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ENVIRONMENTAL MICROBIOLOGY

Contamination Is Not Linked to the Gestational Microbiome

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ABSTRACT Differentiating between contamination and the genuine presence of 16S rRNA genes in gestational tissue samples is the gold standard for supporting the *in utero* colonization hypothesis. During gestation, the fetus undergoes significant physiological changes that may be directly affected by maternal colonization of key bacterial genera. In this study, lab benches, necropsy tables, and air ducts were swabbed at the same time as clinical sampling. The relative and absolute abundance of bacteria present in sheep samples was determined by culture-independent and culture-dependent means. Of 14 healthy pregnant ewes, there was no evidence of any bacteria in the fetal liver, spleen, or brain cortex using culture-independent techniques despite evidence of the presence of bacteria in various locations of the necropsy room used for 11 of these 14 sheep. Of the 336 bacterial genera found in the room swabs, only 12 (5%) were also found in the saliva and vaginal swabs among the three ewes for which bacteria were detected. These 12 taxa represent 1.32% of the relative abundance and approximately 393 16S rRNA copies/swab in these three ewes. Using careful necropsy protocols, bacterial contamination of sheep tissues was avoided. Contamination of saliva and vaginal samples was limited to less than 2% of the bacterial population.

IMPORTANCE Recent evidence for a gestational microbiome suggests that active transfer between mother and fetus *in utero* is possible, and, therefore, actions must be taken to clarify the presence versus absence of these organisms in their respected sources. The value of this study is the differentiation between bacterial DNA identified in the necropsy rooms of animals and bacterial DNA whose origin is purely clinical in nature. We do not know the extent to which microorganisms traverse maternal tissues and infiltrate fetal circulation, so measures taken to control for contamination during sample processing are vital for addressing these concerns.

KEYWORDS gestation, community-level analysis, contamination, environment, microbiome

Studies on placental microbial colonization suggest that healthy pregnancies may harbor a wide variety of bacterial species that benefit both the mother and developing fetus prior to parturition (1). Placental colonization may stem from the maternal diet and can be traced to bacterial communities in other organs and organ systems (2). This observation is based on the understanding that the placenta functions as a critical interface between the maternal and fetal zones. Interestingly, the complex bacterial communities characterized from placental tissues contain organisms that are commonly found in the oral cavity, which has raised the possibility that resident microbial communities in the mother may provide a foundation for establishing a fetal microbiome *in utero*. Several Gram-negative anaerobes, such as *Fusobacterium nucleatum*, have been purported to bind vascular endothelium and induce permeability, which could give rise to increased access by other commensal species (2). Similarly,

Citation Rodriguez MD, Yu KK, Paul ZS, Keller-Wood M, Wood CE, Triplett EW. 2019. Contamination is not linked to the gestational microbiome. Appl Environ Microbiol 85:e01127-19. https://doi.org/10.1128/AEM.01127-19.

Editor Harold L. Drake, University of Bayreuth

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Received 20 May 2019
Accepted 23 July 2019
Accepted manuscript posted online 26 July 2019
Published 17 September 2019

bacterial exposure of the fetus *in utero* results in the presence of live bacteria in meconium of newborn human infants (3).

A recent study by Zarate et al. (4) revealed that the brains of healthy fetal sheep exposed to transient hypoxia were colonized by staphylococci, while control subjects exhibited a relative absence of bacteria. These findings are consistent with nonlethal *in utero* bacterial colonization of fetal tissues other than the gastrointestinal tract. In addition, emphasis has been placed on the placenta as a possible source of these shared microbes.

Despite the compelling nature of these findings, environmental contamination and the possibility of artifactual sequence-based identification of bacteria in tissues and fluids are potential limitations of some studies in the literature. Some investigators have presented hypotheses that contradict the likelihood of transient colonization (5). Depending on methodology, spurious results may arise due to various confounding factors during sample collection, sample processing, and sequencing. One such confounding factor is a low level of bacterial DNA occasionally detected in DNA extraction kits. These bacteria derived from extraction kits are sometimes indistinguishable from those found placental samples (6).

In this study, we propose a possible solution to the problem of "phantom" identification through the use of quantitative measures. Specifically, we propose that the use of a cutoff for a minimum detectable mass of bacterial DNA minimizes or eliminates the problem of false positives in sequencing reactions and subsequent data analyses. Here, by using a threshold test for the quantity of bacterial DNA, we test the hypothesis that bacterial taxa identified in fetal tissues do not match those in the surgery room or air ducts.

RESULTS

Prevalence and identity of bacteria in sheep tissues. Bacteria could be detected through amplification or cultivation in the tissues from 14 ewes. However, fetal and maternal samples from ewes 1, 2, and 17 were likely sterile, with no amplification of 16S rRNA genes from the environment or tissue swabs. No bacterial 16S rRNA amplicons were recovered from tissue samples (fetal/lamb cerebral cortex, fetal/lamb liver, fetal/lamb spleen, and maternal vaginal and oral tissues) acquired from the first set of 12 ewes. No vaginal or oral swabs were collected from those ewes. In ewes 13, 15, and 16 where vaginal and oral swabs were collected, the samples contained sufficient DNA for 16S rRNA gene sequencing (Table 1). Also, bacteria were cultured from these samples and were identified by Sanger sequencing (Table 1). Fifty percent of the organisms cultured were also successfully sequenced from DNA. No cultures were recovered from fetal/lamb cerebral cortex, fetal lung, and fetal spleen samples. However, lower copy numbers of 16S rRNA genes were detectable by quantitative PCR (qPCR) in four fetal/lamb cerebral cortex and fetal/lamb liver samples (see Fig. 2).

