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Vaginal microbiota differences associated with pelvic organ prolapse risk during late gestation in commercial sows†

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Abstract

During the last decade, sow mortality due to pelvic organ prolapse (POP) has increased. To better understand the biology associated with POP, sows were phenotypically assessed and assigned a perineal score (PS) based on presumed POP risk and categorized as PS1 (low), PS2 (moderate), or PS3 (high). The study objective was to identify changes in sow vaginal microbiota that may be associated with POP. The hypothesis is that vaginal microbiota differs between sows with variable risk for POP, and changes in microbiota during late gestation exist between sows with differing risk. Of the 2864 sows scored during gestation week 15, 1.0, 2.7, and 23.4% of PS1, PS2, and PS3 sows, respectively, subsequently experienced POP. Vaginal swabs subjected to 16S rRNA gene sequencing revealed differences in community composition (Bray–Curtis; $P < 0.05$) and individual operational taxonomic unit (OTU) comparisons between vaginal microbiota of PS1 and PS3 sows at gestation week 15. Further, differences ($P < 0.05$) in community composition and OTUs ($Q < 0.05$) were observed in PS3 sows that either did or did not subsequently experience POP. Differences in community structure (alpha diversity measurements; $P < 0.05$), composition ($P < 0.05$), and OTUs ($Q < 0.05$) were observed in gestation week 12 sows scored PS1 compared to week 15 sows scored PS1 or PS3, suggesting that sow vaginal microbiota shifts during late gestation differently as POP risk changes. Collectively, these data demonstrate that sows with greater POP risk have unique vaginal microflora, for which a better understanding could aid in the development of mitigation strategies.

Key words: pelvic organ prolapse, vaginal microbiota, reproduction, pig sow.

Introduction

The swine industry has made significant improvements in several areas of production efficiency, including reproductive performance, throughout the past decade. Unfortunately, during this time, sow mortality has increased with a substantial proportion of the amplified mortality due to pelvic organ prolapse (POP). During a 2018 industry survey, it was discovered that approximately 21% of sow deaths in the USA could be attributed to POP [1]. Sows most commonly experience POP during the peripartum period, which is defined as the period leading up to and shortly after farrowing [2]. Commonly referred to as an anatomical disorder, POP is characterized as a condition where one or more of the pelvic organs presses up against or out of the vagina [3]. While this industry-wide problem is both an animal welfare concern and economic issue, there is currently a lack of understanding of the biological causes of POP, preventing the development of effective mitigation strategies.

Microorganisms harbored on surfaces and in cavities of a host typically participate in a symbiotic relationship that can influence host health. Dysbiosis or changes in the microbiota, particularly within the reproductive tract, have been linked to reproductive dysfunction, and alterations may affect susceptibility to gynecological infections [4]. In humans, the vaginal microbiota can be influenced by sexual development, coitus, personal hygiene, menses, and hormones [4, 5]. During pregnancy, the human vaginal microbiota has been observed to increase in stability, suggesting a protective effect against infections [5, 6].

Pelvic inflammatory disease (PID) and bacterial vaginosis (BV) are microbial infections of the reproductive tract that are linked to vaginal microbiota changes in humans [7, 8]. Symptoms of PID and BV include reproductive dysfunction such as discomfort, inflammation, and infertility, among others. Vaginal microbiota dysfunction has also been linked to reproductive diseases in cattle [9, 10]. Unfortunately, as of now, there is limited knowledge on the vaginal microbiota of sows in general, and current studies have not probed discovery in specific relation to late gestation commercial sows [11–14]. Because of this, it is difficult to describe what is considered to be a healthy microbial population or one of dysbiosis. However, previous work demonstrated that sows with different risk for POP had notable changes in the vaginal microbiota [15]. In order to better understand these changes and validate their association with POP risk, the need to further examine vaginal microbiota across additional production systems, genetic lines, and geographical areas exists. Therefore, additional research is warranted to analyze if and how the vaginal microbiota changes during late gestation in sows preceding POP compared to those that do not experience POP. Thus, the objective of this study was to further define sow vaginal microbiota changes in late gestation in relation to POP, as well as evaluate those changes over the weeks prior to parturition. To accomplish this objective, the current study tested the hypothesis that the vaginal microbiota would differ between sows with variable risk of POP during late gestation, and changes in the microbiota during the last 3 weeks of gestation would exist between sows with differing risk for POP.

Materials and methods

Animals

All experiments involving animals were approved by the Iowa State University (ISU) Institutional Animal Care and Use Committee. This work was conducted on two commercial sow farms (designated farm A and farm B). The farms were part of the same production system with alike genetics, feed, and housing type, and were within one mile from each other. Additionally, farms had a similar health status being porcine reproductive and respiratory syndrome naïve, *Mycoplasma hyopneumoniae* stable, porcine epidemic disease naïve, and influenza A virus stable.

Perineal scoring system

Using an already established perineal scoring system to assess risk of POP in late gestation sows [15], 3035 pregnant sows (gestation weeks 12–15), from two different farms, were categorized during late gestation into three perineal score (PS) classes varying from presumed low to high risk for POP. Sows were scored, one time each week, from gestation week 12 through 15 (based on the week of breeding). The current study was performed over the course of seven consecutive weeks, during Spring of 2019, with each farm being scored on subsequent days, but the same day of each week. Sows were scored only while lying, precluding some sows from being scored each week. Additionally, in a few instances, a small number of sows were removed from the herd prior to farrowing. As before [15], sows presumed low risk for POP were assigned PS1, moderate risk assigned PS2, and high risk were designated PS3. Briefly, to assign the PS, the perineal region was visually evaluated for swelling, redness, and protrusion. A sow lacking swelling, redness, and protrusion was assigned a score of PS1 and considered low risk for POP. Sows with moderate swelling, redness, and protrusion of the perineal area were assigned a PS2 assuming moderate risk for POP. Sows assigned PS3 demonstrated all of the characteristics of severe swelling, redness, and protrusion of the perineal area and were considered high risk for POP.

Sample collection

This study is an expansion of prior work investigating differences in blood parameters between PS1 and PS3 scored sows during gestation week 15 [16]. At both farms, vaginal swabs for microbiota DNA extraction were collected during gestation week 15 from all sows classified as PS3 (wk15PS3; $n = 118$) and parity matched PS1 (wk15PS1; $n = 98$). In farm B only, vaginal swabs were collected from a group of sows assigned PS1 at gestation week 12 (wk12PS1; $n = 37$). Vaginal swabs were collected by aseptically inserting a 7-inch histology brush (2199, Puritan Medical Products) into the vagina and brushing the vaginal orifice for approximately 15 seconds. Swabs were removed and immediately placed in sterile 1X phosphate buffered saline and kept on wet ice for approximately 12–24 hours until processed in the lab. Sample processing involved vortexing for 5 minutes to detach cells followed by centrifugation at $5000 \times g$ at room temperature for 15 minutes to form a pellet. Supernatant was then discarded and the pellet was stored at −80°C until they were used for DNA extraction.

DNA extraction

Tubes containing vaginal microbiota pellets were thawed, and DNA was extracted with a Qiagen DNeasy Powerlyzer Powersoil kit (Qiagen, Germantown, MD) per manufacturer's protocol. Mechanical cell lysis was performed using a Fisher Scientific Beadmill 24. The concentration of the isolated DNA was measured using a Qubit 3.0 Fluorometer.

16S rRNA gene sequencing

16S rRNA gene sequences were amplified from the vaginal swab samples of individual sows. Sample DNA was diluted in sterile water to a concentration between 25 and 75 ng/µl then sent to the ISU DNA facility for sequencing using the Illumina MiSeq platform (Illumina, San Diego, CA). Briefly, the genomic DNA from each sample was amplified using Platinum Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA) with one replicate per sample using universal 16S rRNA gene bacterial primers [515F (5′-GTGYCAGCMGCCGCGGTAA-3′; 26) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′; 27)] amplifying the variable region V4, as previously described [17]. All samples underwent PCR with an initial denaturation step at 94°C for 3 minutes, followed by 45 seconds of denaturing at 94°C, 20 seconds of annealing at 50°C, and 90 seconds of extension at 72°C. This was repeated for 35 total PCR cycles and finished with a 10 minute extension at 72°C. All the PCR products were then purified with the QIAquick 96 PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR bar-coded amplicons were mixed at equal molar ratios and used for Illumina MiSeq paired-end sequencing with 250 bp read length and cluster generation with 10% PhiX control DNA.

Sequence analysis

Sequence analysis was performed with mothur V1.43.0 following the mothur MiSeq Standard Operating Procedure [17]. Barcode sequences, primers, and low-quality sequences were trimmed using a minimum average quality score of 35, with a sliding window size of 50 bp. Chimeric sequences were removed with the "Chimera.vsearch" command. For alignment and taxonomic classification of operational taxonomic units (OTUs), the Silva SSU NR reference database (v138) provided by the mothur website was used. Sequences were clustered into OTUs with a cutoff of 99% 16S rRNA gene similarity (=0.01 distance) and were ordered from most to least abundant. Representative sequences for the 50 most abundant OTUs were classified using NCBI BLAST, to improve classification accuracy. The OTU count table and taxonomy assignments were then imported into Phyloseq (v1.34.0 [18]) and Vegan (v2.5-5[19]) for microbial sequence analysis.

Statistical analysis of PS

Effects of PS, Farm, and the interaction between PS and Farm were assessed in SAS 9.4 (Cary, NC) utilizing a PROC MIXED analysis. Data are considered significant if $P \leq 0.05$ and a tendency for biological meaning if $0.05 < P < 0.10$.

