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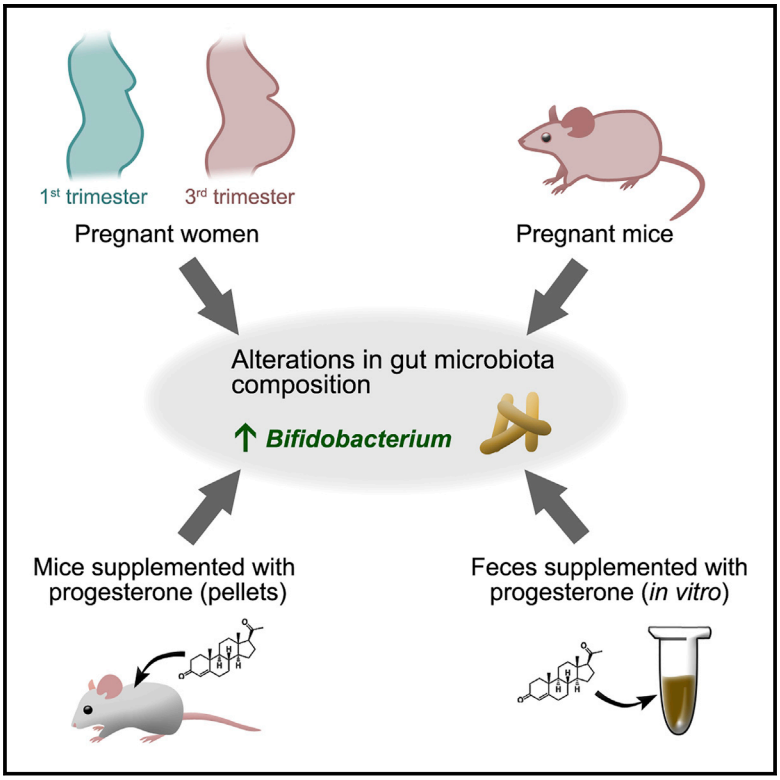
Report

Progesterone Increases *Bifidobacterium* Relative Abundance during Late Pregnancy

Graphical Abstract



Authors

Meital Nuriel-Ohayon, Hadar Neuman, Oren Ziv, ..., Yoram Louzoun, Orly Avni, Omry Koren

Correspondence

omry.koren@biu.ac.il

In Brief

Nuriel-Ohayon et al. demonstrate a dramatic shift in the gut microbial composition of women and mice during late pregnancy, including an increase in the relative abundance of *Bifidobacterium*. Using *in vitro* and *in vivo* experiments, they show that supplementation of progesterone affects the microbial communities, including increasing the relative abundance of *Bifidobacterium*.

Highlights

- *Bifidobacterium* abundance increases in the gut during pregnancy in women and mice
- Progesterone supplementation alters gut microbial composition in mice and *in vitro*
- Progesterone supplementation increases *Bifidobacterium* abundance in mice and *in vitro*
- We suggest that progesterone promotes *Bifidobacterium* growth during late pregnancy

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Progesterone Increases *Bifidobacterium* Relative Abundance during Late Pregnancy

Meital Nuriel-Ohayon,[1] Hadar Neuman,[1,2] Oren Ziv,[1] Anna Belogolovski,[3] Yiftah Barsheshet,[1] Naamah Bloch,[1] Atara Uzan,[1] Roey Lahav,[1] Avi Peretz,[1,4] Sigal Frishman,[5] Moshe Hod,[5] Eran Hadar,[5] Yoram Louzoun,[3] Orly Avni,[1] and Omry Koren[1,6,*]

[1]Azrieli Faculty of Medicine, Bar Ilan University, Safed, Israel
[2]Zefat Academic College, Safed, Israel
[3]Department of Mathematics, Bar-Ilan University, Ramat-Gan, Israel
[4]Clinical Microbiology Laboratory, Baruch Padeh Medical Center, Poriya, Israel
[5]Rabin Medical Center and the Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel
[6]Lead Contact
*Correspondence: omry.koren@biu.ac.il
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SUMMARY

Gestation is accompanied by alterations in the microbial repertoire; however, the mechanisms driving these changes are unknown. Here, we demonstrate a dramatic shift in the gut microbial composition of women and mice during late pregnancy, including an increase in the relative abundance of *Bifidobacterium*. Using *in-vivo*-transplanted pellets, we found that progesterone, the principal gestation hormone, affects the microbial community. The effect of progesterone on the richness of several bacteria species, including *Bifidobacterium*, was also demonstrated *in vitro*, indicating a direct effect. Altogether, our results delineate a model in which progesterone promotes *Bifidobacterium* growth during late pregnancy.

INTRODUCTION

Pregnancy is accompanied by alterations in the oral, skin, vaginal, and gut microbial profiles (Aagaard et al., 2012; DiGiulio et al., 2015; Koren et al., 2012; Nuriel-Ohayon et al., 2016). Among these, the most drastic effects are associated with the gut microbiota, which undergoes changes that partially resemble those observed in metabolic syndrome (Pitlik and Koren, 2017). These changes include increased abundance of Proteobacteria, Actinobacteria, and opportunistic pathogens and a decrease in short chain fatty acid producers and in species richness, all occurring as pregnancy progresses (Koren et al., 2012). Some of the characteristics of pregnancy, e.g., weight gain and low-grade inflammation, were transferrable to germ-free (GF) mice following fecal transplants from pregnant women, indicating a causative role for the gut microbiota in some of the pregnancy phenotypes (Koren et al., 2012).

Progesterone levels increase dramatically during pregnancy, and it plays a crucial role in regulating and maintaining gestation (Mesiano, 2001). Although some links between the endocrine system and the microbiota have been previously described, understanding of the precise interactions between the endocrine system and the microbiota is still limited (Neuman et al., 2015). Here, we demonstrate that progesterone modulates the pregnancy-associated gut microbial composition, including an increase in the relative abundance of *Bifidobacterium*. Thereby, progesterone regulates the microbial composition during pregnancy in a way that may facilitate both the pregnant mother and perhaps also appropriate transmission of beneficial species to the neonate.