Prevalence of bacteria by Illumina MiSeq sequencing in sheep vaginal and oral swabs. Bacteria could be detected through amplification, cultivation, or both in 14 ewes. However, no amplification of 16S rRNA genes from the environment or tissue swabs was observed in fetal and material samples from ewes 1, 2, and 17. No bacterial 16S rRNA amplicons were recovered from tissue samples (fetal/lamb cerebral cortex, fetal/lamb spleen, material vaginal, and maternal oral samples) acquired from the first set of 12 ewes, except for ewes 13, 15, and 16. In these ewes, bacteria were detected only in vaginal and oral swabs using culture-independent PCR amplification and through culturing (Table 1). Fifty percent of the organisms cultured were also successfully sequenced from DNA. No cultures were recovered from fetal/lamb cerebral cortex, fetal lung, and fetal spleen samples. However, lower copy numbers of 16S rRNA genes were detectable by qPCR in four fetal/lamb cerebral cortex and fetal/lamb samples (Fig. 1).

Composition of sheep vaginal and oral bacterial communities. Cultivation-independent 16S rRNA gene amplicon sequencing was performed to characterize microbial communities from vaginal and oral swabs. The most frequently recovered

TABLE 1 Culturing results per animal

Sample type and animal no.[a]	Sequenced[b]	Origin	Species identified
Environmental			
3	Y	Necropsy room	*Aerococcus viridans*
	Y		*Staphylococcus aureus*
	Y		*Klebsiella pneumoniae*
4	Y	Necropsy room	*Staphylococcus aureus*
	Y		*Bacillus cereus*
5	Y	Necropsy room	*Cupriavidus gilardii*
	Y		*Bacillus licheniformis*
	Y		*Staphylococcus aureus*
6	Y	Necropsy room	*Klebsiella pneumoniae*
	Y		*Serratia liquefaciens*
	Y		*Bacillus cereus*
7	Y	Necropsy room	*Escherichia coli*, *Staphylococcus cohnii*, *Bacillus safensis*, *Staphylococcus hominis*, *Escherichia faecalis*
8	Y	Necropsy room	*Bacillus licheniformis*, *Escherichia fergusonii*
9	Y	Necropsy room	*Enterococcus faecalis*
10	Y	Necropsy room	*Staphylococcus epidermidis*, *Bacillus licheniformis*
11	Y	Necropsy room	*Escherichia fergusonii*, *Aerococcus urinaeequi*
12	Y	Necropsy room	*Bacillus safensis*, *Escherichia fergusonii*, *Enterococcus faecalis*
Oral/vaginal swab			
13	Y	Oral swab	*Enterococcus faecalis*
		Vaginal swab	*Clostridium tertium*
14	N	Oral swab	*Clostridium tertium*
		Vaginal swab	*Bacillus zhangzhouensis*
15	Y	Oral swab	*Acidaminococcus fermentans*
		Oral swab	*Mannheimia ruminalis*
		Vaginal swab	*Klebsiella pneumoniae*
		Vaginal swab	*Staphylococcus auricularis*
16	Y	Oral swab	*Corynebacterium coyleae*
		Vaginal swab	*Staphylococcus aureus*

[a]No cultures or DNA was recovered from animals 1, 2, and 17.
[b]Y, yes; N, no.

sequences in the maternal oral cavity were derived from the genera *Mannheimia* (69.5%), *Acidaminococcus* (42.8%), *Moraxella* (23.5%), *Streptococcus* (18.6%), and *Corynebacterium* (12.1%). In contrast, the maternal vaginal canal was dominated by *Cupriavidis* (23.6%) and *Enterococcus* (22.6%) in animal 13 and by *Escherichia/Shigella* in animal 16