Microbial sequence analysis

To compare community structure (alpha diversity measurements) between experimental groups, reads were randomly subsampled to accommodate the sample with the lowest number of reads across data sets (9000 sequences). Measurements of Chao species richness, Shannon Diversity, and Simpson evenness were taken to compare community structures between experimental groups. The means of the experimental group alpha diversity measures were compared using a pooled *t*-test assuming equal variance.

To compare overall microbial community composition, samples were given a Bray–Curtis dissimilarity value and means were then compared using the permutational analysis of variance (PERMANOVA) package built in to Phyloseq. Bray–Curtis dissimilarity was selected because of its ability to compare closely related samples [20].

All plotting was completed using the ggplot2, v2_3.1.1 graphing package [21] in R 4.0.3. Canonical Analysis of Principle Coordinates (CAP) was used to visualize the variation capture by PS, Farm and the interaction between the two.

The absolute abundances of the 100 most abundant OTUs among samples were analyzed using a negative binomial distribution in GLIMMIX procedure of SAS (Version 9.4, SAS Inst., Cary, NC) and were offset by the total library count for a given sample. Using the MULTITEST procedure of SAS, all *P*-values were corrected for false discovery rates. The PROC MIXED procedure of SAS was used to analyze diversity indices. Using Fisher least significant difference test, least squares means were separated, and treatment differences were considered significant if *P* or *Q* values were ≤0.05. For the top 100 OTUs with a *Q* value of ≤0.05, the Log2-fold change (log2FC) between treatment groups were calculated using R and plotted using ggplot2. Three analysis were completed: (1) wk15PS1 sows were compared to wk15PS3, (2) wk15PS3 sows that did experience POP (Yes) compared to wk15PS3 sows that did not experience POP (No), and (3) the subset from farm B at gestation wk12PS1 sows compared to gestation wk15PS1 ($n = 96$) and wk15PS3 ($n = 76$) sows only from farm B, separately.

Additionally, the microbiome sequencing data from Kiefer et al. [15] and this study were combined and processed together in mothur and phyloseq, using the previously mentioned pipeline, to establish shared microbial populations between studies. These studies differ in that they were conducted on different sow farms in different geographical regions and conducted in different years. Shared OTUs between studies were calculated and visualized using the VennDiagram package in R. Overall community structure was visualized using Principal Coordinate Analysis plots generated in phyloseq (mentioned above) and overall community differences were tested using PERMANOVA.

Results

Changes in PS throughout late gestation

On farm observations revealed the earliest time point a sow with a PS3 designation was observable, was approximately gestation week 12. In total, 1563, 2046, 2492, and 2864 sows during gestation weeks 12, 13, 14, and 15, respectively, were scored across both farms. Sows assigned PS1 from gestation week 12 to 15 decreased in incidence from 85.3 to 54.8%, while PS3 assigned sows increased from 0.6 to 4.5% during the same time (Figure 1). Of all sows scored at both farms, PS was influenced ($P < 0.01$) by parity with sows assigned PS1 having an average parity of 1.9 ± 0.1 compared to 3.3 ± 0.2 for PS3 scored sows. However, of sows used for vaginal microbiota sequencing, parity was not different ($P = 0.30$) between wk15PS1 (3.0 ± 0.2) and wk15PS3 (3.3 ± 0.2) as sows were parity matched at the time of sampling. For comparison across gestation weeks 12 and 15, sampling of sows was done only at farm B and only during one breed week for gestation week 12. Within farm B, the parity of wk12PS1 (2.4 ± 0.4) was different ($P < 0.01$) compared to wk15PS3 (3.9 ± 0.2) but was not different ($P = 0.10$) compared to wk15PS1 (3.0 ± 0.2) sows. An insufficient number of sows assigned a PS3 score during week 12 prevented inclusion of wk12PS3 sows in the analysis.

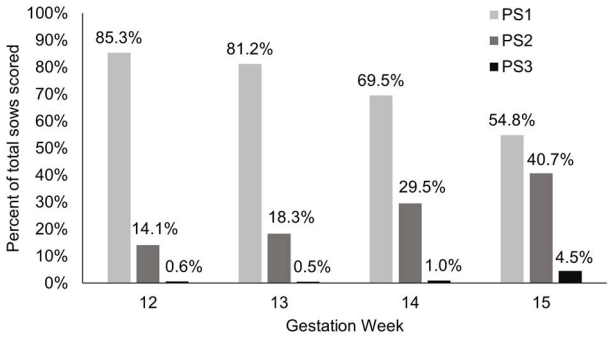

Figure 1. PS changes throughout late gestation. Perineal scoring was conducted on sows during late gestation. Sows assigned a PS1, PS2, and PS3 were presumed low, medium, and high risk, respectively, for POP. A total of 1563, 2046, 2492, and 2864 sows were scored weekly during gestation weeks 12, 13, 14, and 15, respectively. Percentage of sows assigned PS2 or PS3 increased from gestation week 12 to 15 while the percentage of sows assigned PS1 decreased.

Changes in PS during late gestation are associated with differing risk of POP

Of the 2864 sows scored during gestation week 15, 1570, 1166, and 128 were assigned PS1, PS2, and PS3, respectively. As expected, a difference ($P < 0.01$) in POP rates was observed between PS1, PS2, and PS3 assigned sows, with 23.4% of PS3 sows subsequently experiencing POP, while 1.0 and 2.7% of PS1 and PS2 sows, respectively, subsequently experienced POP [16]. With respect to farm influence on POP prevalence, 1.6% of sows scored during week 15 at farm A experienced POP compared to 3.7% of the sows at farm B having the same outcome.

16S rRNA gene amplicon sequencing identified the vaginal microbiota of late gestation sows

A total of 116,112 OTUs were obtained from 261 samples; however, 7216 OTUs remained after quality control and removal of OTUs represented by less than 10 sequences. The average sequencing depth per sample was 21,291 sequences with a standard deviation of 5906 sequences. Bacterial reads made up 99.6% of the reads while 0.34% were archaeal. Twenty-seven and 512 unique phyla and genera, respectively, were represented across the 7216 OTUs used in this dataset. The 50 most abundant vaginal tract OTUs are reported in Table 1.

Similarities in vaginal microbial communities exist between previous and the current study

When comparing the microbial communities of the previous study by Kiefer et al. [15] and the current study, differences ($P = 0.001$) in community composition were detected using PERMANOVA (Figure 2A). In both studies, vaginal swabs were taken at gestation week 15 from PS1 and PS3 sows. Sows across both PS were compared between the initial study [15] and the current. Despite these differences, shared OTUs were observed in both studies with a total of 3227 OTUs present in both studies. Kiefer et al. [15] detected 82 unique OTUs, while the current study detected 4342 unique OTUs (Figure 2B).

Differences in the vaginal microbiota exist between sows at varying risk of POP during week 15 of gestation

When evaluating the average microbial community on a whole-community level at gestation week 15, differences ($P \leq 0.02$) in community composition were detected using PERMANOVA between PS ($R = 0.02$), Farm ($R = 0.03$), and the interaction of PS and Farm ($R = 0.01$) (Figure 3A). Alpha diversity estimators revealed no significant differences in community structure between samples regarding species richness, community evenness, and diversity for the fixed effect of PS (Figure 4A). When evaluating the 100 most abundant OTUs, significant differences for 51, 37, and 3 OTUs between PS, Farm, and the interaction of PS and Farm, respectively, were observed. Of the OTUs that differed due to PS, 18 were more abundant in PS1 sows and 33 were more abundant in PS3 sows (Table 2). Select OTUs of interest include increases ($Q \leq 0.01$) in *Clostridium* (OTU 3, 5, 7, 18, and 50), *Streptococcus* (OTU 4 and 32), and *Treponema* (OTU 47) in the PS3 sows. *Staphylococcus agnetis* (OTU 57) had the highest log2 fold change (log2FC) in microbes that were more abundant in PS3 sows. Farm also had an effect ($Q \leq 0.05$) on the microbiota with 14 OTUs more abundant on farm B and 23 more abundant on farm A (data not shown). When evaluating the interaction of PS and Farm, OTU 25, 39, and 85 were different ($Q \leq 0.03$, dat a not shown).

For sows with increased risk of POP, those experiencing POP have differences in vaginal microbiota compared to those that did not

When evaluating the vaginal microbiota of PS3 sows at gestation week 15 that did and did not subsequently experience POP, differences were observed ($P < 0.01$) in community composition based on POP outcome ($R = 0.02$) and Farm ($R = 0.04$), but no interaction ($R < 0.01$) was detected (Figure 3B). Alpha diversity estimators revealed no significant differences in community structure between samples regarding species richness, community evenness, and diversity (Figure 4B). When evaluating the 100 most abundant OTUs, only 2 OTUs affiliated to the genera *Actinobacillus* (OTU 1) and *Veillonella* (OTU 12) were observed to be increased ($Q \leq 0.01$) in sows not experiencing POP compared to those that did (Table 3).

The vaginal microbiota community differs between sows at low and high risk for POP during gestation week 15 compared to sows at low risk for POP at gestation week 12

In effort to understand the vaginal microbiota change over time, a subset of sows from farm B were sampled at 12 and 15 weeks of gestation. Microbial communities from wk12PS1 sows compared to wk15PS1 and wk15PS3 sows were evaluated within farm B only. Differences were observed ($P < 0.01$, $R = 0.05$) in community composition between wk12PS1 and wk15PS1 as well as between wk12PS1 and wk15PS3 based on CAP (Figure 3C). Alpha diversity estimators revealed differences ($P \leq 0.03$) in community structure between samples regarding species richness, community evenness, and diversity (Figure 4C). Shannon and Simpson indices revealed wk12PS1 sows tended to be different ($P < 0.06$) from wk15PS1 sows and were different ($P = 0.01$) from wk15PS3 sows.