RESULTS

Pregnancy-Associated Microbiota of Women and Mice Is Enriched with *Bifidobacterium*

It has previously been demonstrated by sequencing the V2 region of the 16S rRNA gene that the gut microbiota changes throughout gestation in women (Koren et al., 2012). Because we currently know that V2 primers fail to identify several species in the gut microbiome (Sim et al., 2012), to increase the species coverage, we sequenced the V4 region, an additional variable region in the 16S rRNA gene. Our analysis of a new sample set consisting of 35 women in both their 1st and 3rd trimesters (Table S1) demonstrated significant differences in 3rd trimester microbiota as compared to the 1st trimester (Figure 1A). By sequencing the V4 region, we were able to observe a significant enrichment in genera such as *Neisseria*, *Blautia*, *Collinsella*, and *Bifidobacterium* (Figures 1A and 1B) as pregnancy progressed. We also observed a decrease in the levels of *Clostridium*, *Dehalobacterium*, and an unclassified *Bacteroidales*. The most abundant changes at the genus level are summarized in Figure S1.

To study the mechanisms underlying the interaction between pregnancy and the microbiota, we next used a murine model. As expected, as pregnancy progressed, pregnant female mice gained weight as compared to their control counterparts (Figure S2A), and dynamic changes in the beta (between samples) diversity of the gut microbial composition were observed at embryonic day 18 (E18) of pregnancy (Figure 2A). Changes were observed at several taxonomic levels when comparing E18 versus E0 (Figure 2B; Figure S2B). As we observed in women, the levels of the genus *Bifidobacterium* were significantly elevated (~4.3-fold higher average when compared to non-pregnant controls; Figure 2C). These results demonstrate

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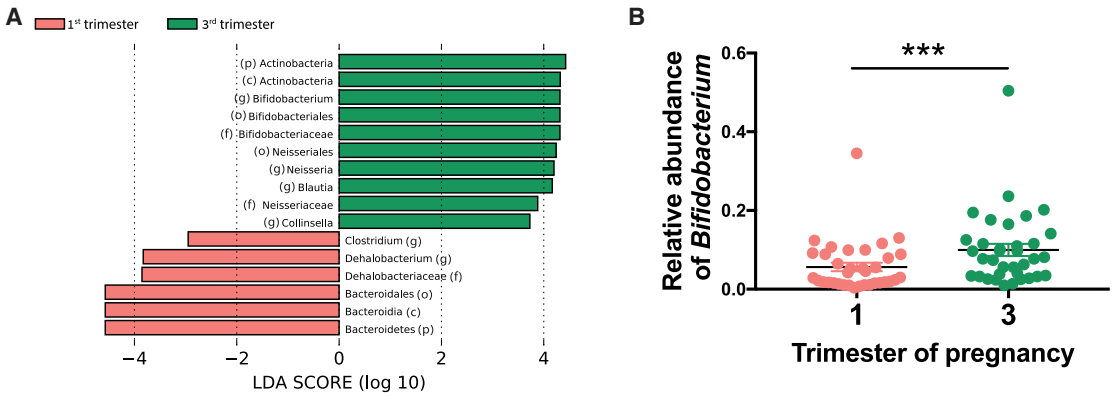

Figure 1. The Gut Microbiota of Pregnant Women Changes during Pregnancy

(A) Linear Discriminant Analysis Effect Size (LEfSe) of the most dominant bacteria in the gut microbiota of women in the first trimester (red) and third trimester (green) of their pregnancies (n = 35; all linear discriminant analysis [LDA] scores, >2.5). Taxonomy levels: (p), phylum; (c), class; (o), order; (f), family; (g), genus. (B) Relative abundance of *Bifidobacterium.* Data shown represent relative abundance for 6,200 randomly selected sequences/sample. Data are represented as mean ± SEM. Asterisks indicate significance (***p < 0.001) as determined by a paired two-sided Student's t test.

that *Bifidobacterium* responds to the late pregnancy conditions in mice as in women.

Progesterone Enriches the Presence of Bifidobacterium *In Vivo*

To explore the potential contribution of progesterone to the observed changes in gut microbial compositions during pregnancy, we implanted progesterone subcutaneously (releasing ca. 1.67 mg/day) as well as placebo pellets, for 21 days in female mice. Progesterone treatment successfully raised serum progesterone levels versus the placebo treatment, as measured on days 11 and 21 (Figure S3).

Microbial analysis by 16S rRNA gene sequencing of stool samples from progesterone- versus placebo-treated mice revealed distinct microbial compositions. Linear Discriminant Analysis Effect Size (LEfSe) characterization of the microbiota on day 18 revealed that the relative abundance of Archaea was significantly elevated in the progesterone-treated group versus placebo due to an increased abundance of *Methanobrevibacter*. Additional genera that were more abundant in the progesterone-treated group included *Lachnospira* and unclassified *Clostridiaceae* (Figure 3A). Most significantly, progesterone supplementation led to increased relative abundance of *Bifidobacterium* (0.23% versus 0.0007% of total bacteria on day 18, p = 0.0007; Figures 3A and 3B). To tease out which bacterial changes were most probably due to progesterone, we compared the microbiota of pregnant mice with that of progesterone- and placebo-treated mice. We searched for bacteria that increased in the first two groups and decreased in the placebo group. A heatmap comparing the relative abundance of microbial changes (Figure 3C) demonstrated *Turicibacter* (previously shown to also increase in pregnant rats and mice [Elderman et al., 2018; Khan et al., 2016]), *Anaeroplasma*, and *Bifidobacterium* as the bacteria that were most likely influenced by the increased levels of progesterone. Together, these findings strongly suggest that some of the pregnancy-associated microbial alterations, such as an increased relative abundance of *Bifidobacterium,* are mediated by progesterone.

