
RI + *BP*	0.670
RI + Flagellins + *BP*	0.802

Figure 1. The importance of serum flagellin levels and gut bacteria in determination of live birth outcome of FET

(A) β and α diversities of the gut microbiota from subjects grouped as with (yes) or without (no) live birth evaluated by non-metric multidimensional scaling (NMDS) and Shannon index, respectively.

(B) Mean relative abundances of gut bacteria with a significant difference in the linear discriminant analysis effect size between the two groups.

(C) Ranking of the importance of serum flagellins and the abundance of different gut bacteria in determining the live birth outcome of FET using random forest classification. The prevalence of these bacteria in the cohort is shown in the right panel.

(D) Receiver operating characteristic curves and the values of the corresponding area under curve (AUC) yielded by different combinations of classifiers. Data are represented as mean ± s.e.m. (N = 30 for with live birth and N = 31 for with live birth group.). $p < 0.05$ statisically significant.

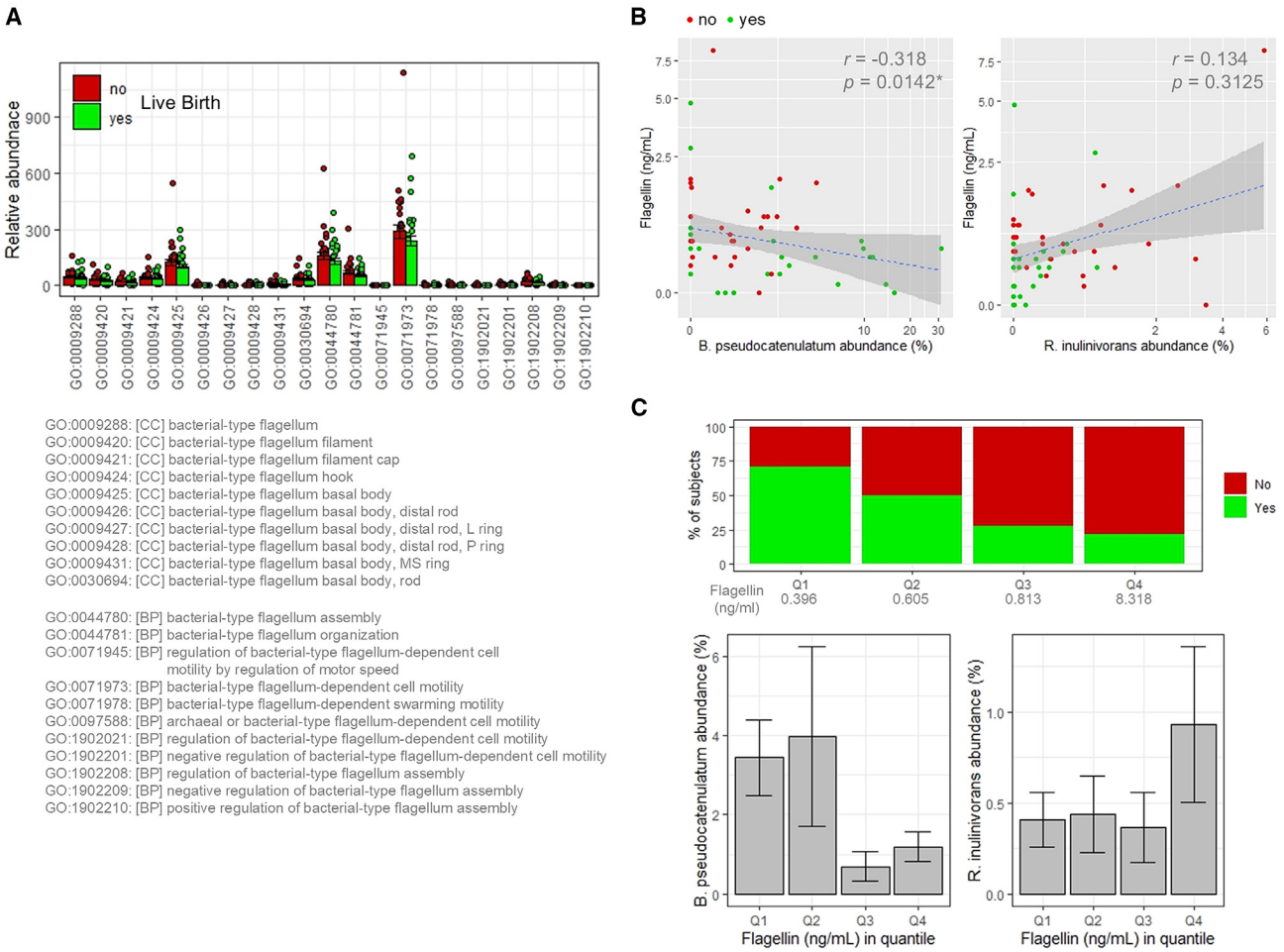


Figure 2. Correlations between the abundances of various gut bacteria and serum flagellin level

(A) Relative abundances of flagellum-related gene ontology (GO terms) of gut microbiota in women with (yes) and without (no) attaining live birth upon FET.

(B) Scatterplot depicting spearman correlations between the abundances of *R. inulinivorans* and *B. pseudocatenulatum*, and serum flagellin level adjusted with age and BMI.

(C) The percentage of subjects with (yes) and without (no) live birth and the abundance of these two bacteria among the cohorts divided by quartiles based on serum flagellin level. The cutoffs and the number of subjects with and without live birth in each quartile in the upper panel. Data are represented as mean ± s.e.m.