Table 1. The 50 most abundant microbes in vaginal samples from late gestation sows.

				NCBI BLAST		
OTU[1]	Relative abundance[2] (%)	Phylum	Taxonomy (Silva v138)[3]	Classification	Similarity (%)	Accession no.
OTU 1	6.62	*Proteobacteria*	*Actinobacillus*	*Actinobacillus porcinus* strain 35NTS	99.6	FJ437063.1
OTU 2	5.83	*Firmicutes*	*Turicibacter*	*Turicibacter sanguinis* strain MOL361	99.6	CP053187.1
OTU 3	4.90	*Firmicutes*	*Clostridium_sensu_stricto_1*	*Clostridium cellulovorans* strain 22rA	98.4	KF528156.1
OTU 4	4.74	*Firmicutes*	*Streptococcus*	*Streptococcus dysgalactiae* subsp. *equisimilis* strain TPCH-A88	99.6	CP053074.1
OTU 5	4.38	*Firmicutes*	*Clostridium_sensu_stricto_1*	*Clostridium moniliforme* strain 2055	99.2	NR_104892.1
OTU 6	4.27	*Firmicutes*	*Romboutsia*	*Romboutsia timonensis* strain DR1	97.6	NR_144740.1
OTU 7	3.08	*Firmicutes*	*Clostridium_sensu_stricto_1*	*Clostridium chauvoei* strain SBP	98.8	CP027286.1
OTU 8	2.84	*Firmicutes*	*Anaerococcus*	*Anaerococcus prevotii* DSM 20548	99.6	CP001708.1
OTU 9	2.41	*Firmicutes*	*Terrisporobacter*	*Terrisporobacter petrolearius* strain LAM0A37	99.2	NR_137408.1
OTU 10	2.14	*Actinobacteriota*	*Corynebacterium*	*Corynebacterium xerosis* strain GS	100	CP032788.1
OTU 11	1.41	*Fusobacteriota*	*Fusobacterium*	*Fusobacterium gastrosuis* strain CDWK1	99.6	NR_146837.2
OTU 12	1.27	*Firmicutes*	*Veillonella*	*Veillonella caviae* strain PV1	99.2	NR_025762.1
OTU 13	1.17	*Firmicutes*	*Streptococcus*	*Streptococcus suis strain* SS-CLA1926B	99.6	MT256098.1
OTU 14	1.14	*Firmicutes*	*Terrisporobacter*	*Terrisporobacter glycolicus* strain RD-1	98.8	MN733184.1
OTU 15	1.05	*Firmicutes*	*Finegoldia*	*Finegoldia magna* strain FDAARGOS	99.2	CP054000.1
OTU 16	1.04	*Firmicutes*	*Nosocomiicoccus*	*Staphylococcaceae* bacterium NML 99-ST-011	100	AY841364.1
OTU 17	1.04	*Firmicutes*	*Parvimonas*	*Parvimonas micra* strain KCOM 2339	98.4	MT982357.1
OTU 18	1.00	*Firmicutes*	*Clostridium_sensu_stricto_1*	*Clostridium butyricum* strain 5467	100	MT510294.1
OTU 19	0.88	*Firmicutes*	*Peptostreptococcus*	*Peptostreptococcus* sp. DSM 106284	100	MN537513.1
OTU 20	0.87	*Firmicutes*	*Kurthia*	*Kurthia gibsonii* strain EMB4	99.6	KY048434.1
OTU 21	0.81	*Bacteroidota*	*Bacteroides*	*Bacteroides massiliensis* V081	96.4	LC515611.1
OTU 22	0.80	*Euryarchaeota*	*Methanobrevibacter*	*Methanobrevibacter* sp. YE315	100	CP010834.1
OTU 23	0.78	*Bacteroidota*	*Porphyromonas*	*Porphyromonas levii* DSM 23370	90.5	NR_113089.1
OTU 24	0.77	*Firmicutes*	*Staphylococcus*	*Staphylococcus simulans* strain D14	99.6	MT568571.1
OTU 25	0.75	*Firmicutes*	*Gallicola*	*Peptoniphilaceae* bacterium SIT14	97.6	LN870299.1
OTU 26	0.74	*Actinobacteriota*	*Corynebacterium*	*Corynebacterium maris* strain Coryn-1	99.6	NR_121700.2

Continued

Table 1. Continued

OTU[1]	Relative abundance[2] (%)	Phylum	Taxonomy (Silva v138)[3]	NCBI BLAST		
				Classification	Similarity (%)	Accession no.
OTU 27	0.71	*Firmicutes*	*Anaerococcus*	*Anaerococcus prevotii* DSM 20548	98.4	CP001708.1
OTU 28	0.67	*Bacteroidota*	*Porphyromonas*	*Porphyromonas somerae* strain KA00683	99.2	KP192301.1
OTU 29	0.65	*Proteobacteria*	*Escherichia-Shigella*	*Escherichia fergusonii* strain SPK	99.6	MW624513.1
OTU 30	0.59	*Firmicutes*	*Facklamia*	*Facklamia tabacinasalis* strain GF112B	99.6	NR_026482.1
OTU 31	0.59	*Proteobacteria*	*Actinobacillus*	*Actinobacillus rossii* strain JF2167	99.6	AY465365.1
OTU 32	0.58	*Firmicutes*	*Streptococcus*	*Streptococcus pasteurianus* strain 2323	99.6	MT604782.1
OTU 33	0.57	*Firmicutes*	*Ezakiella*	*Bacteroides coagulans* strain EUH 581–73	94.5	NR_104900.1
OTU 34	0.55	*Firmicutes*	*Anaerococcus*	*Anaerococcus* sp. strain AGMB00486	99.6	MT568623.1
OTU 35	0.49	*Actinobacteriota*	*Corynebacterium*	*Corynebacterium phoceense* strain JZ R-177	99.6	MH119724.1
OTU 36	0.47	*Firmicutes*	*Lactobacillus*	*Lactobacillus amylovorus* strain 5081	99.6	MT459395.1
OTU 37	0.47	*Bacteroidota*	*Porphyromonas*	*Porphyromonas endodontalis*	96.1	LT680662.1
OTU 38	0.46	*Actinobacteriota*	*Corynebacterium*	*Corynebacterium stationis* strain VRD1 333 N7	99.6	MN840614.1
OTU 39	0.46	*Firmicutes*	*Clostridium_sensu_stricto_1*	*Clostridium perfringens* strain 3116	99.6	MT613499.1
OTU 40	0.45	*Firmicutes*	*Peptococcus*	*Peptococcus simiae* strain M108	99.6	NR_153710.1
OTU 41	0.45	*Firmicutes*	*Peptoniphilus*	*Peptoniphilus* sp. strain AGMB00490	99.6	MT396160.1
OTU 42	0.40	*Actinobacteriota*	*Corynebacterium*	*Corynebacterium amycolatum* strain 1MR	99.6	MT423433.1
OTU 43	0.40	*Bacteroidota*	*Prevotellaceae_UCG-001*	*Duncaniella freteri* strain TLL-A3	84.6	NR_170509.1
OTU 44	0.38	*Firmicutes*	*Staphylococcaceae_unclassified*	*Jeotgalicoccus schoeneichii* strain 140805-STR-02	99.6	NR_151981.1
OTU 45	0.38	*Firmicutes*	*Peptoniphilus*	*Peptoniphilus* sp. 1804121828	99.6	MK945758.1
OTU 46	0.37	*Actinobacteriota*	*Corynebacterium*	*Corynebacterium pollutisoli* strain VDS11	99.6	NR_151947.1
OTU 47	0.37	*Spirochaetota*	*Treponema*	*Treponema bryantii*	99.6	AB849328.1
OTU 48	0.37	*Firmicutes*	*Streptococcus*	*Streptococcus hyovaginalis* strain TRG26	99.6	MH329638.1
OTU 49	0.35	*Firmicutes*	*Peptoniphilus*	*Peptoniphilus harei* strain DCW_SL_25A	99.6	MK424030.1
OTU 50	0.35	*Firmicutes*	*Clostridium_sensu_stricto_1*	*C. cellulovorans* strain 22rA	99.6	KF528156.1

[1]Individual microbes were assigned in order of abundance and classified into an operational taxonomic unit (OTU).
[2]Relative abundance of the specific OTU in the vaginal swabs collected in this study.
[3]Taxonomy was assigned using Silva SSU NR reference database (v138).