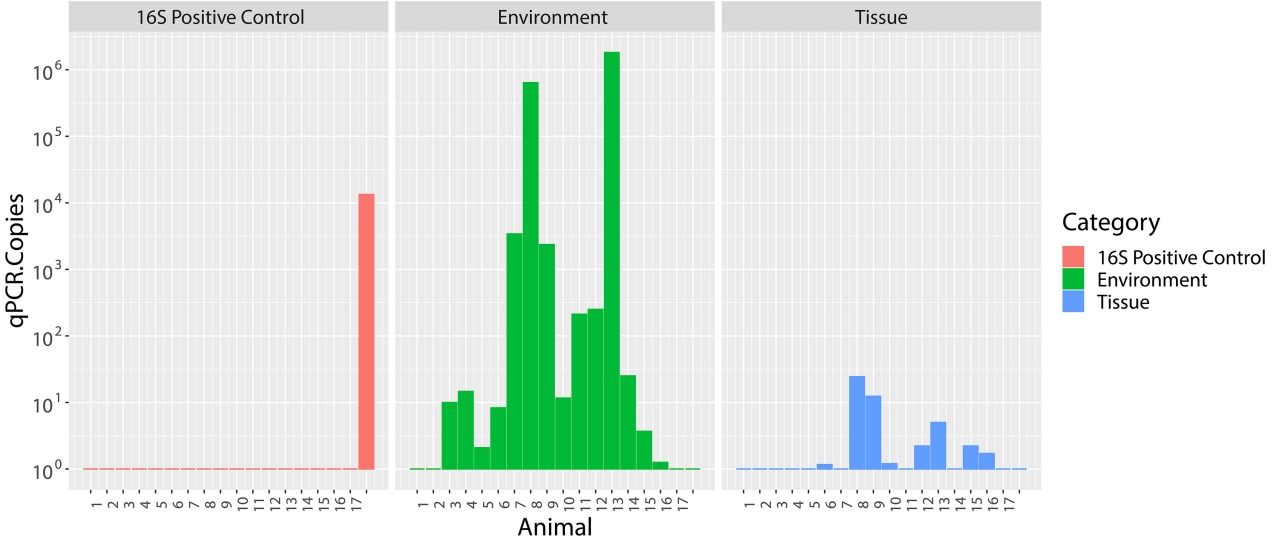

FIG 1 qPCR counts of 16S rRNA genes in environmental swabs and tissues (amplified or nonamplified). Values on the *y* axis are the $\log_{10}$ counts of 16S copies per gram of tissue and per gram of swab biomass.

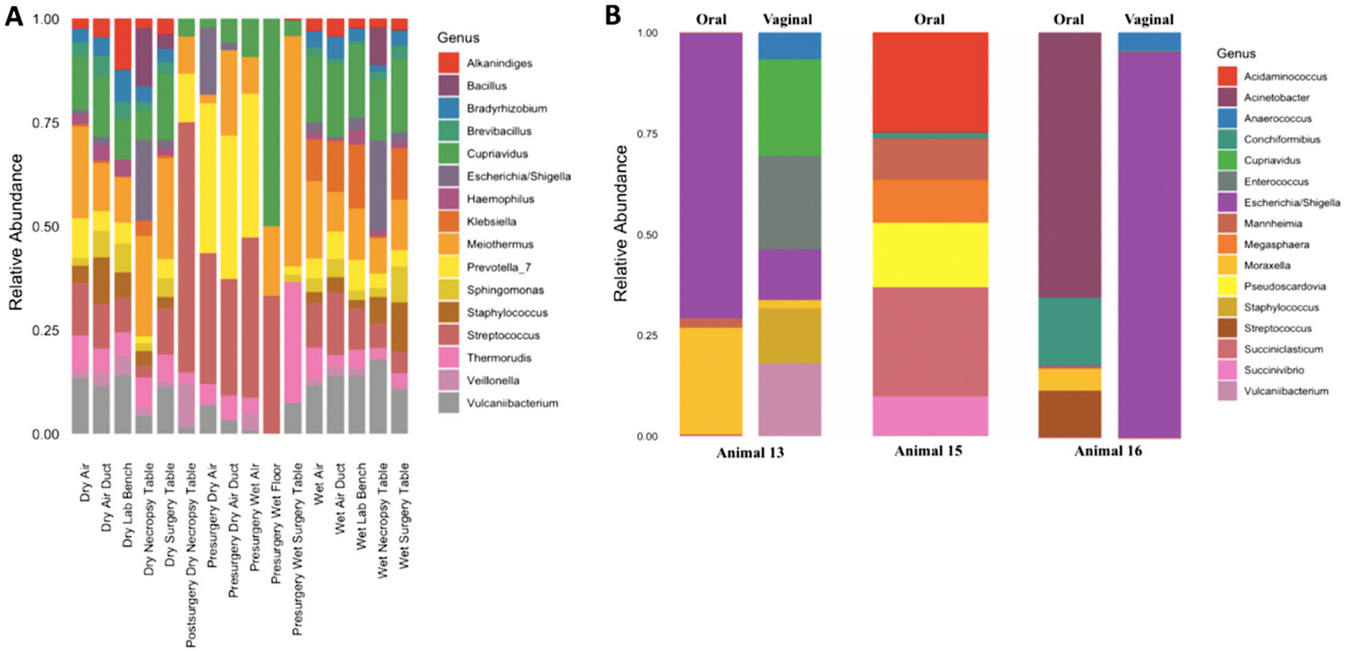

FIG 2 Relative 16S rRNA gene abundance across all necropsy sampling sites for animals 1 to 16 (A) compared to abundances in oral/vaginal swabs taken from animals 13, 15, and 16 (B).

(95.3%) (Fig. 2). The cultures acquired from these samples that were identical to the culture-independent results include *Mannheimia ruminalis*, *Acidaminococcus fermentans*, *Streptococcus ferus*, and *Staphylococcus aureus* (Table 1). Cultures of *Cupriavidus*, *Meiothermus*, and *Veillonella* were also found in the room swabs, but they were significantly low in abundance (less than 0.002% across both vaginal and oral sites), supporting the notion that contamination originating from the room was low in the vaginal and oral sites. The only exception was the maternal vaginal sample collected from animal 13. In this sample, the observed relative abundance of *Cupriavidus gilardii* was 20%. However, none of these contaminants were present in the mouth or vagina of ewes 15 and 16.