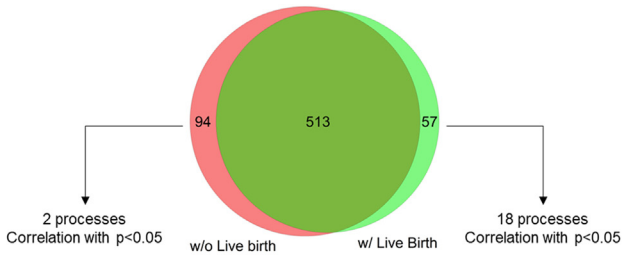
*Statistically significant.

from further analysis (Figure 3A, Table S3A). Among the 57 terms unique to the with live birth group, 18 biological processes were negatively correlated with serum flagellin level with an unadjusted p value <0.05 (Figure 3A, Table S3B). By contrast, 94 terms were exclusive to the without live birth group, and only 2 of them were correlated with serum flagellin level with an unadjusted p value <0.05 (Figure 3A, Table S3C). In those 18 processes, 6 processes represented biosynthetic processes, in which 3 processes showed significant correlations with serum flagellin level after adjustment for multiple comparisons, namely, the cysteine biosynthetic process via cystathionine (GO:0019343), GDP-mannose (GO:0009298), and glucan (GO:0009250) biosynthesis (Table S3B). The cysteine biosynthetic process via cystathionine (GO:0019343) showed the strongest correlation with serum flagellin level with a correlation coefficient of −0.475 (Figure 3B, Table S3B, Figure S3), and *B. pseudocatenulatum* was the key bacteria contributing to its readout (Table S3B).

Besides, *B. pseudocatenulatum* was also the major contributor to α-glucan (GO:0030979) and the creatine (GO:0006601) biosynthesis (Table S3B). Among the remaining 12 biological processes, only creatinine catabolic process (GO:0006602) and carboxylic acid metabolic process (GO:0019752) were contributed mainly by *B. pseudocatenulatum*. However, only ~11% of the readout of the carboxylic acid metabolic process (GO:0019752) was derived from *B. pseudocatenulatum,* and this process was concurrently contributed by 76 identified bacteria in total (Table S3B).

Next, we further examined the GO terms representing enzymes related to the three biosynthetic processes showing significant correlations with serum flagellin level, only the activities of cystathionine β-synthase (GO:0004122) under GO:0019343

A



B

GO term	*r* value	*p* value
GO:0019343: [BP] cysteine biosynthetic process via cystathionine	-0.475	$1.5\times10^{-4*}$
GO:0004122: [MF] cystathionine β-synthase (CBS) activity	-0.488	$8.77\times10^{-5*}$
GO:0004123: [MF] cystathionine γ-lyase (CGL) activity	-0.026	0.8464
GO:0004121: [MF] cystathionine β-lyase (CBL) activity	-0.232	0.0773
GO:0003962: [MF] cystathionine γ-synthase (CGS) activity	-0.176	0.1828

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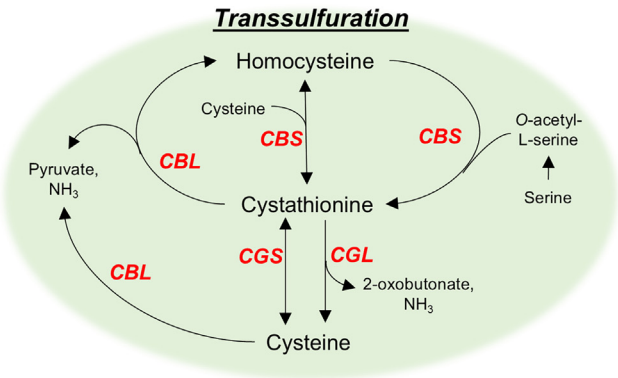

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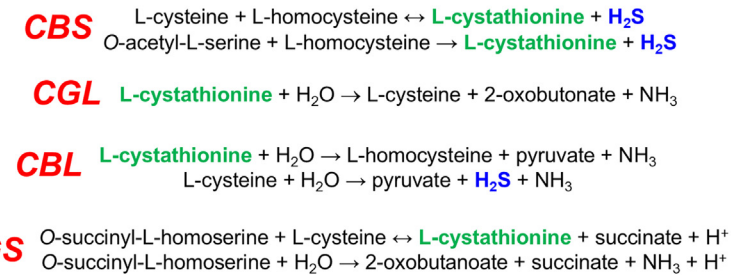


Figure 3. Correlations between the GO terms readouts and serum flagellin level

(A) Venn diagram showing the number of gene ontology (GO) terms derived from bacteria whose abundance in feces was higher in with (w/) or without (w/o) live birth groups.

(B) Spearman correlations of GO terms representing the enzymes related to cystathionine metabolism and serum flagellin level adjusted with age and BMI.

(C) Schematic diagram illustrating transsulfuration pathways in gut bacteria.

(D) Chemical reactions related to the biosynthesis and degradation of cystathionine. *Statistically significant.

and phosphomannomutase (GO:0004615) under GO:0009298 were significantly and negatively correlated with serum flagellin level (Figure 3B, Table S3D, Figure S3). Unlike cystathionine β-synthase activity, which showed a comparable correlation coefficient as GO:0019343, the correlation of phosphomannomutase activity with serum flagellin level was weaker than that of GO:0009298 (Table S3D), suggesting the uniqueness of cystathionine β-synthase.