Progesterone Directly Increases the Relative Abundance of *Bifidobacterium In-Vitro*

To assess a potential direct effect of progesterone on the microbiota, we carried out an *in vitro* experiment where either progesterone or PBS (control) were added to fecal slurry from a female mouse and incubated at 37°C under anaerobic conditions for 11 days. Dramatic effects on microbiota composition, measured by unweighted UniFrac, were observed in the cultures grown with progesterone (Figure 4A). In addition, progesterone also significantly influenced alpha diversity, as the microbial composition following progesterone treatment was significantly less diverse than that of the control (Figures 4B and 4C; Table S2). Some of the genera that increased during the progesterone treatment were *Coprococcus, Faecalibacterium*, *Bacteroides*, *Ruminococcus*, *Veillonella*, *Sutterella*, and *Lactobacillus* (Table S2). Of specific interest, progesterone treatment dramatically increased the relative abundance of *Bifidobacterium* (16.13% in progesterone versus 2.49% in control, p = 0.0043; Figure 4D).

DISCUSSION

Significant alterations in the gut microbiota between the 1st and 3rd trimesters of pregnancy have been documented, including changes in diversity, certain phyla, and specific genera (Koren et al., 2012). However, due to the choice of the sequenced variable region (region V2 of the 16S rRNA), not all of the bacterial changes were previously identified. We, therefore, wished to test a different region of the 16S rRNA gene in analysis of the microbial populations in a cohort of pregnant women from Israel. To this end, we analyzed the gut microbiota of 35 women in their 1st and 3rd trimesters of pregnancy, in a pairwise manner, using the primers for the V4 region of the 16S rRNA gene. We found that the generally dominant bacteria during pregnancy were *Blautia*, *Bifidobacterium* (important for human milk oligosaccharide (HMO) degradation [Nuriel-Ohayon et al., 2016]), unclassified *Ruminococcaceae*, *Bacteroides*, unclassified *Lachnospiraceae*, unclassified *Clostridiales*, *Akkermansia*,

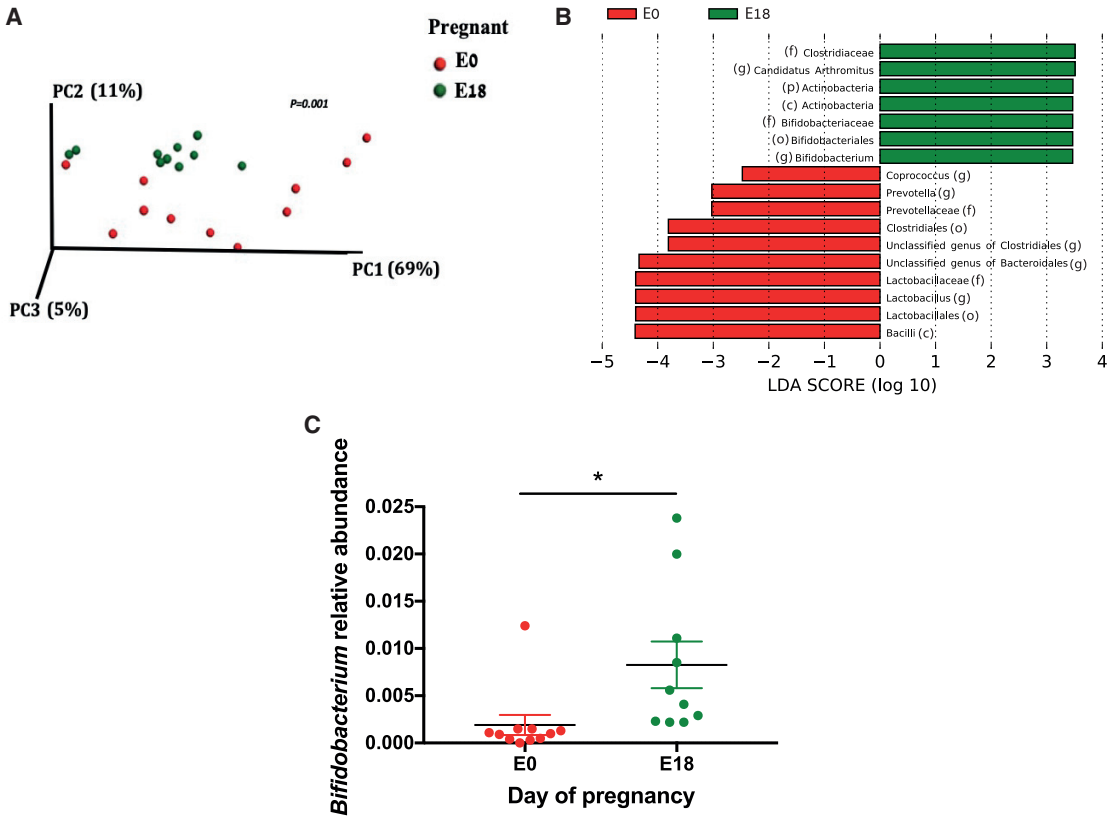

Figure 2. Pregnancy in Mice Is Accompanied by Changes in the Gut Microbiota

(A) Principal coordinates analysis (PcoA) plot based on Weighted UniFrac distances of microbiotas from E0, prior to pregnancy (red), and E18, late pregnancy (green).

(B) Linear Discriminant Analysis Effect Size (LEfSe) of the most dominant bacteria (based on relative abundance) in the gut microbiota of mice on E0 (red) and E18 (green) of their pregnancies.

(C) *Bifidobacterium* relative abundance in E0 versus E18. Data are represented as mean ± SEM (n = 11 in the control group and n = 10 in the pregnancy group). Asterisks indicate significance (*p < 0.05) as determined by a paired two-sided Student's t test.

Faecalibacterium, *Ruminococcus*, and *Prevotella* (known to metabolize estradiol and progesterone [Kornman and Loesche, 1982]) (Figure S1). The genera that increased most significantly in the 3rd versus 1st trimester were *Bifidobacterium*, *Neisseria*, *Blautia*, *and Collinsella*. On the other hand, the genera *Dehalobacterium* and *Clostridium* as well as the Bacteroidales order were significantly higher in the 1st trimester versus the 3rd.

To further identify the pregnancy-associated microbial-sensing signals, we studied pregnant mice. As expected, the mice gained weight during pregnancy. *Bifidobacterium* was the only genus that was elevated in the gut microbiota of both women and mice in the late stages of pregnancy, suggesting that *Bifidobacterium* responds to the conditions of late pregnancy in mice as in women. It is important to note that although in both human and mouse models we observed an increase in the relative abundance of *Bifidobacterium*, the natural abundance of this species during a non-pregnancy state differs between mice and humans (~0.004% versus ~5%, respectively).