Comparison of sheep and surgical room bacteria. A total of 11,150 amplicon sequence variants (ASVs) were identified in environmental samples (following rarefication). Of these, only 733 ASVs paired with sequences that matched ASVs identified in tissue samples. Total nonrarefied ASVs were determined for each site and animal (Table 2). Approximately 6% of microbial taxa identified in the environment was shared with associated tissue samples (Fig. 3). Quantitative PCR analysis showed that the matched ASVs were in very low abundance in tissue samples. The average 16S rRNA gene copy number and relative abundance of top matches were determined (Table 3). As mentioned previously, the primary matches found across all environmental sampling sites (i.e., *Meiothermus*, *Cupriavidus*, *Veillonella*, and *Deinococcus*) were not successfully cultured in any tissue sample acquired during necropsy. Microbial community structure was assessed by calculating Bray-Curtis distances between all samples and identifying clustering patterns by sample type (tissue or environment). Bray-Curtis distances were calculated using the most abundant ASVs at the taxonomic level of genus. Differences between the oral and vaginal groups and the necropsy room samples were significant (permutational multivariate analysis of variance [PERMANOVA] $P < 0.001$) (Fig. 4).

Microbial taxa differ between prenecropsy and postnecropsy samplings. Wet and dry swab samples were taken before and after necropsies in order to assess any changes in microbial taxa before and after sanitization. Only the necropsy samples of ewes 5 and 13 amplified bacterial products, suggesting that the sterile technique used was successful in eliminating contamination completely in most cases. The dominant

TABLE 2 Total nonrarefied ASVs per sampling site by animal

Animal no.	No. of ASVs by site or sampling time[a]											
	Dry necropsy table	Wet necropsy table	Wet surgery table	Dry surgery table	Wet air duct	Dry air duct	Wet lab bench	Dry lab bench	Presurgery	Postsurgery	Oral cavity	Vaginal cavity
1	0	0	0	0	0	0	0	0	0	0		
2	0	0	0	0	0	0	0	0	0	0		
3	0	0	8	0	0	0	0	0	0	0		
4	10	15	15	13	18	0	16	0	0	0		
5	13	0	0	0	32	11	0	16	0	0		
6	10	8	8	12	3	12	7	7	0	0		
7	0	22	16	0	20	30	24	17	0	0		
8	35	34	33	24	23	28	58	0	0	0		
9	25	0	15	15	0	12	0	19	0	0		
10	0	0	0	14	0	0	19	0	0	0		
11	0	23	15	14	18	17	13	0	0	0		
12	5	0	0	0	8	7	13	0	0	0		
13	0	7	0	0	8	11	8	0	50	11	4	19
14	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0	10	0
16	0	0	0	0	0	0	0		0	0	28	25
17	0	0	0	0	0	0	0	0	0	0	0	0

[a]Oral and vaginal swabs were taken only from ewes 13 to 17.

organisms found under presurgical conditions included *Meiothermus* and *Cupriavidus*, which were also prevalent in necropsy table, surgery table, and wet and dry air ducts (Fig. 5). In contrast, postnecropsy samples revealed two dominant organisms that were cleaned following presurgery: *Paracoccus* and *Oribacterium*.

DISCUSSION

The results of the present study demonstrate that incidental contamination of tissues collected during a sterile necropsy procedure does not account for bacterial DNA isolated and sequenced from fetal tissues when a protocol that prevents or minimizes sequencing errors based on low-input DNA mass is used. Controversy surrounding the validity of DNA sequencing in placenta (2, 7) and other tissues (8, 9) is based on whether incidental contamination from laboratory surfaces, instruments, or even from airborne sources can account for false detection and sequencing of bacterial DNA. This may occur as sample carryover, contamination of reagents, or some other source of artifact in the amplification or sequencing process (10, 11). Lauder and colleagues (6) raised this as a possible explanation of discovery of bacterial DNA in tissues thought to be sterile. In recognition of the fact that one can sometimes obtain sequences from