When evaluating the top 100 OTUs, a total of 20 OTUs were different ($Q < 0.05$, $P < 0.05$) between wk12PS1 and wk15PS1 sows, of which 10 were more abundant in wk12PS1 and 10 more abundant in wk15PS1 (Table 4). *Streptococcus* (OTU 4 and 32), *Porphyromonas* (OTU 23 and 51), *Staphylococcus* (OTU 24), *Gallicola* (OTU 25 and 98), *Ezakiella* (OTU 33), *Peptococcus* (OTU 40), and *Peptoniphilus* (OTU 45) were observed to be higher in abundance in wk12PS1 sows compared to wk15PS1. Conversely, *Porphyromonas* (OTU 28 and 84), *Staphylococcus* (OTU 57), *Anaerococcus* (OTU 61 and 70), *Enterococcus* (OTU 63), *Campylobacter* (OTU 76),

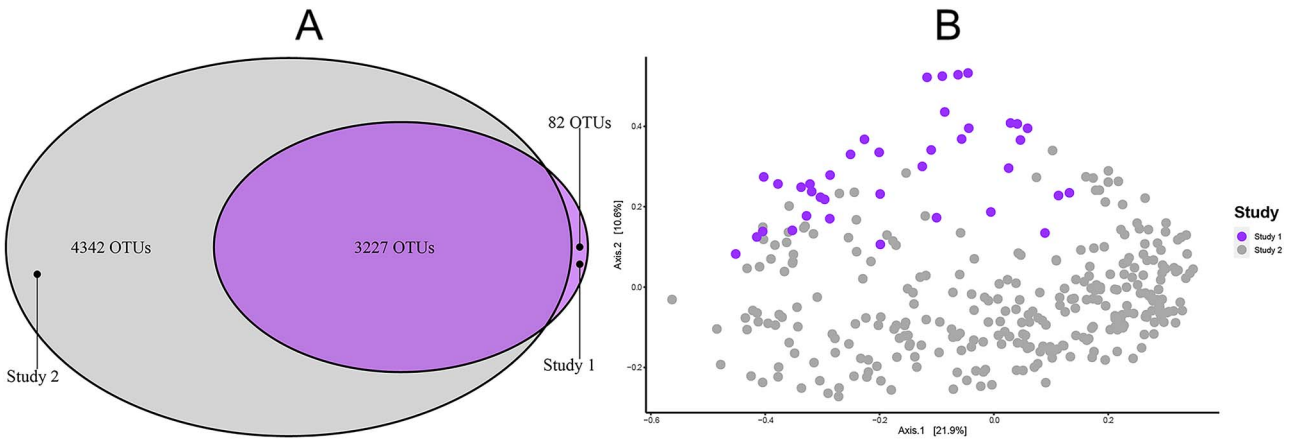

Figure 2. Microbial community comparison between two different studies analyzing the vaginal microbiota of sows in relation to pelvic organ prolapse. (A) The similarities and differences between the initial study [15] (Study 1, purple) and the current study (Study 2, gray) is illustrated by a Venn diagram. A total of 3227 operational taxonomic units (OTUs) were shared across both studies. There were 82 OTUs unique to the initial study [15], and 4342 unique to the current study. (B) The PCOA displays the differences in beta diversity of the vaginal microbial communities of sows from the two different studies. Each point is an individual sow's vaginal microbial population with the vaginal microbial communities of sows from the initial study [15] denoted in purple (Study 1), while sows from the current study are represented in gray (Study 2). All points represent Bray–Curtis dissimilarity measures for each sample.

Lachnospiraceae (OTU 78), *Trueperella* (OTU 89), and *Corynebacterium* (OTU 94) were all higher in abundance in wk15PS1 sows.

A total of 40 OTUs were different ($Q < 0.05$, $P < 0.05$) between wk12PS1 and wk15PS3 sows, when evaluating the most abundant 100 OTUs. Of these, 23 OTUs were more abundant in wk12PS1 sows and 17 OTUs more abundant in wk15PS3 (Table 5). *Streptococcus* (OTU 4, 13, 32, and 48), *Clostridium* (OTU 5), *Romboutsia* (OTU 6), *Corynebacterium* (OTU 10 and 53), *Veillonella* (OTU 12), *Finegoldia* (OTU 15), *Methanobrevibacter* (OTU 22), *Porphyromonas* (OTU 23 and 51), *Staphylococcus* (OTU 24), *Gallicola* (OTU 25), *Escherichia* (OTU 29), *Ezakiella* (OTU 33), *Peptococcus* (OTU 40), *Peptoniphilus* (OTU 41 and 45), and *Anaerococcus* (OTU 54, 61, and 70) were more abundant in wk12PS1 compared to wk15PS3 sows. In comparison the vaginal microbiota of wk15PS3 sows had a higher abundance of *Kurthia* (OTU 20), *Porphyromonas* (OTU 28 and 84), *Anaerococcus* (OTU 34), *Corynebacterium* (OTU 38, 74, and 94), *Staphylococcus* (OTU 57), *Enterococcus* (OTU 63), *Facklamia* (OTU 72), *Campylobacter* (OTU 76 and 100), *Lachnospiraceae* (OTU 78), *Peptostreptococcus* (OTU 81), *Trueperella* (OTU 89), *Peptoniphilus* (OTU 96), and *Gallicola* (OTU 98) compared to wk12PS1 sows.

Discussion

The U.S. swine industry has experienced marked improvements in efficiency within the last decade; however, an increasing mortality rate, of which 21% is due to POP [1], is a major animal welfare and productivity concern. The POP prevalence in sows is higher than reported for other livestock species and the biological underpinnings of why this may be occurring in commercial sow farms remain unclear. Developing successful mitigation strategies requires a better understanding of the biological events preceding POP. This study tested the hypothesis that the vaginal microbiota differs between sows with variable risk for POP during late gestation and that microbial population shifts during the last 3 weeks of gestation were different between sows acquiring opposing risk levels for POP as sows completed gestation. Understanding the vaginal microbiota and its relation to animal health and reproduction is an emerging field in swine physiology [11–15].

In general, the limited knowledge of the vaginal microbiota in sows, particularly during late gestation, in commercial production systems makes it difficult to describe what is considered a normal and/or healthy microbial population. Thus, one objective of the current study was to evaluate if findings from previous work could be validated [15]. This study differs from Kiefer et al. [15] in that the two studies were conducted in different years, in different geographical locations, with different genetic lines of sows, management, and health statuses. Nonetheless, similar observations were detected in assessing the microbial differences in sows differing in PS across the two studies thereby validating previous results [15]. When comparing the shared OTUs, 3227 OTUs were present in both studies. Of these, *Actinobacillus porcinus*, *Duncaniella*, *Porphyromonas somerae*, *Treponema bryantii*, and *Veillonella caviae* were of interest due to the similarity of changes in relation to POP risk across both studies.

Identifying OTUs with similar representation across both studies may provide novel description of the core vaginal microbiota of swine and more specifically the relationship between the vaginal microbiota with pregnancy and POP risk. While these studies expand the current characterization of the sow vaginal microbiome, a greater understanding is needed to strengthen associations with specific reproductive outcomes in sows.

In this dataset, no significant difference in the overall microbial community structure was detected between PS1 and PS3 animals at week 15 nor between animals that subsequently did or did not experience POP. However, wk12PS1 animals had significantly higher community evenness and diversity when compared to wk15PS3 animals. Dramatic changes in alpha diversity measurements such as Chao species richness and Simpson Evenness have been linked to infection of a single pathogen [22, 23] or dysbiosis of the microbial community [4]. This suggests that POP may not be influenced by infection from a single organism but by a multitude of organisms or factors not detected using these tools. However, community composition differences were detected using PERMANOVA on Bray–Curtis dissimilarity measurements, which accounts for both