In vivo and *in vitro* experiments demonstrated the causative role of progesterone on *Bifidobacterium* proliferation. It is important to emphasize that the selection of anaerobic conditions and a specific growth medium may have restricted our *in vitro* observations to only a subset of the true gut microbial potential and may have promoted the growth of species that do not normally thrive within the gut. Furthermore, the progesterone concentration used in the *in vitro* experiment (80 ng/mL) was initially higher than physiological levels in mice, perhaps more comparable to levels in human pregnancy (Kumar and Magon, 2012) and may not have stayed stable throughout the experiment. Nonetheless, the differences between groups were robust enough to lead to the conclusion that progesterone has a broad effect on microbiota composition, which deserves further study.

Female hormones have previously been described to promote the proliferation of multiple bacterial species. For example, several bacterial species, such as *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, and *Escherichia coli*, were isolated from the follicular fluid during *in vitro* fertilization (IVF) (Pelzer et al., 2012), and addition of *in vitro* progesterone to the follicular fluid stimulated the growth of *Bifidobacterium* spp. among others species (Pelzer et al., 2012). The low

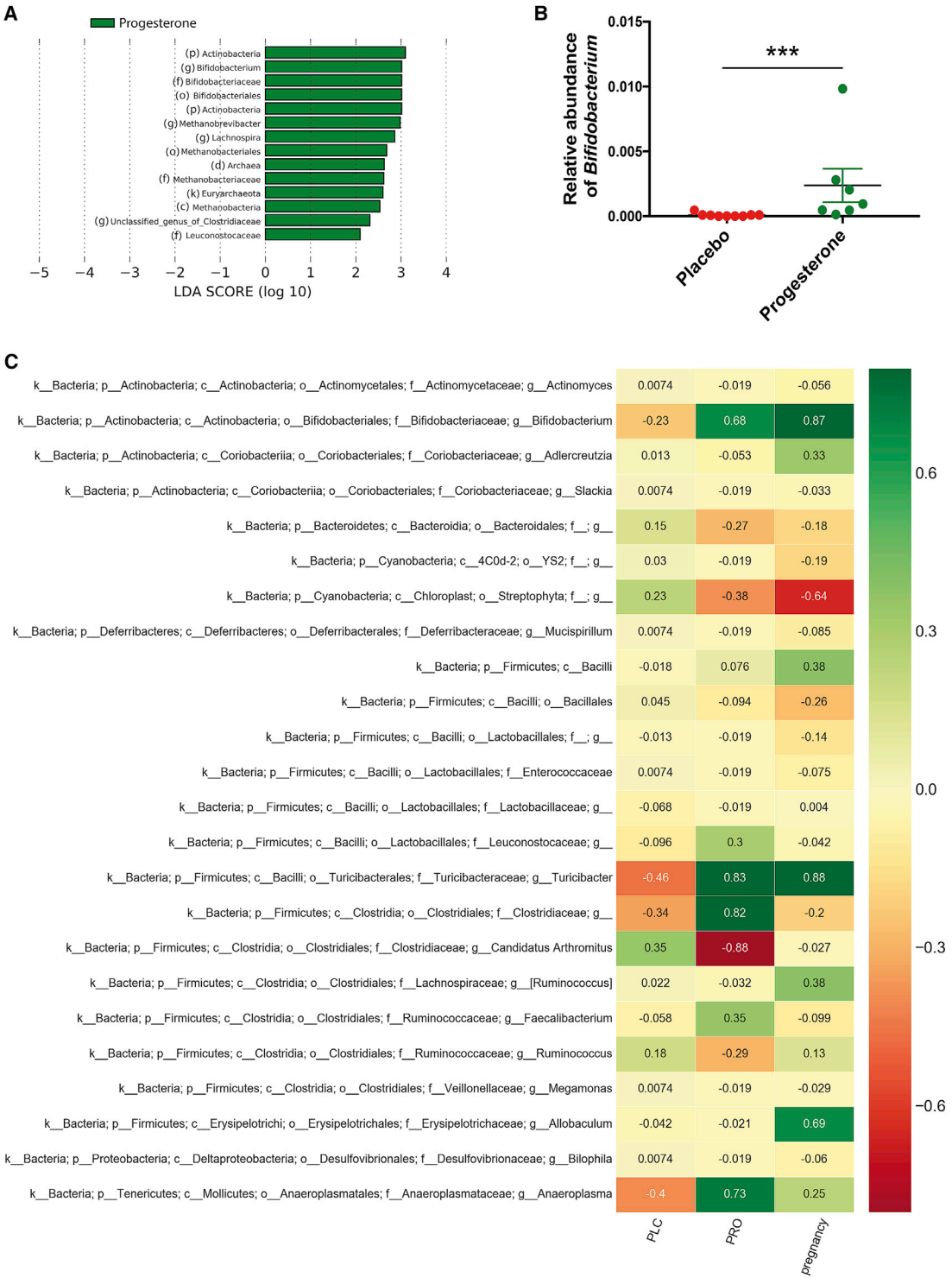

Figure 3. Progesterone Alters the Bacterial Composition in Mice

(A) Linear Discriminant Analysis Effect Size (LEfSe) of the most dominant microbes in progesterone treated females after 18 days. Biomarkers of progesterone (green) were ranked and plotted by effect size (all LDA scores, >2.5). Taxonomy levels: (d), domain; (k), kingdom; (p), phylum; (c), class; (o), order; (f), family; (g), genus.

(B) Relative abundance of *Bifidobacterium*.

(C) Heatmap comparing the relative abundance of the different bacteria in different experimental groups: pregnancy, progesterone (PRO), placebo (PLC). Data are represented as mean ± SEM (n = 7 in the progesterone group and n = 9 in the placebo group) Asterisks indicate significance (***$p < 0.001$) as determined by the Mann-Whitney test.