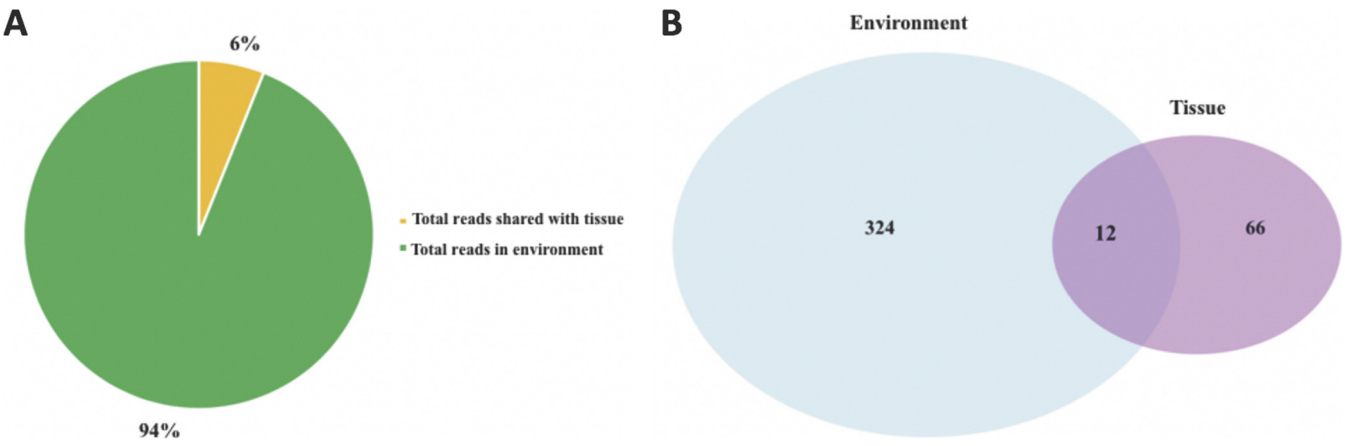

FIG 3 (A) Percentages of total reads found in environmental samples only and in both the environment and tissues (shared). (B) Presence/absence of top 12 contaminants present across all samples.

TABLE 3 Total 16S rRNA gene copies and percent relative abundance of the top 12 contaminants in associated tissue samples

Contaminant	Relative abundance by animal and sample type											
	Sheep 13				Sheep 15				Sheep 16			
	Oral		Vaginal		Oral		Vaginal		Oral		Vaginal	
	16S rRNA copy no.	% abundance	16S rRNA copy no.	% abundance	16S rRNA copy no.	% abundance	16S rRNA copy no.	% abundance	16S rRNA copy no.	% abundance	16S rRNA copy no.	% abundance
Campylobacter concisus	0	0	1.10e+02	0.0147	0	0	0	0	0	0	0	0
Cupriavidus gilardii	0	0	0	0	0	0	0	0	3.51e+02	0.00415	0	0
Deinococcus geothermalis	0	0	1.10e+02	0.0176	0	0	0	0	0	0	0	0
Fusobacterium nucleatum	0	0	0	0	0	0	0	0	0	0	0	0
Meiothermus silvanus	0	0	1.10e+04	0.155	0	0	0	0	0	0	0	0
Meiothermus chliarophilus	0	0	0	0	0	0	0	0	3.51e+02	0.00829	1.20e+03	0.0101
Olsenella umbonata	2.38e+2	0.0217	2.38e+02	0.0029	2.38e+02	0.00546	0	0	0	0	0	0
Prevotella melaninogenica	0	0	2.38e+03	0.114	0	0	0	0	0	0	1.20e+03	0.0303
Veillonella atypica	0	0		0.0381	0	0	0	0	0	0	0	0
Veillonella dispar	0	0	0	0	0	0	0	0	0	0	1.20e+03	0.0101
Veillonella tobetsuensis	0	0	2.38e+02	0.0264	0	0	0	0	0	0	0	0
Veillonella ogosae	0	0	0	0	0	0	0	0	0	0	0	0

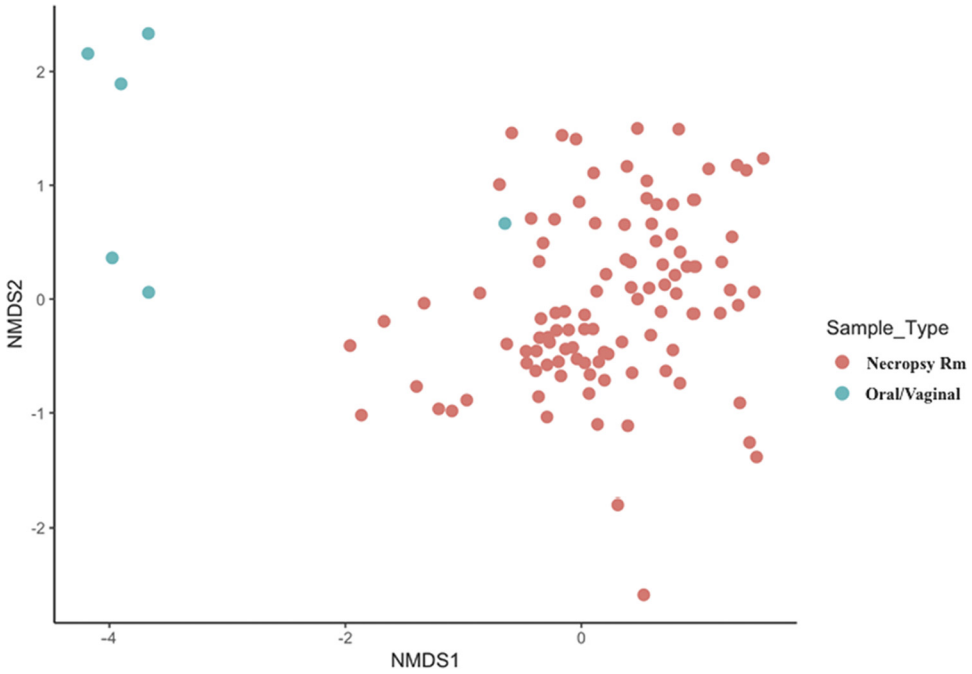

FIG 4 Nonmetric multidimensional scaling (NMDS) ordination plot (Bray-Curtis value of >0.2) depicting relatedness of bacterial communities between environmental and tissue samples ($R = 0.37$, $P = 0.045$). Rm, room.