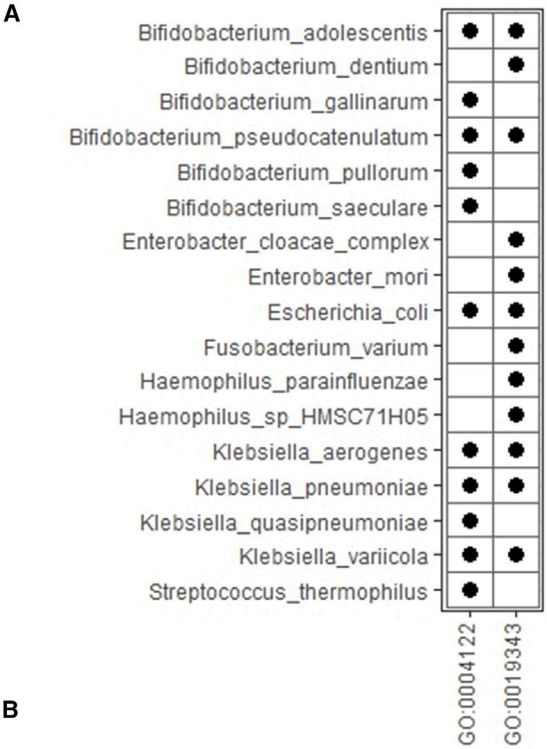
Enzymes in cysteine biosynthesis via cystathionine and serum flagellin concentration

Cysteine biosynthesis via cystathionine is the key process of the transsulfuration pathway (Figure 3C). Gut bacteria can utilize cysteine to produce hydrogen sulfide, (H_2S) which in adequate concentration, helps to maintain gut integrity.[17,18] The GO terms representing other enzymes related to the transsulfuration pathway, including cystathionine β-lyase (GO:0004121) and cystathionine γ-synthase (GO:0003962), were also examined (Figure 3C). The cystathionine β-lyase activity (GO: 0004121) showed a moderate but insignificant correlation with serum flagellin levels (Figure 3B). The comparison of the products yielded by the reactions catalyzed by cystathionine β-synthase and cystathionine β-lyase showed that H_2S was another common product beside cystathionine (Figure 3D).

Cystathionine β-synthase activity in *Bifidobacterium* and serum flagellin concentration

The taxonomical and functional profiles that indicated the abundance of *B. pseudocatenulatum* and cysteine biosynthesis via cystathionine in the gut microbiota were the most significant parameters that were negatively correlated with serum flagellin level, and, therefore, the relationship between these two was further explored. The readout of GO terms, cysteine biosynthetic process via cystathionine (GO:0019343), and cystathionine β-synthase activity (GO:0004122), were attributed to 17 bacterial species detected in this cohort, and 6 of them belonged to the genus *Bifidobacterium* (Figure 4A). Among these 17 species, only *B. pseudocatenulatum*, *B. adolescentis*, and *Escherichia coli* were present in more than half of the subjects in this cohort (Figure 4A). The correlation coefficient between the abundance of GO:0004122 derived from *B. pseudocatenulatum* and serum flagellin level was −0.319 (Table S4) and was strengthened to −0.462 upon the addition of those readings contributed by *B. adolescentis* (Figure 4B). The combination of readouts from all the detectable species in *Bifidobacterium* similarly enhanced the correlation (Figure 4B).

A



Species	Prevalence (%)
Bifidobacterium adolescentis	55.74
Bifidobacterium dentium	8.20
Bifidobacterium gallinarum	1.64
Bifidobacterium pseudocatenulatum	73.77
Bifidobacterium pullorum	1.64
Bifidobacterium saeculare	1.64
Enterobacter cloacae complex	11.48
Enterobacter mori	3.28
Escherichia coli	86.89
Fusobacterium varium	24.59
Haemophilus parainfluenzae	34.43
Haemophilus sp. HMSC71H05	8.20
Klebsiella aerogenes	3.28
Klebsiella pneumoniae	45.90
Klebsiella quasipneumoniae	27.87
Klebsiella variicola	31.15
Streptococcus thermophilus	8.20

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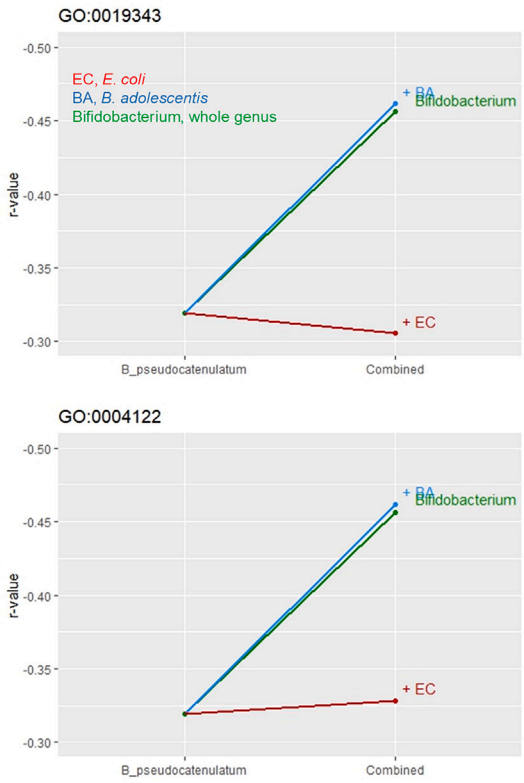

C

Abundance of *Bifidobacterium pseudocatenulatum*		
	***r* value**	***p* value**
CRP	-0.015	0.9091
IFNγ	-0.044	0.7391
MCP-1	-0.338	0.0088*
SAA	0.008	0.9522
Flagellin	-0.318	0.0142*

GO:0004122 from *Bifidobacterium pseudocatenulatum*		
	***r* value**	***p* value**
CRP	-0.015	0.9081
IFNγ	-0.059	0.6579
MCP-1	-0.357	0.0055*
SAA	0.003	0.9815
Flagellin	-0.319	0.0137*