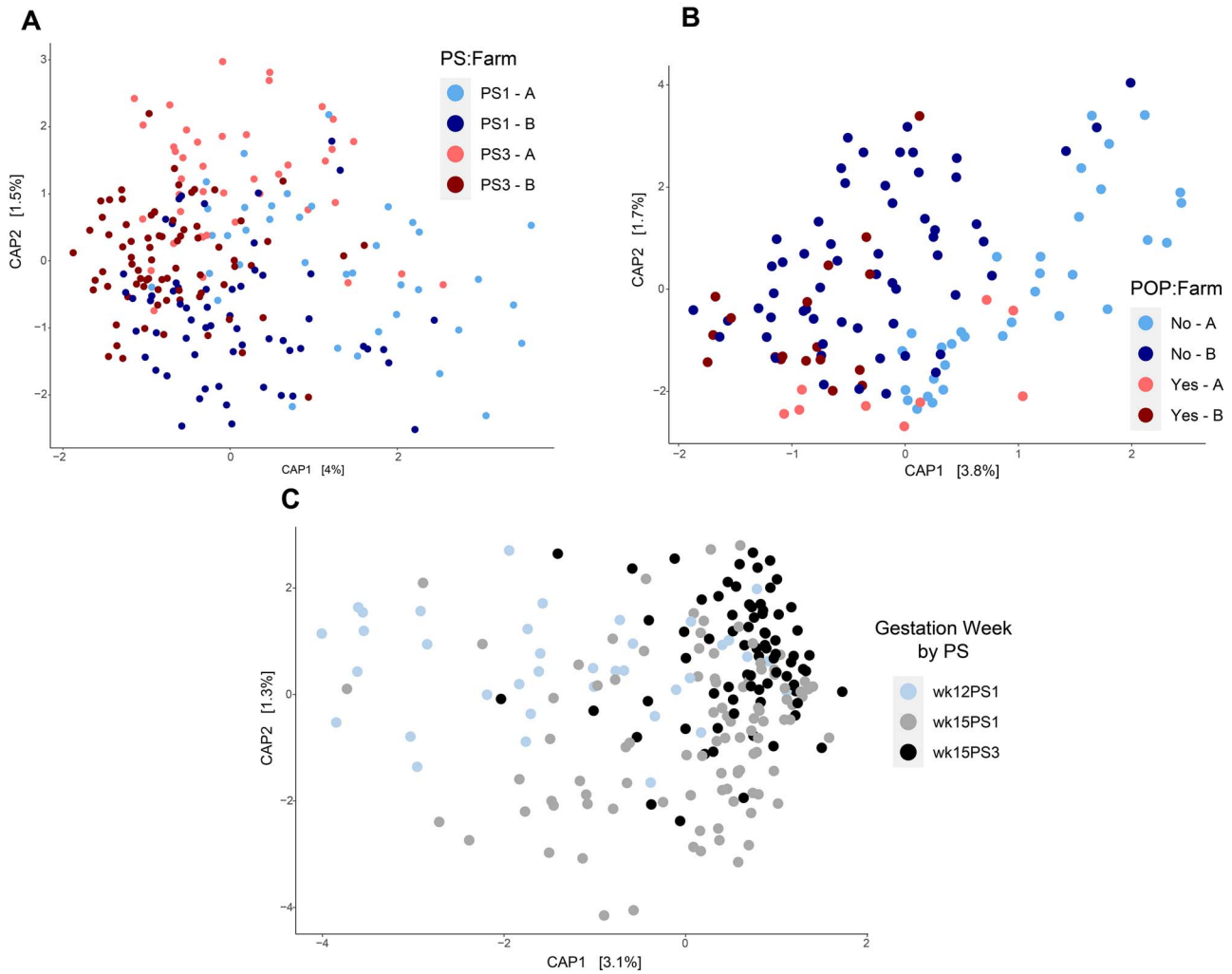

Figure 3. Vaginal microbial community comparisons. CAP showing the maximum variation of beta-diversity between vaginal microbiota communities. (A) CAP from sows with assumed low (PS1, $n = 101$) or high (PS3, $n = 121$) risk for POP during gestation week 15 (days 108–115) from two separate farms. Statistical differences ($P < 0.05$) were detected in overall microbial communities between PS, Farm, and the interaction of PS and Farm. (B) CAP from a subset of sows that subsequently did (Yes; $n = 28$) or did not (No; $n = 93$) experienced POP that also were scored PS3. Statistical differences ($P < 0.05$) were detected in overall microbial communities between POP outcome and Farm. (C) CAP using sows from Farm B only, at gestation week 12 scored PS1 (wk12PS1, $n = 39$), gestation week 15 PS1 (wk15PS1, $n = 61$), and gestation week 15 PS3 (wk15PS3, $n = 78$). Statistical differences ($P < 0.05$) were detected in overall microbial communities between gestation week, score, and the interaction of week and score. All sample points were based on Bray–Curtis dissimilarities of the overall composition of microbial communities.

presence/absence of species and their overall abundance, between both PS and gestation week comparisons. This significant difference signifies distinctions in bacterial members and the abundance between experimental groups, which prompted further investigation on an individual taxonomic level to identify which members of the community differed between groups.

When evaluating specific OTUs within the microbial community structure between sows at differing risk for POP, there were distinct differences. A number of *Peptoniphilus* OTUs were more abundant in PS1 scored sows within the current study. Although the function of *Peptoniphilus* in the vaginal microbiota is currently unknown, a higher abundance of *Peptoniphilus* was observed in the vaginal microbiota of thermoneutral pregnant sows compared to those experiencing heat stress [12], demonstrating its prior detection in sows and differential abundance in response to environmental changes. However, *Peptoniphilus* (OTU 96) was increased in the wk15PS3 sows compared to the wk12PS1 sows, which is contrary to the majority of *Peptoniphilus* OTUs being increased in PS1 sows. It is important to make distinctions within this genus because certain species may have positive or negative associations to reproductive function in swine.

A higher abundance of several *Porphyromonas* OTUs including *P. somerae* was noted in PS1 sows in this study confirming discoveries from previous work [15], suggesting the plausibility that some *Porphyromonas* members could have a beneficial influence on the vaginal microbiota in late gestation sows. However, increases in *P. somerae* can have a pathogenic effect in humans [24] and have been linked to uterine disease in cattle [25]. It is known that microbial populations and abundance can be altered depending on the host species and could also function differently within different host species as well as across different host tissues within a species [26]. *Porphyromonas* is an organism of interest because of its higher

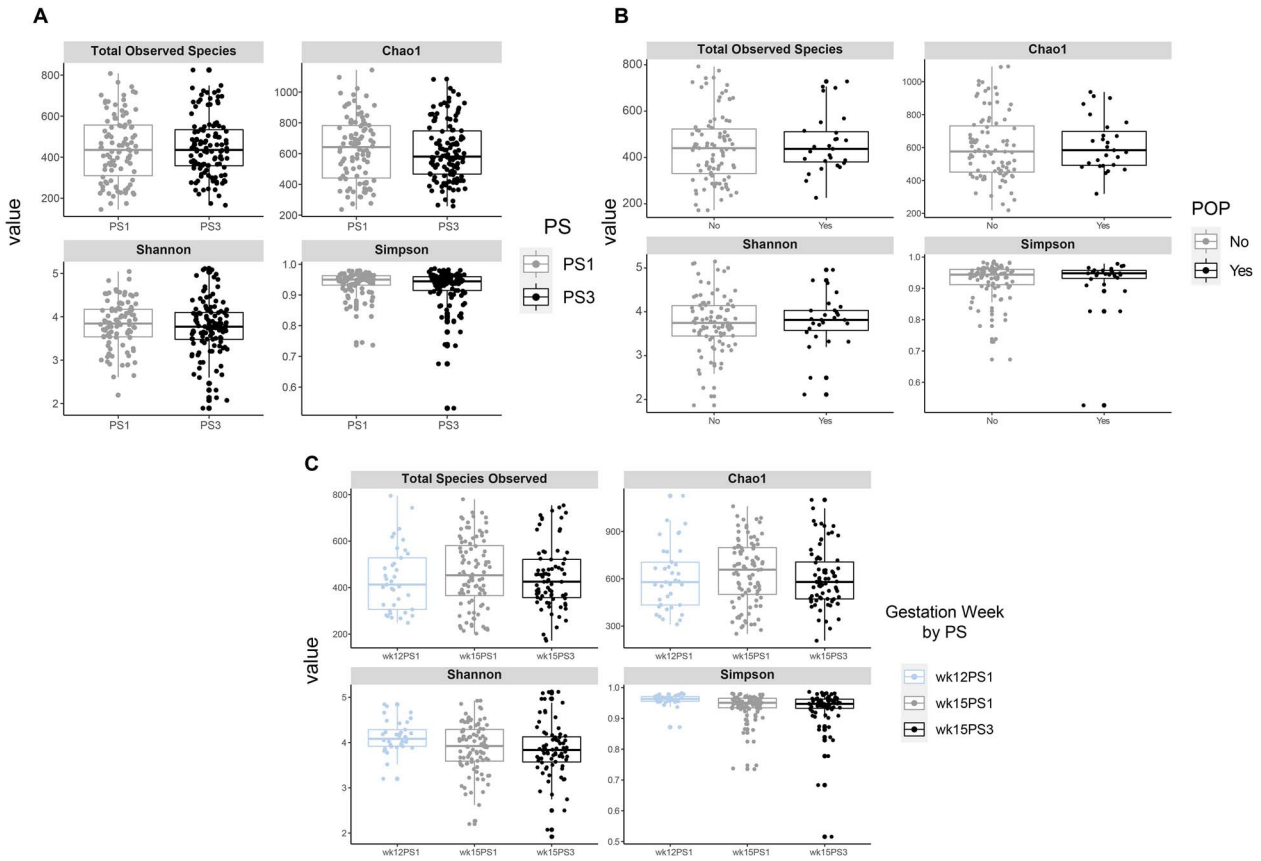

Figure 4. Alpha diversity of the vaginal microbiota for sows during late gestation. The above graphics compare alpha diversity measurements species evenness (Simpson), richness (TSO, Chao1), and diversity (Shannon) across the different variables within this study. (A) Alpha diversity measurements for sow vaginal microbiota at low (PS1; gray points, $n = 101$) or high (PS3; black points, $n = 121$) risk for POP at gestation week 15 (days 108–115). (B) Alpha diversity measurements for sows that subsequently did (Yes; black points, $n = 28$) or did not (No; gray points, $n = 93$) experience POP that also were scored PS3. (C) Alpha diversity measurements for sows from farm B at gestation week 12 scored PS1 (wk12PS1; light blue points, $n = 39$), gestation week 15 PS1 (wk15PS1; gray points, $n = 61$), and gestation week 15 PS3 (wk15PS3; black points, $n = 78$). Shannon and Simpson diversity were observed to be significantly different ($P \leq 0.03$) with differences between week 12 sows and both week 15 PS1 and PS3 sows.

abundance in sows at low risk for POP compared to high risk; however, having been described as pathogenic in other species indicates that further investigation into its function within the swine reproductive tract during late gestation is warranted.

Several *Anaerococcus* OTUs were also observed to be greater in the PS1 sows compared to those at high risk for POP (PS3). Interestingly, *Anaerococcus* was more abundant in the vagina of thermoneutral pregnant sows compared to sows exposed to heat stress, suggesting that thermal stress can alter specific vagina microbiota [12]. *Anaerococcus* has also been identified in swine at other stages of production as well, being increased in the fecal microbiota of weaned piglets that tended to be heavier than their counterparts [27]. Observations of the *Anaerococcus* differences within the vaginal microbiota in sows with low risk for POP (PS1) are conflicting with studies conducted in humans in relation to PID and BV, emphasizing the need for better defining this microbe in sows and its function with respect to reproductive health. Again, this may be a genus for which distinct species have opposing effects on POP risk and should be explored further.