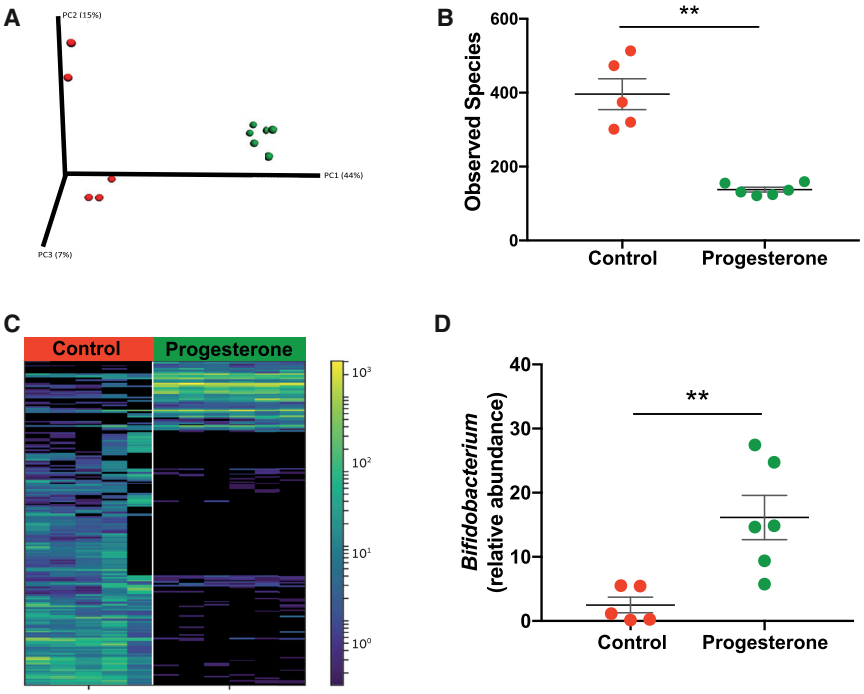

Figure 4. Progesterone Increases the Relative Abundance of the Genus *Bifidobacterium* and Decreases Alpha Diversity *In Vitro*

(A) Unweighted UniFrac distances of microbiotas from control (red) or progesterone (green) supplemented cultures.

(B) Alpha diversity based on observed species for 10,130 randomly selected sequences/sample.

(C) A heatmap (created using Calour) of the operational taxonomic units (OTUs) that significantly differed between progesterone and control treatments. The relative abundance is represented by a color gradient (blue, low abundance; yellow, high abundance).

(D) Relative abundance of *Bifidobacterium*. Data shown represent relative abundance for 10,130 randomly selected sequences/sample. Data are represented as mean ± SEM (n = 5 in the control group and n = 6 in the progesterone group. Asterisks indicate significance (**$p < 0.01$) as determined by the Mann-Whitney test.

abundance of Bifidobacteria may reflect a dysregulated hormonal equilibrium.

Bifidobacterium may be important for positive pregnancy outcomes, as reduced relative abundance of *Bifidobacterium* is associated with preterm birth (Dahl et al., 2017). Additionally, *Bifidobacterium* may be beneficial for the pregnant mother by moderating weight gain, improving insulin sensitivity and glucose tolerance, and boosting the immune system. In a study in which *Bifidobacterium breve* was given to mice fed a high-fat diet, the probiotic decreased weight gain (Kondo et al., 2010). Interestingly, even a sterilized culture of Bifidobacteria managed to suppress fat accumulation, improve insulin resistance, and lower blood glucose levels in mice on a high-fat diet (Kikuchi et al., 2018). Other studies have shown that oral administration of *Bifidobacterium* spp. improved insulin resistance and glucose tolerance in mice and rat cells by reducing inflammation and affecting both glucose and lipid metabolism (Kim et al., 2014; Le et al., 2014). In terms of effects on the immune system, Bifidobacteria have been shown to interact with human immune cells and to modulate specific pathways, involving innate and adaptive immune processes (Ruiz et al., 2017). Colonization of mice with *Bifidobacterium bifidum* resulted in increased levels of interleukin 6 (IL-6) and IL-8 cytokines, presumably through nuclear factor κB (NF-κB) activation (Turroni et al., 2014). Supplementation of *Bifidobacterium* has also been shown to elevate fecal levels of immunoglobulin A in young women (Kabeerdoss et al., 2011), perhaps playing defense roles in the gastrointestinal, respiratory, and genitourinary tracts (Woof and Kerr, 2006).

Bifidobacteria are clearly critical members of the newborn microbiota repertoire, as they are lactic-acid-producing bacteria that have the ability to metabolize HMOs (Bäckhed et al., 2015; Turroni et al., 2017). It has also been shown that Bifidobacteria are passed from mother to infant during vaginal birth because specific *Bifidobacterium* species from the mother's prenatal feces have been found in the feces of infants born vaginally but not by cesarean delivery (Makino et al., 2011; Nuriel-Ohayon et al., 2016). They are also transferred through breast milk (Nuriel-Ohayon et al., 2016). The passage of Bifidobacteria from mother to infant may explain the importance of high levels of this genus during late pregnancy; besides its ability to degrade HMOs, Bifidobacteria also have an important role in the maturation of the immune system in the first period of life, as studies have shown that reduced levels of Bifidobacteria in newborns are linked with disease states (Ruiz et al., 2017). Therefore, we hypothesize that the elevation in *Bifidobacterium* during late pregnancy may not only be beneficial for pregnancy but may also reflect an evolutionary process of preparation for birth and lactation.

What could be the mechanisms underlying the selective growth of *Bifidobacterium* in the presence of progesterone both *in vivo* and *in vitro*? One option might be due to the presence of the enzyme hydroxysteroid dehydrogenase (HSD) which is involved in steroid metabolism and is abundant in members of the Actinobacteria phylum (Kisiela et al., 2012), specifically in Bifidobacteria (see Method Details). Alternatively, a yet unidentified *Bifidobacterium* regulator senses progesterone.

In summary, our study demonstrates an increase in the relative abundance of *Bifidobacterium* in the 3rd trimester of pregnancy in both humans and mice, as well as in models of progesterone supplementation. These findings, which provide insights into the understanding of the relationship between hormones and the gut microbiota during pregnancy, may be relevant not only for pregnancy but also for other conditions in which hormones are involved, including progesterone supplementation as a component of fertility treatments or therapy in women at menopause.

STAR★METHODS

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

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.03.075.