D

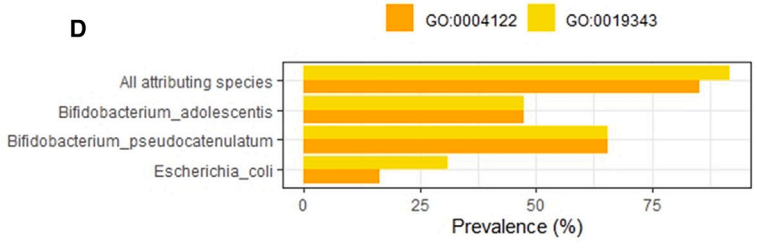

Figure 4. GO terms of cystathionine β-synthase in the genus of *Bifidobacterium*

(A) Species contributing to the readouts of gene ontology (GO) terms for cysteine biosynthetic process via cystathionine (GO:0019343) and cystathionine β-synthase activity (GO:0004122) and their prevalences in the cohort.

(legend continued on next page)

Furthermore, moderate but significant negative correlations between the abundance of and readout of GO:0004122 derived from *B. pseudocatenulatum* and serum MCP-1 level were observed (Figures 4C and S4).

As a comparison, the correlations of serum MCP-1 and flagellin levels with GO terms representing cysteine biosynthesis via serine (GO:0006535 and GO:0009001) and utilization of cysteine via glutathione biosynthesis (GO:0006750 and GO:0004357) derived from *B. pseudocatenulatum* were also evaluated, but no significant correlation was observed (Figure S5). Moreover, although *Escherichia. coli* was a highly prevalent species in this cohort (Figure 4A), the contribution of *E. coli* to the readout of GO:0004122 was detected in less than 30% of the total participants (Figures 4B and 4D). Taken together, the cystathionine β-synthase activity of *Bifidobacterium* is associated with a decreased serum flagellin level, thus decreasing the risk of unsuccessful live birth upon FET.

DISCUSSION

This observational study indicates that serum concentration of flagellin and fecal abundance of *R. inulinivorans* and *B. pseudocatenulatum* are important factors for predicting the live birth outcome of a FET cycle. The activity of cystathionine β-synthase in *B. pseudocatenulatum* is significantly and negatively correlated to flagellin and MCP-1 levels in the circulation. Although the causative relationship between these three factors and live birth attainment after FET requires further investigation, our findings suggest that gut microbiota may be a suitable treatment target for improving the live birth rate upon FET.

An elevation of bacterial constituents in the circulation often reflects a decreased integrity of the gut, where trillions of microbes inhabit. The gut barrier is upheld by epithelial tight junctions and mucosal immunity.[10] Owing to the different natures of flagellin, a bacterial protein, and LPS, a polysaccharide, the mechanisms of their infiltration into the circulation are different. Women without live birth after FET in this study showed higher serum levels of flagellin but not LPS, which implicated a subtle change in mucosal immunity rather than systemic damage at the gut epithelium. Given the fact that the participants in this cohort were generally healthy individuals, damage to the gut epithelium was not anticipated. Flagellin as an antigen can trigger adaptive immunity, and the penetration of flagellated bacteria is usually blocked by the induced anti-flagellin antibodies.[19] However, our previous and other studies have found that certain types of flagellin derived from gut microbiota can escape host surveillance and enter the circulation.[15,20] On the other hand, a significantly higher SAA level was observed in women with live births after FET, and a negative correlation between serum SAA and flagellin levels was observed in this study ($r = -0.235$, $p = 0.066$). Although it did not reach statistical significance, it is noteworthy to investigate whether SAA can bind to flagellin in circulation like it does to LPS, which has been previously reported to promote LPS clearance.[21]

Roseburia species are recognized as beneficial species due to their capacity to produce butyrate.[22,23] Their roles as flagellated species in the gut microbiota are also suggested to be favorable characteristics in disease treatment.[24] The activation of TLR5 in intestinal dendritic cells by *R. intestinalis* enhances the differentiation of regulatory T (Treg) cells, resulting in immunomodulation against Crohn's disease.[24] By contrast, in this study, we observed a higher abundance of *R. inulinivorans* in women who failed to attain live birth after FET (Figure 1B). The individuals in the 4th quartile of serum flagellin level had the highest abundance of *R. inulinivorans.* Nonetheless, our data do not conclude whether the increased abundance of *R. inulinivorans* was a cause or consequence of elevated serum flagellin levels. Compared with *R. intestinalis*, *R. inulinivorans* is less frequently studied. A study reported several distinctive features of *R. inulinivorans* that differ from other *Roseburia* species and complement other species to maintain the gut ecosystem.[25] The detection of flagellin in circulation implicates the penetration of flagellins, which can be caused by weakened mucosal immunity. Whether elevation of *R. inulinivorans* is an indicator of weakened intestinal immunity or a result of the compensatory mechanism of an altered gut ecosystem in subjects without live birth attainment from FET requires further investigation.

Bifidobacterium species have been widely used as probiotics. Although many diseases are associated with the reduced abundance of these species, the mechanisms by which they exert beneficial effects are largely unknown.[26] Our data suggest that the abundance of *B. pseudocatenulatum* in the gut is negatively correlated with serum flagellin level and higher in women attaining live birth after FET. Such a beneficial relationship appears to be strongly associated with cystathionine β-synthase activity. The chemical reactions catalyzed by cystathionine β-synthase produce not only cystathionine but also hydrogen sulfide. There are studies suggesting the role of hydrogen sulfide in preserving gut integrity.[17,18] Other than the cystathionine β-synthase activity, the α-glucan biosynthetic process that showed a significant negative correlation with serum flagellin level (Table S3B) was mainly contributed by *Bifidobacterium* species (Tables S3B and S5). Glucans are also suggested to be beneficial for maintaining gut symbiosis.[27,28] We speculate that the beneficial effect of *B. pseudocatenulatum* toward live birth attainment upon FET is possibly mediated by the production of hydrogen sulfide and glucans, resulting in improved gut integrity and limited system inflammation caused by the penetration of bacterial materials. The potential causative relationship is noteworthy for future studies.