While 20 OTUs were observed to be increased in low-risk sows (PS1), 33 OTUs were in greater abundance in high-risk sows (PS3). Among those, several OTUs of the genus *Corynebacterium* were found to be higher in PS3 sows. The function of *Corynebacterium* in the pig vaginal microbiota is, however, currently unexplored and warrants future investigation. By comparison however, *Corynebacterium* has been observed to have a negative effect on fertility in cattle [28] and has been observed in higher abundance in cattle with uterine infections [29] and those suffering from endometritis [30]. In addition, several OTUs of *Clostridium* cluster I were detected to be more abundant in PS3 scored sows. Interestingly, while both the *Corynebacterium* and *Clostridium* cluster I OTUs were detected in high abundance previously, they were not significantly different with respect to POP risk [15]. However, *Clostridium* cluster I has been demonstrated to be more abundant in the vaginal microbiota of sows with endometritis [11], suggesting a possible negative role of *Clostridium* cluster I with pig reproductive health.

Increases in *Duncaniella*, *Streptococcus dysgalactiae*, and *T. bryantii* were observed in sows assigned PS3 in the current study, which is consistent with the initial characterization of sows with elevated risk for POP [15]. *Duncaniella* is a genus within the Prevotellaceae family and Prevotellaceae have been detected to be increased in the fecal microbiota of women suffering from

Table 2. Differences in OTUs between vaginal microbiota of PS1 and PS3 sows during gestation week 15.

		NCBI BLAST				
OTU[1]	Taxonomy (Silva v138)[2]	Classification	Similarity (%)	PS[3]	Log2FC[4]	*Q*-value
OTU 2	*Turicibacter*	*Turicibacter sanguinis* strain MOL361	99.6	PS3	0.32	<0.01
OTU 3	*Clostridium_sensu_stricto_1*	*Clostridium cellulovorans* strain 22rA	98.4	PS3	0.31	<0.01
OTU 4	*Streptococcus*	*Streptococcus dysgalactiae* subsp. *equisimilis* strain TPCH-A88	99.6	PS3	0.34	<0.01
OTU 5	*Clostridium_sensu_stricto_1*	*Clostridium moniliforme* strain 2055	99.2	PS3	0.36	<0.01
OTU 6	*Romboutsia*	*Romboutsia timonensis* strain DR1	97.6	PS3	0.32	<0.01
OTU 7	*Clostridium_sensu_stricto_1*	*Clostridium chauvoei* strain SBP	98.8	PS3	0.31	<0.01
OTU 9	*Terrisporobacter*	*Terrisporobacter petrolearius* strain LAM0A37	99.2	PS3	0.30	<0.01
OTU 10	*Corynebacterium*	*Corynebacterium xerosis* strain GS	100	PS3	0.72	<0.01
OTU 14	*Terrisporobacter*	*Terrisporobacter glycolicus* strain RD-1	98.8	PS3	0.32	<0.01
OTU 16	*Nosocomiicoccus*	*Staphylococcaceae* bacterium NML 99-ST-011	100	PS3	0.42	<0.01
OTU 18	*Clostridium_sensu_stricto_1*	*Clostridium butyricum* strain 5467	100	PS3	0.34	<0.01
OTU 20	*Kurthia*	*Kurthia gibsonii* strain EMB4	99.6	PS3	0.35	0.01
OTU 22	*Methanobrevibacter*	*Methanobrevibacter* sp. YE315	100	PS3	0.38	<0.01
OTU 23	*Porphyromonas*	*Porphyromonas levii* DSM 23370	90.5	PS1	1.21	<0.01
OTU 26	*Corynebacterium*	*Corynebacterium maris* strain Coryn-1	99.6	PS3	0.57	<0.01
OTU 27	*Anaerococcus*	*Anaerococcus prevotii* DSM 20548	98.4	PS3	0.57	<0.01
OTU 28	*Porphyromonas*	*Porphyromonas somerae* strain KA00683	99.2	PS3	2.07	<0.01
OTU 30	*Facklamia*	*Facklamia tabacinasalis* strain GF112B	99.6	PS3	0.32	0.02
OTU 32	*Streptococcus*	*Streptococcus pasteurianus* strain 2323	99.6	PS3	0.36	<0.01
OTU 33	*Ezakiella*	*Bacteroides coagulans* strain EUH 581–73	94.5	PS1	2.10	<0.01
OTU 34	*Anaerococcus*	*Anaerococcus* sp. strain AGMB00486	99.6	PS1	1.09	<0.01
OTU 35	*Corynebacterium*	*Corynebacterium phoceense* strain JZ R-177	99.6	PS3	0.49	0.02
OTU 40	*Peptococcus*	*Peptococcus simiae* strain M108	99.6	PS1	1.66	<0.01
OTU 41	*Peptoniphilus*	*Peptoniphilus* sp. strain AGMB00490	99.6	PS1	1.89	<0.01
OTU 43	*Prevotellaceae_UCG-001*	*Duncaniella freteri* strain TLL-A3	84.6	PS3	0.45	0.01
OTU 44	*Staphylococcaceae_unclassified*	*Jeotgalicoccus schoeneichii* strain 140805-STR-02	99.6	PS3	0.42	0.01
OTU 45	*Peptoniphilus*	*Peptoniphilus* sp. 1804121828	99.6	PS1	0.71	<0.01
OTU 46	*Corynebacterium*	*Corynebacterium pollutisoli* strain VDS11	99.6	PS3	0.58	0.01
OTU 47	*Treponema*	*Treponema bryantii*	99.6	PS3	0.61	<0.01
OTU 50	*Clostridium_sensu_stricto_1*	*C. cellulovorans* strain 22rA	99.6	PS3	0.34	<0.01

Continued

Table 2. Continued

		NCBI BLAST				
OTU[1]	Taxonomy (Silva v138)[2]	Classification	Similarity (%)	PS[3]	Log2FC[4]	*Q*-value
OTU 51	*Porphyromonas*	*Porphyromonas canoris* strain JCM 16132	92.5	PS1	2.36	<0.01
OTU 53	*Corynebacterium*	*Corynebacterium callunae* strain AS67	99.2	PS3	0.49	0.02
OTU 54	*Anaerococcus*	*Anaerococcus* sp. Marseille-P3915	98.4	PS1	2.45	<0.01
OTU 55	*Corynebacterium*	*Corynebacterium urealyticum* strain 2431	99.6	PS3	0.72	<0.01
OTU 57	*Staphylococcus*	*Staphylococcus agnetis* strain PR5962A	99.6	PS3	2.81	<0.01
OTU 60	*Atopostipes*	*Atopostipes* sp. strain ZH16	99.6	PS3	0.40	0.01
OTU 61	*Anaerococcus*	*Anaerococcus nagyae* strain ENR0686	94.8	PS1	1.21	<0.01
OTU 62	*Lachnospiraceae_UCG-007*	*Lachnotalea glycerini* strain DLD10	96.8	PS3	0.33	0.01
OTU 66	*Bifidobacterium*	*Bifidobacterium pseudolongum* A1	100	PS3	0.49	<0.01
OTU 70	*Anaerococcus*	*Anaerococcus* sp. Marseille-P3915	99.6	PS1	2.13	<0.01
OTU 71	*Lactobacillus*	*Lactobacillus vaginalis* strain 17465	99.6	PS3	0.41	< 0.01
OTU 72	*Facklamia*	*Facklamia hominis* strain DNF00119	98.4	PS1	1.19	<0.01
OTU 73	*Firmicutes_unclassified*	*Clostridium oryzae* strain KC3	90.1	PS3	0.47	<0.01
OTU 74	*Corynebacterium*	*Corynebacterium glucuronolyticum* strain V17 2011556	99.6	PS1	1.78	<0.01
OTU 76	*Campylobacter*	*Campylobacter ureolyticus* strain LMG 6451	96.5	PS1	2.46	0.01
OTU 81	*Peptostreptococcus*	*Peptostreptococcus anaerobius* strain WH7	91.3	PS1	1.97	<0.01
OTU 84	*Porphyromonas*	*Porphyromonas asaccharolytica* strain HA3347-27	99.6	PS1	1.50	0.02
OTU 89	*Trueperella*	*Trueperella pyogenes* strain TN2	99.6	PS1	1.07	0.01
OTU 96	*Peptoniphilus*	*Peptoniphilus olsenii* strain WAL 12922	97.2	PS1	1.62	<0.01
OTU 97	*Facklamia*	*F. hominis* strain DNF00119	98.4	PS3	0.46	0.04
OTU 98	*Gallicola*	*Gallicola* sp. RM-6	92.5	PS1	2.75	<0.01

[1]Individual microbes were assigned in order of abundance and classified into an operational taxonomic unit (OTU).
[2]Taxonomy was assigned using Silva SSU NR reference database (v138).
[3]Sows were assigned a PS based on their relative risk of experiencing a POP. Sows assigned PS1 were presumed low risk for POP and sows assigned PS3 were presumed high risk for POP. Specific OTUs are more abundant in sows with the PS indicated.
[4]Log2 fold change.

gestational diabetes mellitus when blood sugar is increased [31]. Interestingly, glucose and its derivatives are increased in circulation of sows with elevated risk of POP [15] indicating a possible biological relationship that could be further explored.