samples lacking DNA or with sequence copy numbers below the limit of detection, we designed our DNA sequencing protocol to include requirement of detection of a minimum amount of bacterial DNA in each sample using endpoint PCR. Accordingly, samples that contain a quantifiable mass of DNA but which lack enough of the DNA of the bacterial 16S rRNA gene are not subjected to sequencing. Here, microbial DNA recovered from negative controls (blank swabs) was not sufficient for downstream methods and was not discernible by gel electrophoresis and was thus negligible (below 10 copies per gram). Using this protocol, we find (i) that not all samples, from environment or from tissue, contain enough bacterial DNA to sequence; (ii) that samples sometimes contain bacterial DNA but do not produce live bacteria, as seen in

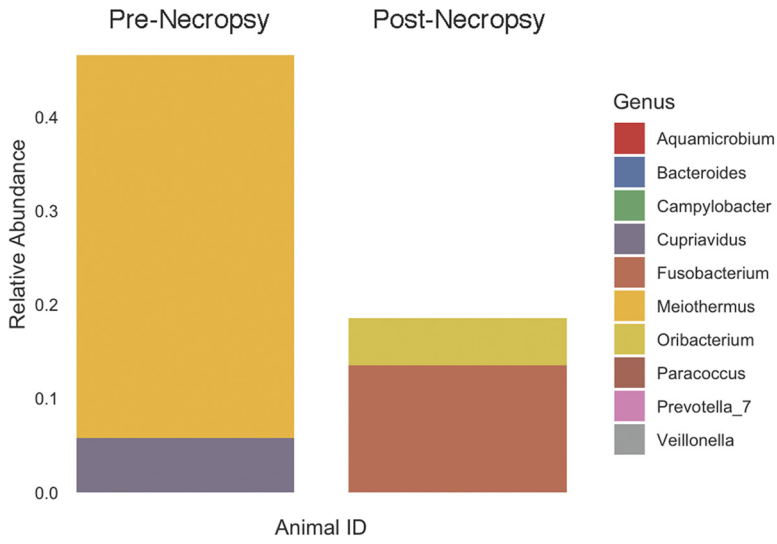

FIG 5 Total relative 16S rRNA gene abundance of the 10 most common organisms by genus in environmental swab samples taken prenecropsy and postnecropsy.

culture; (iii) that there is some minor overlap between environment and tissue and that this overlap does not explain the presence of the most abundant bacteria in either sample.

Paracoccus was found in the postnecropsy room samples. *Paracoccus denitrificans* is an organism that is typically found in soil and in this context could stem from debris tracked in by the sheep before the start of the necropsy. *Oribacterium* is a commensal bacterium frequently identified in oral samples. Given that oral swabs were taken, this could mean carryover between sheep and human via handling and during swabbing. Overall, these organisms were not found prior to surgery, suggesting that they originated in either the animal subjects or their caretakers. In essence, inconsistency between these two scenarios indicates that the original contaminants (*Meiothermus* and *Cupriavidus*) were eradicated following sanitization, which further demonstrates the importance of sterility during these experiments.

We have recently reported evidence that bacterial cell components can be found in the late-gestation fetus after inoculation of the mother with live bacteria. In that study, intravenous inoculation of pregnant ewes with 100 CFU of green fluorescent protein (GFP)-expressing *Staphylococcus aureus* resulted in the detection of GFP plasmid DNA and GFP protein in placenta and fetal tissues 4 to 6 days later (12). The inoculation did not cause any clinical signs of infection or septicemia in the ewes (which maintained normal body temperature and food intake). We were not able to culture live GFP-expressing bacteria from the fetal tissues, suggesting that we were observing and quantifying remnants of bacterial cells. These data provide proof of principle that the fetus can be exposed to bacteria or to components of bacteria in maternal blood. Nevertheless, our data support the notion that small numbers of bacteria can be cleared but that antigens from the bacteria find their way into the fetus. We do not know if the GFP-expressing bacteria in our inoculation experiments entered the fetus as live organisms or as remnants after being killed by the maternal immune system.

We also know that some alterations in the physiological environment of the fetus can result in the release of bacteria from mother to fetus. Exposure of late-gestation pregnant sheep to transient hypoxia resulted in the appearance of bacteria in the fetal brain that matched bacteria in the placenta (13). While hypoxia-induced active exposure of the fetal brain was subclinical, it was likely made possible by the exposure of the ewes to bacteria in the environment (4). We suspect that the transfer of bacteria (live and/or dead) from mother to fetus occurs in multiple species, including humans. We have reported that meconium of newborn human infants contains live and sequence-identifiable bacteria, with approximately 1/3 of infants producing sterile meconium and the remainder producing meconium containing live bacteria (3). We argue that, despite differences in placental structure, exposure of the fetus *in utero* to bacteria or their cellular components is a relatively common event. This tenet is illustrated by a study in gnotobiotic pregnant mice exposed to bacteria by maternal gavage. In that study, the exposure of the mother to bacteria altered the immunology of the neonatal gastrointestinal tract (14).