(B) Spearman correlation between serum flagellin level and the readouts of GO:0019343 and GO:0004122 attributed by *B. pseudocatenulatum* alone or in combination with *B. adolescentis*, all *Bifidobacterium* or *E. coli* adjusted with age and BMI.

(C) Spearman correlation between the serum levels of various cytokines and flagellin and the abundance and readout of GO:0004122 attributed by *B. pseudocatenulatum* adjusted with age and BMI.

(D) The prevalences of all and the most prevalent species contributing to the readouts of GO:0019343 and GO:0004122 in this cohort. CRP, C-reactive protein; IFNγ, interferon-γ; MCP1, monocyte chemoattractant protein-1; SAA, serum amyloid A. *Statistically significant.

Limitations of the study

There are limitations of this observational study, including the inability to prove the causative nature of flagellin in preventing live birth attainment and the relatively small number of subjects. It is possible that the serum level of flagellin is an indicator of a change in immunity. Hence, further investigations with larger sample sizes and experimental approaches to delineate the role of flagellin in FET will validate whether circulating flagellin can serve as a treatment target to improve the live birth rate upon FET or is merely an indicator of systemic inflammation.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Connie W. Woo (cwhwoo@hotmail.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The gut microbiota data have been deposited at NCBI Sequence Read Archive (SRA) BioProject database and are publicly available as of the date of publication. Accession number is listed in the key resources table.
- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.

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AUTHOR CONTRIBUTIONS

Conceptualization, C.W.W.; methodology, K.T.C.C., J.H.C.Y., C.W.W., and R.H.W.L.; validation, K.T.C.C., J.H.C.Y., C.W.W., and R.H.W.L.; formal analysis, K.T.C.C., J.H.C.Y., C.W.W., and R.H.W.L.; investigation, L.C.Y., W.Y.-L.W., J.C., S.W.M.C., and K.W.C. resources, K.W.C, R.H.W.L., and C.W.W.; data curation, R.H.W.L. and C.W.W.; writing—original draft, R.H.W.L. and C.W.W.; writing—review and editing, R.H.W.L. and C.W.W.; visualization, C.W.W.; supervision, R.H.W.L. and C.W.W.; funding acquisition, C.W.W., K.T.C.C., and J.H.C.Y. contributed equally to this study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

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REFERENCES

1. World Health Organization (2023). Infertility prevalence estimates, 1990–2021 (World Health Organization).
2. Njagi, P., Groot, W., Arsenijevic, J., Dyer, S., Mburu, G., and Kiarie, J. (2023). Financial costs of assisted reproductive technology for patients in low- and middle-income countries: a systematic review. Hum. Reprod. Open *2023*, hoad007. https://doi.org/10.1093/hropen/hoad007.
3. Society for Assisted Reproductive Technology. Final National Summary Report for 2021. https://sartcorsonline.com/Csr/Public?ClinicPKID=0&reportingYear=2021&newReport=True.
4. Adebayo, F.O., Ameh, N., Adesiyun, A.G., Ekele, B.A., and Wada, I. (2023). Correlation of female age with outcome of IVF in a low-resource setting. Int. J. Gynaecol. Obstet. *161*, 283–288. https://doi.org/10.1002/ijgo.14545.
5. Bui, B.N., van Hoogenhuijze, N., Viveen, M., Mol, F., Teklenburg, G., de Bruin, J.-P., Besselink, D., Brentjens, L.S., Mackens, S., Rogers, M.R.C., et al. (2023). The endometrial microbiota of women with or without a live birth within 12 months after a first failed IVF/ICSI cycle. Sci. Rep. *13*, 3444. https://doi.org/10.1038/s41598-023-30591-2.
6. Diaz-Martínez, M.D.C., Bernabeu, A., Lledó, B., Carratalá-Munuera, C., Quesada, J.A., Lozano, F.M., Ruiz, V., Morales, R., Llácer, J., Ten, J., et al. (2021). Impact of the Vaginal and Endometrial Microbiome Pattern on Assisted Reproduction Outcomes. J. Clin. Med. *10*, 4063. https://doi.org/10.3390/jcm10184063.
7. Thanaboonyawat, I., Pothisan, S., Petyim, S., and Laokirkkiat, P. (2023). Pregnancy outcomes after vaginal probiotic supplementation before frozen embryo transfer: a randomized controlled study. Sci. Rep. *13*, 11892. https://doi.org/10.1038/s41598-023-39078-6.
8. Jepsen, I.E., Saxtorph, M.H., Englund, A.L.M., Petersen, K.B., Wissing, M.L.M., Hviid, T.V.F., and Macklon, N. (2022). Probiotic treatment with specific lactobacilli does not improve an unfavorable vaginal microbiota prior to fertility treatment—A randomized, double-blinded, placebo-controlled trial. Front. Endocrinol. *13*, 1057022. https://doi.org/10.3389/fendo.2022.1057022.
9. Vexø, L.E., Stormlund, S., Landersoe, S.K., Jørgensen, H.L., Humaidan, P., Bergh, C., Englund, A.L.M., Klajnbard, A., Bogstad, J.W., Freiesleben, N.I.C., et al. (2023). Low-grade inflammation is negatively associated with live birth in women undergoing IVF. Reprod. Biomed. Online *46*, 302–311. https://doi.org/10.1016/j.rbmo.2022.10.004.
10. Ghosh, S.S., Wang, J., Yannie, P.J., and Ghosh, S. (2020). Intestinal Barrier Dysfunction, LPS Translocation, and Disease Development. J. Endocr. Soc. *4*, bvz039. https://doi.org/10.1210/jendso/bvz039.
11. Berg, R.D. (1996). The indigenous gastrointestinal microflora. Trends Microbiol. *4*, 430–435. https://doi.org/10.1016/0966-842X(96)10057-3.
12. Candelli, M., Franza, L., Pignataro, G., Ojetti, V., Covino, M., Piccioni, A., Gasbarrini, A., and Franceschi, F. (2021). Interaction between Lipopolysaccharide and Gut Microbiota in Inflammatory Bowel Diseases. Int. J. Mol. Sci. *22*, 6242. https://doi.org/10.3390/ijms22126242.
13. Thevaranjan, N., Puchta, A., Schulz, C., Naidoo, A., Szamosi, J.C., Verschoor, C.P., Loukov, D., Schenck, L.P., Jury, J., Foley, K.P., et al.