Mucin acts as a barrier to pathogens in the reproductive tract, and its degradation could lead to inflammation, potentially due to lipopolysaccharide (LPS) [31]. Gram-negative bacteria are known to produce LPS, which is a glycolipid surface molecule on most Gram-negative bacteria, and a well-known immune system stimulant [32]. Interestingly, *Prevotella* is a species that can degrade mucin and thereby increase cellular permeability [33]. Elevated *Prevotella* in the sow reproductive tract could theoretically increase permeability in the reproductive tract as well, which would be expected to elicit some level of an immunological response. In support of this posit,

Table 3. Differences in OTUs between vaginal microbiota of sows during gestation week 15 assigned PS3 that subsequently did or did not experience POP

		NCBI BLAST				
OTU[1]	Taxonomy (Silva v138)[2]	Classification	Similarity (%)	POP outcome[3]	Log2FC[4]	Q-value
OTU 1	*Actinobacillus*	*Actinobacillus porcinus* strain 35NTS	99.6	No	0.88	<0.01
OTU 12	*Veillonella*	*Veillonella caviae* strain PV1	99.2	No	1.22	0.01

[1]Individual microbes were assigned in order of abundance and classified into an operational taxonomic unit (OTU).
[2]Taxonomy was assigned using Silva SSU NR reference database (v138).
[3]POP outcome refers to whether a PS3 sow at high risk for pelvic organ prolapse (POP) did (Yes) or did not (No) subsequently experience POP. Specific OTUs are more abundant in outcome designated.
[4]Log2 fold change.

LPS binding protein (LBP), a marker of inflammation, is increased in sows at high risk of POP [16]. Furthermore, in the vaginal microbiota of women suffering from BV, a higher abundance of *Prevotella* has also been noted strengthening the connection of this particular species of bacteria to compromised reproductive tract health [34].

Streptococcus dysgalactiae is an additional microbe of interest, particularly as it can possess virulence factors, and is associated with several diseases in humans and animals [35–38]. Virulence factors are products of bacteria that aid in eluding host defenses [39]. *Streptococcus dysgalactiae* is considered a pathogen in humans when found in the female genital tract and is associated with reproductive dysfunction in equids [40, 41]. Observations in this study, consistent with previous work, have found *S. dysgalactiae* to be increased in the vaginal microbiota of late gestation sows at high risk of POP [15] making it a noteworthy target for further investigation to decipher its potential role in affecting POP risk.

Similar to *S. dysgalactiae*, *Treponema* has also been linked to mammalian reproductive disorders [42, 43], as well as diseases that cause inflammation in the swine intestinal tract [44]. These findings demonstrate increased *Treponema* in the vaginal microbiota in PS3 scored sows, consistent with previous work [15], and provides a potential explanation of accompanied increases in biomarkers of inflammation (i.e. LBP) in PS3 sows [16]. However, *Treponema* has been shown to have a negative correlation with cytokines [45]. These differing results may be explained by different host species, pregnancy status, and sample type used for evaluation.

The OTU with the greatest log2FC increase in PS3 sows in this study was *S. agnetis*. *Staphylococcus agnetis* is an emerging pathogen in poultry and may have an effect on collagen and fibronectin [46, 47]. Collagen plays a role in the structure of the muscles and tissue in the female pelvic floor and reproductive tract [48]. Thus, interfering with the host animal's connective tissue may be a possible mechanism through which *S. agnetis* could be associated with POP. This, however, will require verification in future studies focused on the mechanistic actions of this microbe.

Butyrate is a bacterially produced, short-chained fatty acid that has been shown to have several beneficial effects on the host animal. In the gut, butyrate is shown to upregulate tight-junction proteins in the epithelial layer of the gut, strengthening epithelial integrity and enhancing intestinal barrier function [49–52]. Additionally, butyrate stimulates the proliferation and maturation of intestinal mucosa cells, speeding the process of development or repair after injury of these tissues [53]. Known butyrate producing species, such as *Roseburia* spp., *Lachnospira* spp., *Clostridium* spp., *Ruminococcus* spp., *Coprococcus* spp., *Butyrivibrio* spp., etc., often metabolize lactic acid produced by bacterial species, such as *Lactobacillus*, to butyrate and are often considered beneficial microbes for the reasons mentioned above [54–56]. Comparatively to the gut environment, little research has been conducted on the effect of butyrate-producing bacterial species on the reproductive system, though it may be highly relevant. It has been shown that when exposed to butyrate, porcine granulosa cells alter the secretion of both β-Estradiol (E2) and progesterone (P4) [57]. β-Estradiol and P4 have both been shown to directly affect the vaginal tissue integrity [58–60]. Previous work [16] demonstrated an increase in circulating E2 levels in sows at high risk for POP compared to low risk. Several OTUs within the 100 most abundant (OTU 3, 5, 7, 18, 50, and 73) classified as *Clostridium* spp. and OTU 62 (*Lachnospiraceae_UCG-007*) could potentially have butyrate producing capabilities and were all found to be more abundant in PS3 animals. Additionally, OTUs classified as known lactic acid-producing bacteria (OTU 66, *Bifidobacterium* and OTU 71, *Lactobacillus*) were also more abundant in PS3 animals. It may be possible that, in this porcine model, butyrate production by bacterial species may influence vaginal tissue integrity, subsequently contributing to POP. This work highlights the importance of understanding the impact of butyrate on reproduction and identifies potential microbial targets to combat POP.

Only *A. porcinus* and *V. caviae* were differentially abundant between sows at high risk for POP that subsequently did or did not experience POP. Both bacteria were greater in PS3 scored sows that did not subsequently experience POP compared to those that did. *Veillonella* has been detected in pigs previously and was observed in higher abundance in healthy pig fecal microbiota compared to those with intestinal diseases [16, 61, 62]. In this study, wk15PS1 sows had greater *V. caviae* presence compared to wk12PS1 and was also greater in wk12PS1 compared to wk15PS3 sows. These observations collectively indicate that progression from week 12 of gestation to week 15 is accompanied by increased *V. caviae* in sows that remain low risk for POP, although this progression does not occur if a sow's risk for POP increases during this same time period. Based on these observations and the consistency with prior work [15], *Veillonella* represents a potential beneficial microorganism warranting further exploration to determine potential roles in regulation of the microbiota as it relates to POP risk in sows.

Table 4. Differences in OTUs between vaginal microbiota of gestation week 12 PS1 sows and gestation week 15 PS1 sows

OTU[1]	Taxonomy (Silva v138)[2]	NCBI BLAST		wk12PS1 versus wk15PS1[3]	Log2FC[4]	*P*-value	*Q*-value
		Classification	Similarity (%)				
OTU 4	*Streptococcus*	*Streptococcus dysgalactiae subsp. equisimilis strain TPCH-A88*	99.6	wk12PS1	0.77	<0.01	<0.01
OTU 23	*Porphyromonas*	*Porphyromonas levii DSM 23370*	90.5	wk12PS1	0.83	0.02	<0.01
OTU 24	*Staphylococcus*	*Staphylococcus simulans strain D14*	99.6	wk12PS1	1.21	0.03	0.05
OTU 25	*Gallicola*	*Peptoniphilaceae bacterium SIT14*	97.6	wk12PS1	0.50	0.02	<0.01
OTU 28	*Porphyromonas*	*Porphyromonas somerae strain KA00683*	99.2	wk15PS1	1.00	<0.01	<0.01
OTU 32	*Streptococcus*	*Streptococcus pasteurianus strain 2323*	99.6	wk12PS1	0.47	<0.01	0.01
OTU 33	*Ezakiella*	*Bacteroides coagulans strain EUH 581–73*	94.5	wk12PS1	1.13	0.02	<0.01
OTU 40	*Peptococcus*	*Peptococcus simiae strain M108*	99.6	wk12PS1	0.89	<0.01	<0.01
OTU 45	*Peptoniphilus*	*Peptoniphilus sp. 1804121828*	99.6	wk12PS1	0.49	<0.01	<0.01
OTU 51	*Porphyromonas*	*Porphyromonas canoris strain JCM 16132*	92.5	wk12PS1	1.37	<0.01	<0.01
OTU 57	*Staphylococcus*	*Staphylococcus agnetis strain PR5962A*	99.6	wk15PS1	3.48	0.01	<0.01
OTU 61	*Anaerococcus*	*Anaerococcus nagyae strain ENR0686*	94.8	wk15PS1	0.76	0.01	<0.01
OTU 63	*Enterococcus*	*Enterococcus faecium strain FFNL3053*	99.6	wk15PS1	1.18	<0.01	<0.01
OTU 70	*Anaerococcus*	*Anaerococcus sp. Marseille-P3915*	99.6	wk15PS1	0.74	0.02	<0.01
OTU 76	*Campylobacter*	*Campylobacter ureolyticus strain LMG 6451*	96.5	wk15PS1	2.27	<0.01	<0.01
OTU 78	*Lachnospiraceae_ XPB1014_group*	*Lachnospiraceae bacterium CA63*	94.1	wk15PS1	0.51	<0.01	0.02
OTU 84	*Porphyromonas*	*Porphyromonas asaccharolytica strain HA3347–27*	99.6	wk15PS1	1.23	0.03	<0.01
OTU 89	*Trueperella*	*Trueperella pyogenes strain TN2*	99.6	wk15PS1	0.92	0.02	<0.01
OTU 94	*Corynebacterium*	*Corynebacterium kutscheri strain NCTC3655*	99.6	wk15PS1	0.40	0.03	0.03
OTU 98	*Gallicola*	*Gallicola sp. RM-6*	92.5	wk12PS1	1.57	0.01	<0.01

[1]Individual microbes were assigned in order of abundance and classified into an operational taxonomic unit (OTU).
[2]Taxonomy was assigned using Silva SSU NR reference database (v138).
[3]Sows were assigned a PS based on their relative risk of experiencing a POP. Sows assigned PS1 were presumed low risk for POP and compared between gestation week 12 (wk12PS1) and 15 (wk15PS1). Specific OTUs are more abundant in sows with the week and PS indicated.
[4]Log2 fold change.