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AUTHOR CONTRIBUTIONS

Experiments were designed by M.N.O., O.Z., H.N., and O.K. Samples from pregnant women were collected by S.F., E.H., and M.H. All experiments were performed by M.N.O. Data were analyzed by M.N.O., A.B., Y.L., and H.N. A.U., A.P., O.Z., and R.L. assisted with the progesterone pellets study. N.B., O.A., and Y.B. helped in discussion of data. M.N.O., H.N., O.A., and O.K. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
DEPO-PROVERA™	Pfizer	NA
Progesterone pellets- 35mg	Innovative Research of America	Cat# P-131
Placebo (control) for Progesterone pellets- 35mg	Innovative Research of America	Cat# C-111
Critical Commercial Assays		
MO BIO PoweSoil DNA isolation kit	MO BIO Laboratories	Cat# 12888
Deposited Data		
16S metagenomics raw sequence data	This paper	ENA project no: PRJEB31104
Experimental Models: Organisms/Strains		
SPF Mice/Swiss Webster	Taconic Biosciences	Model# SW-F/SW-M
Germ-free Mice/Swiss Webster	Taconic Biosciences	Model# SW-F/SW-M
Oligonucleotides		
16S 515 Forward primer	IDT syntezza	(Caporaso et al., 2012)
16S 806 Reverse primer	IDT syntezza	(Caporaso et al., 2012)
Software and Algorithms		
GraphPad Prism v7	Graphpad Software	https://www.graphpad.com/scientific-software/prism/
Calour	(Jiang et al., 2017)	https://github.com/biocore/calour, https://github.com/amnona/EZCalour
QIIME version 1.8.0	(Caporaso et al., 2010)	http://qiime.org/1.8.0/install/install.html
GreenGenes database	(DeSantis et al., 2006)	http://greengenes.lbl.gov/Download/OTUs/
LEfSe	(Segata et al., 2011)	http://huttenhower.sph.harvard.edu/galaxy/

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Omry Koren (Omry.Koren@biu.ac.il).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human Subjects and data collection

Fecal samples were collected from 35 healthy pregnant Israeli women between the ages of 23–42 years, with no pregnancy complications. All procedures involving human subjects were approved by the institutional review board of Clalit health services (Approval number 0135-15) and Rabin medical center (Approval number 0263-15), and informed consent was obtained. Fecal samples were collected at 12-14 weeks (1st trimester) and 34-36 weeks (3rd trimester). Fresh fecal samples were collected using FecesCatcher (ZymoResearch, Tustin, CA), and then frozen immediately at −80°C in sterile 50 mL tubes until DNA purification.

Experimental animals

All mice used in the study were 8-10 week-old dams of the Swiss Webster strain. Mice were housed and bred in the same room, under specific pathogen- free conditions with a 12h light/dark cycle and maintained at 22°C ± 1. Pregnant dams were single-housed, whereas all other mice were group housed (2-3 mice/ cage). All mice were given free access to food and water and were fed from the same food batch (Harlan-Teklad, Madison, WI). In each experiment, mice were randomly assigned to experimental groups. The day of randomization into groups was considered as day 0. Blood samples were collected from the facial vein into 1ml heparin tubes kept on ice (Greiner bio-one, Frickenhausen, Germany). Plasma was separated by centrifugation at 1,500 g for 20 mins at 4°C, and kept frozen at −20°C until analysis. All experimental procedures were approved by the Bar-Ilan University Institutional Animal Care and Use Committee (protocol numbers 40-06-2016, 39-06-2016 and 20-04-2015)

METHOD DETAILS

Pregnancy mouse model

Fecal samples from 8-week old female Swiss Webster mice were randomly assigned to two treatment groups: (a) control-naive (n = 11), and (b) mated (n = 10). Pregnancy was confirmed in the mated group by the appearance of a vaginal plug that defined day E1 of gestation. Fecal samples were collected at days E0 and E18 for 16S rRNA gene sequence analysis and frozen immediately at −80°C.

Progesterone implanted mouse model

Female mice were randomly assigned into one of two groups that were implanted with pellets releasing: (a) 35mg progesterone (n = 7) or (b) placebo pellets containing only matrix without progesterone (n = 9)(Innovative Research of America, Sarasota, FL). Pellets containing progesterone or placebo pellets were implanted subcutaneously in the lateral side of the neck between the ear and the shoulder of the mice and lasted 21 days.

Throughout the experiment, fecal samples were collected on days 0 and 11 for gut microbiota analysis. Plasma levels of progesterone were measured by competitive chemiluminescent immunoassay using IMMULITE 2000 (Diagnostic product corporation). Samples were stored at −80°C prior to the assay.

Fecal microbiota analysis

DNA was extracted from all fecal samples using the PowerSoil DNA extraction kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions following a preliminary step of beadbeating for 2 minutes and elution in 50 μl. Purified DNA was PCR amplified using PrimeSTAR Max (Takara-Clontech, Shiga, Japan) for the variable V4 region (using 515F-806R barcoded primers) of the 16S rRNA gene, as previously described (Caporaso et al., 2012). Amplicons were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA) and subsequently quantified using Quant-It Picogreen dsDNA quantitation kit (Invitrogen, Carlsbad, CA). Equimolar amounts of DNA from individual samples were pooled and sequenced using the Illumina MiSeq platform at the Genomic Center of the Bar-Ilan University, at the Azrieli Faculty of Medicine.

In vitro anaerobic bacterial culture

Fecal matter from a naive adult Swiss Webster female was resuspended in 6 mL PBS in an anaerobic chamber (90% N_2, 5% CO_2, 5% H_2), vortexed for 5 minutes, and allowed to settle by gravity for 5 min. The supernatant was divided into two tubes: 2.5 mL of the supernatant was added to a tube containing Depo Provera (medroxyprogesterone acetate, Pfizer, Belgium) to a final concentration of 80 ng/ml (n = 6), or an equal volume of PBS (n = 5). Suspensions were transferred into anaerobic blood culture bottles enriched with soybean-casein digest broth. Vials were incubated at 37°C. After 11 days, 1 mL of bacterial culture was centrifuged (10,000 RPM for 5 minutes) and bacterial cells were harvested for microbiota composition analysis using the PowerSoil DNA extraction kit (MoBio, Carlsbad, CA).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using PRISM 7.0a. Weight and progesterone values were compared between groups using multiple t tests. All data were expressed as mean ± SEM. Asterisks in figures indicate significance (*$p < 0.05$, **$p < 0.01$, **$p < 0.001$). False discovery rate (FDR) adjusted p values were calculated for multiple comparisons. The number of mice per group is annotated in the figure legends.