(2017). Age-Associated Microbial Dysbiosis Promotes Intestinal Permeability, Systemic Inflammation, and Macrophage Dysfunction. Cell Host Microbe *21*, 455–466.e4. https://doi.org/10.1016/j.chom.2017.03.002.

14. Eaves-Pyles, T., Murthy, K., Liaudet, L., Virág, L., Ross, G., Soriano, F.G., Szabó, C., and Salzman, A.L. (2001). Flagellin, a Novel Mediator of *Salmonella* -Induced Epithelial Activation and Systemic Inflammation: IκBα Degradation, Induction of Nitric Oxide Synthase, Induction of Proinflammatory Mediators, and Cardiovascular Dysfunction. J. Immunol. *166*, 1248–1260. https://doi.org/10.4049/jimmunol.166.2.1248.
15. Clasen, S.J., Bell, M.E.W., Borbón, A., Lee, D.H., Henseler, Z.M., de la Cuesta-Zuluaga, J., Parys, K., Zou, J., Wang, Y., Altmannova, V., et al. (2023). Silent recognition of flagellins from human gut commensal bacteria by Toll-like receptor 5. Sci Immunol *8*, eabq7001. https://doi.org/10.1126/sciimmunol.abq7001.
16. Willis, S.K., Hatch, E.E., Laursen, A.S., Wesselink, A.K., Mikkelsen, E.M., Tucker, K.L., Rothman, K.J., Mumford, S.L., and Wise, L.A. (2022). Dietary patterns and fecundability in 2 prospective preconception cohorts. Am. J. Clin. Nutr. *116*, 1441–1451. https://doi.org/10.1093/ajcn/nqac213.
17. Motta, J.-P., Flannigan, K.L., Agbor, T.A., Beatty, J.K., Blackler, R.W., Workentine, M.L., Da Silva, G.J., Wang, R., Buret, A.G., and Wallace, J.L. (2015). Hydrogen Sulfide Protects from Colitis and Restores Intestinal Microbiota Biofilm and Mucus Production. Inflamm. Bowel Dis. *21*, 1006–1017. https://doi.org/10.1097/MIB.0000000000000345.
18. Zhao, H., Yan, R., Zhou, X., Ji, F., and Zhang, B. (2016). Hydrogen sulfide improves colonic barrier integrity in DSS-induced inflammation in Caco-2 cells and mice. Int. Immunopharmacol. *39*, 121–127. https://doi.org/10.1016/j.intimp.2016.07.020.
19. Tran, H.Q., Ley, R.E., Gewirtz, A.T., and Chassaing, B. (2019). Flagellin-elicited adaptive immunity suppresses flagellated microbiota and vaccinates against chronic inflammatory diseases. Nat. Commun. *10*, 5650. https://doi.org/10.1038/s41467-019-13538-y.
20. Yiu, J.H.C., Cai, J., Cheung, S.W.M., Chin, K.T.-C., Chan, C.F., Ma, E.S., Sharma, R., Dorweiler, B., and Woo, C.W. (2023). The association between flagellin producers in the gut microbiota and HDL-C level in humans. Front. Microbiomes *2*, 1287369. https://doi.org/10.3389/frmbi.2023.1287369.
21. Cheng, N., Liang, Y., Du, X., and Ye, R.D. (2018). Serum amyloid A promotes LPS clearance and suppresses LPS -induced inflammation and tissue injury. EMBO Rep. *19*, e45517. https://doi.org/10.15252/embr.201745517.
22. Duncan, S.H., Hold, G.L., Barcenilla, A., Stewart, C.S., and Flint, H.J. (2002). Roseburia intestinalis sp. nov., a novel saccharolytic, butyrate-producing bacterium from human faeces. Int. J. Syst. Evol. Microbiol. *52*, 1615–1620. https://doi.org/10.1099/00207713-52-5-1615.
23. Tamanai-Shacoori, Z., Smida, I., Bousarghin, L., Loreal, O., Meuric, V., Fong, S.B., Bonnaure-Mallet, M., and Jolivet-Gougeon, A. (2017). *Roseburia* spp.: a marker of health? Future Microbiol. *12*, 157–170. https://doi.org/10.2217/fmb-2016-0130.
24. Shen, Z., Luo, W., Tan, B., Nie, K., Deng, M., Wu, S., Xiao, M., Wu, X., Meng, X., Tong, T., et al. (2022). Roseburia intestinalis stimulates TLR5-dependent intestinal immunity against Crohn's disease. EBioMedicine *85*, 104285. https://doi.org/10.1016/j.ebiom.2022.104285.
25. Hillman, E.T., Kozik, A.J., Hooker, C.A., Burnett, J.L., Heo, Y., Kiesel, V.A., Nevins, C.J., Oshiro, J.M.K.I., Robins, M.M., Thakkar, R.D., et al. (2020). Comparative genomics of the genus Roseburia reveals divergent biosynthetic pathways that may influence colonic competition among species. Microb. Genom. *6*, mgen000399. https://doi.org/10.1099/mgen.0.000399.
26. Derrien, M., Turroni, F., Ventura, M., and van Sinderen, D. (2022). Insights into endogenous Bifidobacterium species in the human gut microbiota during adulthood. Trends Microbiol. *30*, 940–947. https://doi.org/10.1016/j.tim.2022.04.004.