We found small amounts of bacterial DNA in a subset of fetal tissues. If the paradigm of Lauder et al. (6) (i.e., bacterial DNA in tissues is the result of contamination) held true in our experiments with our requirement for quantitation before sequencing, we would have found bacterial DNA in all tissues, and the sequences would have matched the bacterial DNA found in the environment. We argue that our results demonstrate that, under the correct conditions, true bacterial DNA can be found in tissues that are deemed "sterile" on the basis of negative cultures.

One condition in which bacterial DNA in tissue matched that in the environment was identified. Necropsies were performed, and samples were collected for two fetuses known to have died *in utero* from an intrauterine infection. In this case, the identified bacteria were found in the environment as well as in the tissues, indicating spread of the bacteria from the fetus to the environment. These were the only two necropsies for which contamination was observed, and in this case, it was the animal contaminating the environment.

TABLE 4 Sampling and treatment by animal

Tissue source and animal no.[a]	State of harvest[b]	Treatment
Fetal/lamb		
1	2-Day neonate	Ampicillin during surgery and for 5 days postsurgery
2	Possible infection	Ampicillin during and after surgery
3	2-Day neonate	Ampicillin during surgery (13 days), stopped 8 days prior to delivery; lamb received ampicillin after birth
4	Catheter infection	Ampicillin at time of necropsy
5	Stillborn	Ampicillin prior to and after surgery, not at necropsy
6	Infection	Ampicillin at time of necropsy
7	Healthy	No ampicillin at time of necropsy
8	Maternal complication	Ampicillin prior to and during necropsy
9	Live/healthy	No ampicillin at time of necropsy
10	Infection	Ceftiofur sodium every day including day of necropsy
11	2-Day neonate	Ceftiofur sodium 9 days before birth, ampicillin 2 days postsurgery
12	2-Day neonate	Cefazolin 2 days before delivery, ampicillin at surgery and 5 days postsurgery
Fetal/lamb and maternal		
13	Live/healthy	Ceftiofur sodium 4 days prior to surgery and during and after surgery
14	Catheter infection	Ceftiofur sodium 4 days prior to and during and after surgery
15	Live/healthy	Cefazolin at surgery and 2 days postsurgery
16	2-Day neonate	Ceftiofur sodium 7 days before delivery
17	1.5 h old	Ceftiofur sodium during surgery and 2 days postsurgery

[a]All fetal/lamb tissues were collected from liver, lung, cerebral cortex, spleen, and stool. All maternal tissues were collected from oral and vaginal swabs. For all animals, environmental samples were collected from necropsy tables (wet and dry), lab benches (wet and dry), and air ducts (wet and dry).
[b]Condition of the ewe and lamb/fetus at the time of necropsy.

These results strongly suggest that the bacterial DNA found in fetal tissues is not the result of contamination from the surgery. The amount of bacterial DNA in fetal tissues does not necessarily match the abundance of live bacteria in the tissue; some (or under specific conditions, all) of the sequenced 16S rRNA genes might be residual DNA in the tissue, released from dead or partially degraded bacteria. Nevertheless, the results of our study support the conclusion that the fetus is exposed to the bacteria in the maternal environment and that sequence-identifiable bacterial DNA in fetal tissues is not artifactual.

MATERIALS AND METHODS

Surgical techniques and sample collection. All animal use was approved by the University of Florida Animal Care and Use committee. Seventeen pregnant ewes of known gestational age were sampled for this study; all ewes and their fetuses/lambs had undergone surgery to place fetal catheters and/or telemetry devices for study in other protocols (Table 4). Ewes were housed and euthanized, and tissues were sampled as described by Yu et al. (12). For all animals, the tissues were collected from the fetus/lamb, including liver, lung, spleen, and stool. At necropsy of the pregnant ewes, the uterus was exposed and incised, and the fetus was removed for collection of fetal liver, spleen, and cerebral cortex samples. Lamb samples (5 days postnatal) were also collected.

In all cases, tissue samples were dissected on a sterile surface using instruments that were baked at 260°C for at least 12 h, and tissue samples were collected in sterile culture tubes and snap-frozen in liquid nitrogen. Surgical instruments were autoclaved prior to each surgery, and gloves were changed at appropriate intervals to prevent cross-contamination between tissues. All snap-frozen tissues were stored at −80°C. Contact surfaces on the necropsy tables were swabbed before and during necropsy procedures. The tables, lab benches, and air ducts were sampled with sterile swabs (wet and dry). Sterile swabs were dipped in sterile nuclease-free water before contact with the desired surfaces. Oral and vaginal cavities were swabbed in four of the ewes, numbers 13 to 17. Environmental sites included the necropsy room, floors, walls, wet and dry air ducts, air around fetal and maternal organs, and necropsy tables, in addition to maternal and fetal tissue sites (Table 4). Swab tips were cut, placed in sterile 1.5-ml microcentrifuge tubes, and stored at 4°C until culturing or extraction later that day.