Bioinformatics and microbiome analysis

Microbial communities were analyzed using QIIME version 1.8.0 (Caporaso et al., 2010). Paired–end sequences were grouped into operational taxonomic units (OTUs) using the GreenGenes (DeSantis et al., 2006) database, and sequences with a similarity of 97% or greater were grouped into the same OTU. Chimeric sequences were removed, and rarefaction was carried out using 10,000 and 6,200 sequences per sample (for mouse and human pregnancies, respectively), 10,130 sequences per sample (for *in-vitro* studies), and 13,000 sequences per sample (for the mouse pellet experiments). Beta diversity was measured using UniFrac (Lozupone and Knight, 2005). Linear discriminant analysis (LDA) of the effect size (LEfSe) was used to identify significantly differentiating OTUs and genera among groups (Segata et al., 2011).

Calour analysis

For identification of differentially abundant bacteria between the control and progesterone groups, samples were rarified to 10,130 reads/sample, and all OTUs with less than 10 reads total over all the samples were filtered out. Differentially abundant bacteria were identified using the rank-mean test statistic (i.e., difference in the means of the rank transformed frequencies of each OTU) compared to 1,000 random permutations. False discovery rate was controlled using the dsFDR test (Jiang et al., 2017), which is a

permutation-based FDR control algorithm, designed to handle sparse data (such as microbiome abundance tables), with a threshold of 0.1. The resulting 205 OTUs were sorted according to the effect size (mean-rank difference). The differential abundance analysis and heatmap plot were prepared using Calour (Xu et al., 2019).

Normalization

Given the large variation in OTU values, we transformed these values to Z scores by adding a minimal value to each OTU level (0.01) and calculating the 10-basis log of each value. Statistical Whitening was then performed on the table, by removing the average and dividing by the standard deviation of each OTU.

Correlations

ANOVA was performed to find the most significant difference between bacteria in each condition (progesterone/placebo/pregnancy) and only bacteria with a p value less than 0.05 were considered significant (Figure 3C).

Bioinformatic analysis of HSD sequences

We extracted the *Bifidobacterium longum* HSD gene sequence from Ensembl bacteria (Kersey et al., 2018) (ENA|CEF11898.1). This sequence was blasted (Altschul et al., 1990) against all nucleotide collections and while having a high similarity with the *Bifidobacterium longum* complete genome (accession number: NC_004307.2), and the *Bifidobacterium breve* genome, no similarities were found between the HSD gene in the *Bifidobacterium* to this gene in other species, such as human, mouse, or even other Actinobacteria (*Mycobacterium*).

DATA AND SOFTWARE AVAILABILITY

The accession number for the 16S rRNA sequencing data reported in this paper is ENA: PRJEB31104.

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Supplemental Information

Progesterone Increases *Bifidobacterium* Relative Abundance during Late Pregnancy

Meital Nuriel-Ohayon, Hadar Neuman, Oren Ziv, Anna Belogolovski, Yiftah Barsheshet, Naamah Bloch, Atara Uzan, Roey Lahav, Avi Peretz, Sigal Frishman, Moshe Hod, Eran Hadar, Yoram Louzoun, Orly Avni, and Omry Koren

Figure S1

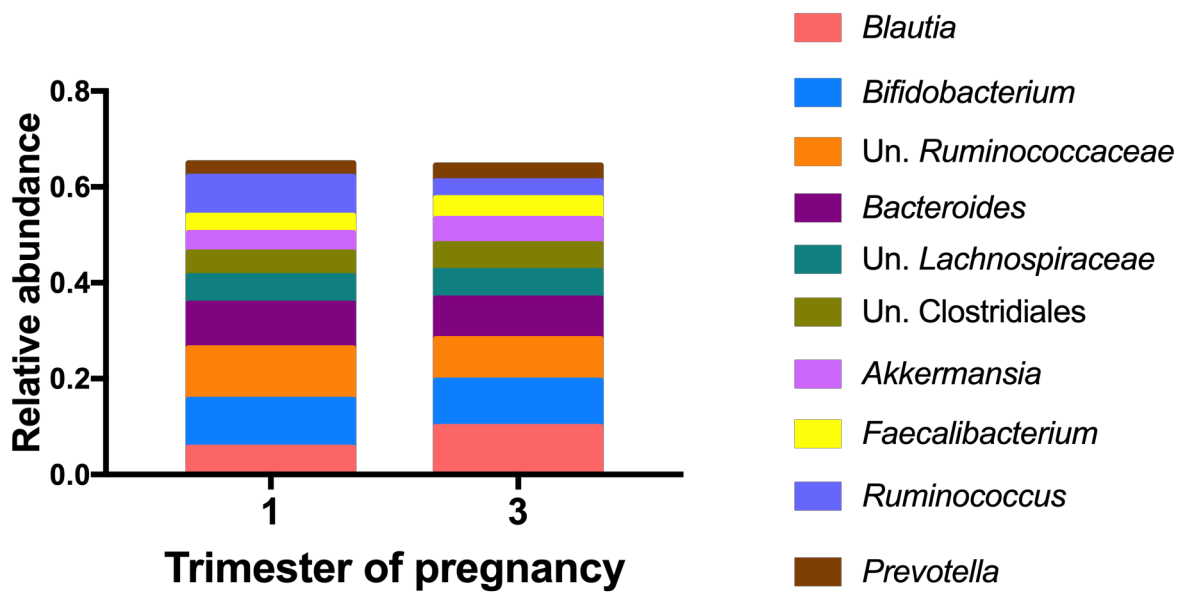

Figure S1. Most abundant gut genera in pregnant women, Related to Figure 1. Taxa plots present the 10 most abundant genera on average of pregnant women (n=35) on the 1st and 3rd trimesters.