27. De Felice, B., Damiano, S., Montanino, C., Del Buono, A., La Rosa, G., Guida, B., and Santillo, M. (2020). Effect of beta- and alpha-glucans on immune modulating factors expression in enterocyte-like Caco-2 and goblet-like LS 174T cells. Int. J. Biol. Macromol. *153*, 600–607. https://doi.org/10.1016/j.ijbiomac.2020.03.046.
28. Gangoiti, J., Corwin, S.F., Lamothe, L.M., Vafiadi, C., Hamaker, B.R., and Dijkhuizen, L. (2020). Synthesis of novel α-glucans with potential health benefits through controlled glucose release in the human gastrointestinal tract. Crit. Rev. Food Sci. Nutr. *60*, 123–146. https://doi.org/10.1080/10408398.2018.1516621.
29. Yiu, J.H.C., Chan, K.S., Cheung, J., Li, J., Liu, Y., Wang, Y., Fung, W.W.L., Cai, J., Cheung, S.W.M., Dorweiler, B., et al. (2020). Gut Microbiota-Associated Activation of TLR5 Induces Apolipoprotein A1 Production in the Liver. Circ. Res. *127*, 1236–1252. https://doi.org/10.1161/CIRCRESAHA.120.317362.
30. Cook, L., Lisko, D.J., Wong, M.Q., Garcia, R.V., Himmel, M.E., Seidman, E.G., Bressler, B., Levings, M.K., and Steiner, T.S. (2020). Analysis of Flagellin-Specific Adaptive Immunity Reveals Links to Dysbiosis in Patients With Inflammatory Bowel Disease. Cell. Mol. Gastroenterol. Hepatol. *9*, 485–506. https://doi.org/10.1016/j.jcmgh.2019.11.012.
31. Beghini, F., McIver, L.J., Blanco-Míguez, A., Dubois, L., Asnicar, F., Maharjan, S., Mailyan, A., Manghi, P., Scholz, M., Thomas, A.M., et al. (2021). Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bobakery 3. Elife *10*, e65088. https://doi.org/10.7554/eLife.65088.
32. Parks, D.H., Tyson, G.W., Hugenholtz, P., and Beiko, R.G. (2014). STAMP: statistical analysis of taxonomic and functional profiles. Bioinformatics *30*, 3123–3124. https://doi.org/10.1093/bioinformatics/btu494.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-human IgG secondary horseradish peroxidase-conjugated antibodies	Jackson ImmunoResearch	Cat#109-035-088; RRID: AB_2337584
anti-human IgA secondary horseradish peroxidase-conjugated antibodies	Jackson ImmunoResearch	Cat#109-035-011; RRID: AB_2337580
Biological samples		
Human serum	The University of Hong Kong-Queen Mary Hospital	N/A
Human feces	The University of Hong Kong-Queen Mary Hospital	N/A
Chemicals, peptides, and recombinant proteins		
ZymoBIOMICS Microbial Community Standard	Zymo Research	Cat#D6300
QUANTI-Blue™	InvivoGen	Cat#rep-qbs2
Purified flagellin from *Salmonella* typhimurium	Enzo Life Sciences	Cat #ALX-522-058-3010
Critical commercial assays		
OMNIgene·GUT Collection Kits	DNA Genotek	Cat#OM-200
QIAamp PowerFecal Pro DNA Kit	Qiagen	Cat#51804
LAL assay	HyCult Biotech	Cat#HIT302
IL-1β	R&D System	Cat#DY201
IFNγ	R&D System	Cat#DY285B
MCP1	R&D System	Cat#DY279
CRP	R&D System	Cat#DY1707
SAA	R&D System	Cat#DY3019
ultrapure flagellin from *Bacillus subtilis*	InvivoGen	Cat#tlrl-pbsfla
ultrapure flagellin from *Salmonella* typhimurium	InvivoGen	Cat#tlrl-epstfla
Deposited data		
Raw sequences of gut microbiota analysis	NCBI Sequence Read Archive (SRA) https://www.ncbi.nlm.nih.gov/bioproject/?term=991649	PRJNA991649
Experimental models: Cell lines		
HEK-Dual™-hTLR5 reporter cell line	InvivoGen	Cat. #hkd-htlr5ni
Software and algorithms		
Kneaddata	Harvard School of Public Health https://github.com/biobakery/biobakery/wiki/kneaddata	N/A
MetaPhlAn	Duy Tin Truong, Nicola Segata and Curtis Huttenhower https://github.com/biobakery/biobakery/wiki/metaphlan3	Version 3.0
HUMAnN	Harvard School of Public Health https://github.com/biobakery/humann	Version 3.0
R studio	R Project for Statistical Computing and Graphing https://www.r-project.org	Version 3.6.2
STAMP	Dalhousie University https://beikolab.cs.dal.ca/software/STAMP	Version 2.1.3
SPSS	IBM	Version 26
MedCal	MedCalc Software Ltd	Version 22

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Ethics statement

This study was approved by the Institutional Review Board, the University of Hong Kong – Hospital Authority Hong Kong West Cluster (reference number: UW 19-340) in April 2019. Written informed consent was obtained from all participating women.