The vaginal microbiota is known to change throughout gestation in humans [5], and this may also happen in sows. In this study, the sample size was small for individual sows assigned PS3 during week 15 after previously being assigned PS1 during week 12; however, the results of this work suggest a temporal assessment of sows during gestation may be beneficial to better understand normal changes to vaginal microbial populations and shifts as reproductive disorders emerge. Of similar interest was the observation of differences between farms given that they were geographically close and very similarly managed with respect to genetics, nutrition, veterinary care, etc. Differences in the microbiota of sows between farms in this study coupled with the observation of differences in POP incidence rate create a compelling area of further investigation to determine what other factors (i.e. water source, within barn environmental differences, etc.) may influence the observed variation across farms.

Collectively, this study validates the phenotypic PS system to identify sows at higher risk for POP. Bacterial candidates of interest were identified consistent with prior work and may be associated

Table 5. Differences in OTUs between vaginal microbiota of gestation week 12 PS1 sows and gestation week 15 PS3 sows

OTU[1]	Taxonomy (Silva v138)[2]	NCBI BLAST		wk12PS1 versus wk15PS3[3]	Log2FC[4]	*P*-value	*Q*-value
		Classification	Similarity (%)				
OTU 4	*Streptococcus*	*Streptococcus dysgalactiae subsp. equisimilis strain TPCH-A88*	99.6	wk12PS1	0.96	<0.01	<0.01
OTU 5	*Clostridium_sensu_stricto_1*	*Clostridium moniliforme strain 2055*	99.2	wk12PS1	0.34	<0.01	0.03
OTU 6	*Romboutsia*	*Romboutsia timonensis strain DR1*	97.6	wk12PS1	0.31	0.01	0.04
OTU 10	*Corynebacterium*	*Corynebacterium xerosis strain GS*	100	wk12PS1	0.33	0.05	0.02
OTU 12	*Veillonella*	*Veillonella caviae strain PV1*	99.2	wk12PS1	0.41	0.02	0.01
OTU 13	*Streptococcus*	*Streptococcus suis strain SS-CLA1926B*	99.6	wk12PS1	0.60	<0.01	0.03
OTU 15	*Finegoldia*	*Finegoldia magna strain FDAARGOS*	99.2	wk12PS1	0.41	0.02	0.01
OTU 20	*Kurthia*	*Kurthia gibsonii strain EMB4*	99.6	wk15PS3	0.46	<0.01	0.03
OTU 22	*Methanobrevibacter*	*Methanobrevibacter sp. YE315*	100	wk12PS1	0.39	<0.01	0.03
OTU 23	*Porphyromonas*	*Porphyromonas levii DSM 23370*	90.5	wk12PS1	2.84	<0.01	<0.01
OTU 24	*Staphylococcus*	*Staphylococcus simulans strain D14*	99.6	wk12PS1	1.61	0.01	0.05
OTU 25	*Gallicola*	*Peptoniphilaceae bacterium SIT14*	97.6	wk12PS1	1.01	<0.01	<0.01
OTU 28	*Porphyromonas*	*Porphyromonas somerae strain KA00683*	99.2	wk15PS3	2.96	<0.01	<0.01
OTU 29	*Escherichia-Shigella*	*Escherichia fergusonii strain SPK*	99.6	wk12PS1	0.72	<0.01	<0.01
OTU 32	*Streptococcus*	*Streptococcus pasteurianus strain 2323*	99.6	wk12PS1	0.54	<0.01	0.01
OTU 33	*Ezakiella*	*Bacteroides coagulans strain EUH 581–73*	94.5	wk12PS1	3.77	<0.01	<0.01
OTU 34	*Anaerococcus*	*Anaerococcus sp. strain AGMB00486*	99.6	wk15PS3	1.74	<0.01	<0.01
OTU 38	*Corynebacterium*	*Corynebacterium stationis strain VRD1 333 N7*	99.6	wk15PS3	0.36	0.01	0.01
OTU 40	*Peptococcus*	*Peptococcus simiae strain M108*	99.6	wk12PS1	2.65	<0.01	<0.01
OTU 41	*Peptoniphilus*	*Peptoniphilus sp. strain AGMB00490*	99.6	wk12PS1	3.93	<0.01	<0.01
OTU 45	*Peptoniphilus*	*Peptoniphilus sp. 1804121828*	99.6	wk12PS1	1.14	<0.01	<0.01
OTU 48	*Streptococcus*	*Streptococcus hyovaginalis strain TRG26*	99.6	wk12PS1	0.68	0.02	<0.01
OTU 51	*Porphyromonas*	*Porphyromonas canoris strain JCM 16132*	92.5	wk12PS1	4.29	<0.01	<0.01
OTU 53	*Corynebacterium*	*Corynebacterium callunae strain AS67*	99.2	wk12PS1	0.44	0.01	0.01
OTU 54	*Anaerococcus*	*Anaerococcus sp. Marseille-P3915*	98.4	wk12PS1	3.86	<0.01	<0.01
OTU 57	*Staphylococcus*	*Staphylococcus agnetis strain PR5962A*	99.6	wk15PS3	5.87	<0.01	<0.01
OTU 61	*Anaerococcus*	*Anaerococcus nagyae strain ENR0686*	94.8	wk12PS1	2.29	<0.01	<0.01
OTU 63	*Enterococcus*	*Enterococcus faecium strain FFNL3053*	99.6	wk15PS3	1.01	<0.01	<0.01
OTU 70	*Anaerococcus*	*Anaerococcus sp. Marseille-P3915*	99.6	wk12PS1	3.09	<0.01	<0.01
OTU 72	*Facklamia*	*Facklamia hominis strain DNF00119*	98.4	wk15PS3	1.91	<0.01	<0.01

Continued

Table 5. Continued

OTU[1]	Taxonomy (Silva v138)[2]	NCBI BLAST		wk12PS1 versus wk15PS3[3]	Log2FC[4]	*P*-value	*Q*-value
		Classification	Similarity (%)				
OTU 74	*Corynebacterium*	*Corynebacterium glucuronolyticum strain V17 2011556*	99.6	wk15PS3	2.82	<0.01	<0.01
OTU 76	*Campylobacter*	*Campylobacter ureolyticus strain LMG 6451*	96.5	wk15PS3	5.30	<0.01	<0.01
OTU 78	*Lachnospiraceae_XPB1014_group*	*Lachnospiraceae bacterium CA63*	94.1	wk15PS3	0.51	0.01	0.02
OTU 81	*Peptostreptococcus*	*Peptostreptococcus anaerobius strain WH7*	91.3	wk15PS3	2.89	<0.01	<0.01
OTU 84	*Porphyromonas*	*Porphyromonas asaccharolytica strain HA3347–27*	99.6	wk15PS3	2.79	<0.01	<0.01
OTU 89	*Trueperella*	*Trueperella pyogenes strain TN2*	99.6	wk15PS3	2.22	<0.01	<0.01
OTU 94	*Corynebacterium*	*Corynebacterium kutscheri strain NCTC3655*	99.6	wk15PS3	0.57	<0.01	0.03
OTU 96	*Peptoniphilus*	*Peptoniphilus olsenii strain WAL 12922*	97.2	wk15PS3	3.07	<0.01	<0.01
OTU 98	*Gallicola*	*Gallicola sp. RM-6*	92.5	wk15PS3	4.84	<0.01	<0.01
OTU 100	*Campylobacter*	*Campylobacter corcagiensis strain LMG 27932*	99.6	wk15PS3	5.26	<0.01	<0.01

[1]Individual microbes were assigned in order of abundance and classified into an operational taxonomic unit (OTU).
[2]Taxonomy was assigned using Silva SSU NR reference database (v138).
[3]Sows were assigned a PS based on their relative risk of experiencing a POP. Sows assigned PS1 were presumed low risk for POP and sows assigned PS3 were presumed high risk of POP. OTUs were compared between low-risk sows at gestation week 12 (wk12PS1) to high-risk sows at gestation week 15 (wk15PS3). Specific OTUs are more abundant in sows with the week and PS indicated in the column.
[4]Log2 fold change.

with POP, in addition to providing further characterization of the vaginal microbiota of pregnant sows. These data aid in the understanding of the biological association leading up to POP in the U.S. commercial swine herd. Further, additional research that is mechanistic by design is needed to demonstrate POP risk causality of specific microbes to continue moving this research area forward.

Data availability

The 16S rRNA gene sequencing data have been submitted to the NCBI Sequence Read Archive SRA and are available under the BioProject ID PRJNA739728.

Conflict of interest

Any opinion, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the funding agency. No conflicts of interest, financial, or otherwise are declared by the authors.

Authors' contributions

ZEK designed experiments, completed experiments, performed analysis, and wrote the draft of the manuscript. LRK contributed to execution of experiments, data analysis, and edited the manuscript. JMS and ALC contributed to experimental design, execution of experiments, and edited the manuscript. CM-W contributed to execution of experiment and edited the manuscript. AFK contributed to experimental design and edited the manuscript. SS-E contributed to experimental design, data analysis and edited manuscript. JWR contributed to experimental design, project funding, provided project oversight, and edited the manuscript.

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