Figure S2

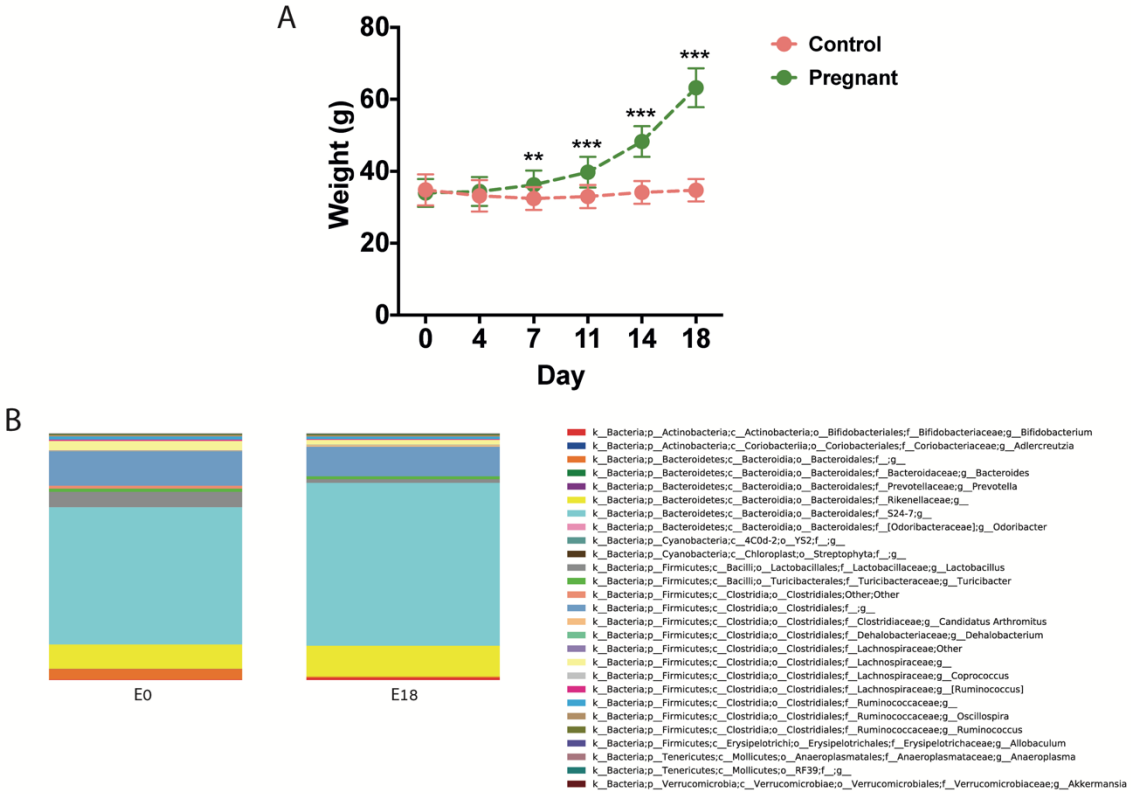

Figure S2. Mice pregnancy is correlated with weight gain and microbiota alterations, Related to Figure 2. **(A)** weight changes in pregnant (green) and age-matched control (red) mice. Data are represented as means ± SEM (n=11 in the control group and n=10 in the pregnancy group) **(B)** Taxa plots presenting most abundant genera of pregnant mice on day E0 and E18.

Figure S3

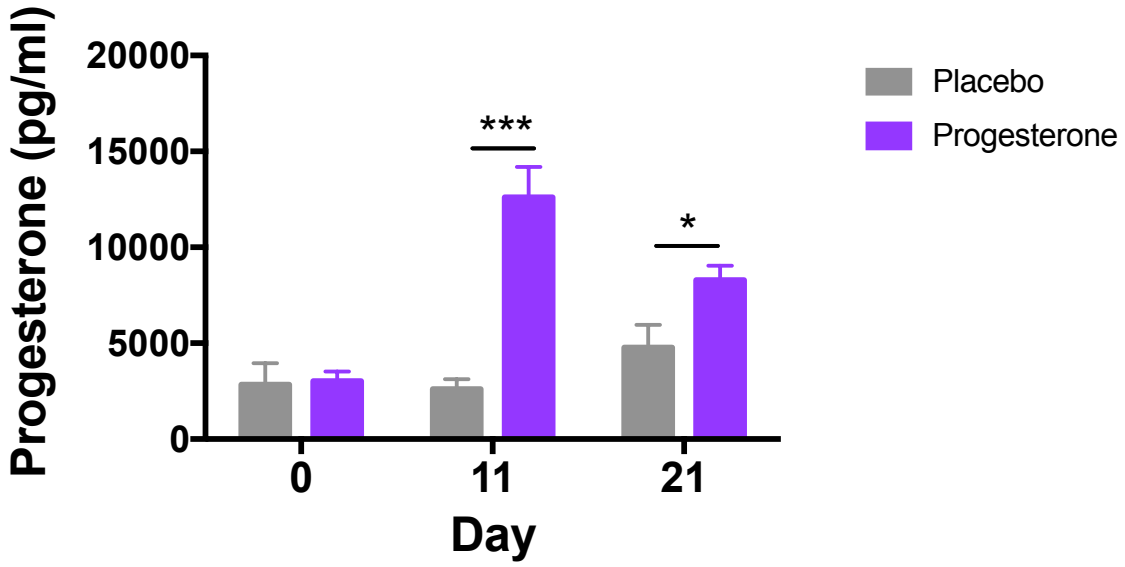

Figure S3. Progesterone levels in mice plasma are elevated due to progesterone pellets, Related to Figure 3. Plasma progesterone levels in mice receiving progesterone pellets (purple) or placebo (gray), as measured on days 0, 11, and 21 of the pellet experiments. Bars represents means ± SEM and asterisks indicate significance (*$p<0.05$, ***$p<0.001$) as determined by two- sided Student's t- test. (n=7 in the progesterone group and n=9 in the placebo group)

Table S1. Characteristics of pregnant women (n=35), Related to Figure 1.

Parameter	Mean	SD
Age (year)	32.7	4.4
BMI	23.4	5.9
Parity	1.05	0.99