Study participants

This observational study recruited 67 women who underwent FET at the Centre of Assisted Reproduction and Embryology, The University of Hong Kong- Queen Mary Hospital (HKU-QMH CARE), which was an assisted reproduction centre in a university-affiliated tertiary hospital in Hong Kong. The participants were recruited between June 2019 and September 2021. Exclusion criteria included a history of antibiotics intake or infection within 7 days, and the presence of known structural pathologies that could reduce the conception rate after FET, such as hydrosalpinx or endometrial polyps. Serum and fecal samples were collected prior to FET. Six participants who did not provide fecal samples prior to FET or provided problematic samples were excluded from the gut microbiota study. The primary outcome was live birth rate, while secondary outcomes included pregnancy rate and miscarriage rate. Samples were allocated to ''with live birth'' and ''without live birth'' groups for analysis.

METHOD DETAILS

Flagellin and LPS measurements

Serum flagellin level was measured using the HEK-Dual^{TM}-hTLR5 reporter cell line (InvivoGen, San Diego, USA) according to the manufacturer's instructions as described previously.[29] 20 μl of 10-times-diluted serum was incubated with 180 μl of cell suspension (65,000 cells/well) for 24 hours. The secreted alkaline phosphatase activity was measured using QUANTI-Blue^{TM} (InvivoGen, San Diego, USA), and the absorbance at 660 nm was measured with a spectrophotometer. Purified flagellin from *Salmonella* typhimurium (Enzo Life Sciences, USA) was used as a standard. Serum lipopolysaccharide level was measured using limulus amoebocyte lysate (LAL) assay (HyCult Biotech, Uden, Netherlands) according to the manufacturer's protocol.

Cytokine ELISA

The proinflammatory cytokines in the serum including IL-1β, IFNγ, MCP1, CRP, and SAA were measured using commercial ELISA kits according to the manufacturer's instructions (R&D System, Minneapolis, USA).

Anti-flagellin IgG and IgA ELISA

The measurement of anti-flagellin antibodies in circulation was performed as previously described, with modifications.[30] ELISA microplates were coated with 1 μg/ml ultrapure flagellin from *Bacillus subtilis* (InvivoGen) or *Salmonella* typhimurium (InvivoGen) with 0.1M carbonate buffer overnight at 4°C, respectively. The wells were then blocked using 3% BSA in tris-buffered saline containing 0.05% tween-20 for 2 hours at room temperature. After blocking, coated wells were incubated with diluted serum samples for an hour, followed by incubation with anti-human IgG or IgA secondary horseradish peroxidase-conjugated antibodies (Jackson ImmunoResearch) for an hour. TMB substrate solution was added for antibody detection. The absorbance was measured at 450 nm.

Fecal DNA preparation for sequencing

Feces were collected using OMNIgene·GUT Collection Kits (DNA Genotek, Canada). Fecal DNA was extracted using the QIAamp PowerFecal Pro DNA Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol, and subjected to whole genome shotgun metagenomic sequencing by the Genomics Core of the Centre for PanorOmic Sciences (CPOS), LKS Faculty of Medicine, the University of Hong Kong. The efficiency of the isolation protocol was verified using a mock microbial community, the ZymoBIOMICS Microbial Community Standard, purchased from Zymo Research (CA, USA). DNA fragments were sequenced using Illumina NovaSeq 6000 (151bp; pair-end).

Gut microbiota analysis

The shotgun metagenomic sequence files were first preprocessed using KneadData (Harvard University, USA) with the default settings, including the removal of human (hg37dec_v0.1) contaminant sequences identified by Bowtie2 using the very-sensitive mode, reads with low quality reads ($Q < 20$), and fragmented short reads (<50 bp). The taxonomic profile was determined using MetaPhlAn 3.0 (Harvard University, USA). This version was built using 99,237 reference genomes representing 16,797 species retrieved from Genbank as of January 2019. The functional profiling was performed using HUMAnN 3.0 (Harvard University, USA) with a non-redundant database, Uniref90 (Version 201901b).[31]

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using R software Version 3.6.2, STAMP (Version 2.1.3), SPSS Statistics (Version 26), and MedCalc (Version 22).[32] A 95% power was achieved for this study on the two independent group designs, to detect a 20% difference

in measured parameters at 5% significance. Normality was checked by the Shapiro-Wilk test. Continuous variables were expressed as medians (25^{th} – 75^{th} percentile) and compared between groups using the Mann-Whitney U test. Categorical variables were expressed as counts (%) and compared between groups by the Fisher's Exact test. Univariate binary logistic regression was used to study the prediction of live birth by flagellin. Repeated k-fold cross validation and a random forest model using classification were applied to determine the importance of bacterial species and serum flagellin levels to predict live birth. The receiver-operating characteristics curve was used to determine the predictive performance on live birth rate. The difference in gut microbiota pattern was evaluated by permutational analysis of variance (PERMANOVA) and non-metric multidimensional scaling (NMDS) using the vegan package in R programme. Spearman partial correlation analysis was performed using the ppcor package in R programme. P values were adjusted by the Benjamini-Hochberg method or false discovery rate (FDR) for multiple comparisons. All significance tests were two-tailed, and the statistical significance level was defined by P values of <0.05.