Necropsy environmental sampling. In five of the surgeries, necropsy room sites were sampled with sterile swabs before and during necropsy. Two sets of autoclaved cotton swabs were used to collect environmental samples. One set was briefly dipped into sterile water before sample collection. The second set was directly used for sample collection after removal from autoclave package. Prenecropsy environmental samples were collected from the necropsy room before the animal entered. Swabs were collected from the necropsy table where the carcass was to be dissected, including the following sites: (i) the necropsy table where the fetus was to be dissected, (ii) the lab bench where samples were to be placed, (iii) the air duct, and (iv) room air. During necropsy, a second set of environmental samples was collected by swabbing of the necropsy tables as well as the air above the fetal body during dissection (fetal air) and the air above the ewe during dissection of the ewe (maternal air) and air duct. Swab heads

were carefully snapped off without contact with the cotton and sealed inside individual sterile 1.5-ml Eppendorf tubes. Tubes were set on ice and stored in 4°C for culturing and DNA extraction which followed later that day.

Swab sampling of animal housing rooms before and after sanitization. To test for the presence of bacteria derived from the animal housing room, swabs were taken from the animal housing room and compared to samples taken before and after necropsy. Two sets of autoclaved cotton swabs were used to collect animal room environmental samples. The first set of swabs was dipped in sterile water, and the second set was used directly from sterile packaging. Swabs were wiped over animal cage floors, cage walls, air duct, and room air. Sampling methods were the same as previously described for swabbing during necropsy.

DNA extraction and 16S rRNA gene amplification. DNA was extracted from 30 mg of cerebral cortex, liver, and intestinal contents ($n = 50$). Whole DNA in tissue samples was extracted using an E.Z.N.A. Tissue DNA kit (Omega-Biotek, Norcross GA), while DNA from environmental swabs was extracted using an E.Z.N.A. Bacterial DNA kit (Omega-Biotek, Norcross, GA). Intestinal contents were processed using an Omega BioTek E.Z.N.A. Stool DNA kit (Omega-Biotek, Norcross, GA). DNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Negative-control swabs were used to assess any potential contamination across samples and kit reagents during this process. Barcoded PCR of the V3 and V4 region of the 16S rRNA gene was done as described previously (15). Swabs and tissue DNA that produced appropriately sized amplicons were submitted for 16S rRNA gene sequencing.

High-throughput, 16S rRNA sequencing was done on an Illumina MiSeq platform at the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida. An average of 11.8 million reads were generated. Read processing and classification were performed as previously described (https://github.com/audy/miseq-16S-pipeline). PandaSeq was used to merge overlapping paired ends (16). Reads were processed using dada2 (17) as described previously to a length of 420 bases (15). Following filtering of primer sequences and chimeras using the dada2 function removeChimeraDenovo, 2.5 million reads and 10,130 operational taxonomic units (OTUs) were discarded from the run. ASV tables were generated using the phyloseq function otu_table. These tables were used for community-level analysis.

Statistical analysis. Shannon diversity data were tested for normality ($P < 0.05$) using a Shapiro-Wilk test (18) and ratio of variances (0.875). Analysis of similarities (ANOSIM) was performed, and nonmetric multidimensional scaling (NMDS) using Bray-Curtis distance (Bray-Curtis value of < 0.2) plots were generated by the phyloseq package. All graphical analyses were performed with R, version 3.3.0 (http://www.R-project.org), and the ggplot2 package (19).

Culturing of environmental swabs and tissue homogenates. Environmental swabs were tested for the presence of live bacteria by culturing on brain heart infusion (BHI) broth, tryptic soy agar (TSA), and TSA–5% sheep blood agar under both anaerobic and aerobic conditions. Swabs were removed from their tubes with sterile tweezers and struck onto medium. Intestinal contents and cerebral cortex samples were also tested. These samples (50 mg) were mechanically homogenized in 500 μl of sterile 1× phosphate-buffered saline (PBS) solution and serially diluted 1:10 before 100 μl of each dilution was spread on solid medium. All plates were incubated at 37°C for approximately 4 days and then screened for the presence of live colonies.

Colony PCR and real-time quantitative PCR. Colony PCR and Sanger sequencing were performed on morphologically distinct colonies cultured per sample by transferring cells to 20 μl of sterile distilled H_2O (dH_2O) and denaturing for 10 min at 95°C, followed by centrifugation at 10,000 rpm for 3 min. Full-length 16S rRNA genes were amplified from the supernatants using forward (8F-AGAGTTTGATCCT GGCTCAG) and reverse (1492R-GGTTACCTTGTTACGACTT) primers as described previously (4). Samples taken from the same colonies were also transferred to 2 ml of BHI broth, incubated overnight, stored in with 50% glycerol, and set in a freezer at −80°C for long-term storage. These cultures were identified to species level using Sanger sequencing (Table 2). Real-time quantitative PCR was performed as described by Nadkarni et al. (20) using a real-time MX3000p PCR system (Stratagene/Thermo Fisher Scientific, MA). Each reaction mixture included a no-template control and *Staphylococcus simulans* DNA as a positive control. The thermal profile was run as described previously (4). All DNA samples were processed in triplicate, and the mean was used for graphical analysis in R Studio (https://www.rstudio.com/) with the ggplot package (https://ggplot2.tidyverse.org/). Averages taken for each set of triplicate values were expressed as an estimate of bacterial genera per gram of host tissue.

Data availability. The data set supporting the results of this article is available in the NCBI Sequence Read Archive (SRA) under accession number PRJNA513299.

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