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The nasopharyngeal, ruminal, and vaginal microbiota and the core taxa shared across these microbiomes in virgin yearling heifers exposed to divergent in utero nutrition during their first trimester of gestation and in pregnant beef heifers in response to mineral supplementation

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ABSTRACT:

Emerging evidence has indicated that microbial transmission from the bovine dam to her fetus may take place before birth, and that the maternal microbiota during pregnancy modulates programming of fetal metabolic and nervous system development, highlighting the potential and extended role of the maternal microbiome in calf health and development. In the present study, we characterized the nasopharyngeal, ruminal and vaginal microbiota from two cohorts of beef heifers managed at the same location: 1) virgin yearling heifers (9 months old) born from dams received gestational diets which resulted in low (LG, n = 22) or medium (MG, n = 23) weight gain during the first 84 days of gestation; and 2) pregnant replacement heifers that received a vitamin and mineral supplement (VTM, n = 17) or not (Control, n = 15) during the first 6 months of gestation. Nasopharyngeal and vaginal swabs as well as ruminal fluid were collected from both cohorts and the microbiota of each sample was assessed using 16S rRNA gene sequencing. In addition to the comparison between treatment groups within each cohort, the similarity of the microbiota of the three sample types were evaluated, and shared taxa amongst these communities were identified. The bacterial genera present in the rumen and vagina that can influence methanogenic archaeal genera were predicted using a stepwise-selected generalized linear mixed model. No significant difference was observed in the alpha and beta diversity in any of the nasopharyngeal, ruminal and vaginal microbiota between LG and MG offspring virgin heifers, or between the control and VTM pregnant heifers ($p > 0.05$). Subtle compositional changes in the vaginal microbiota in yearling heifers, and in the nasopharyngeal and ruminal microbiota of pregnant heifers were detected in response to treatments. Forty-one archaeal and bacterial OTUs were shared by over 60% of all samples from both virgin and pregnant heifers. Two taxa within the *Methanobrevibacter* genus were identified as core taxa and this genus was more relatively

abundant in pregnant heifers compared to virgin heifers. Among the 25 top genera, *Prevotella* and *Prevotella* UCG-003 (negative) and *Christensenellaceae R-7* group (positive) were predicted to have a significant effect on ruminal *Methanobrevibacter* spp. The results of this study indicate that there is little impact of divergent gestational nutrition during the first trimester on the calf microbiome at 9 months postnatal, and that VTM supplementation during pregnancy may not alter the maternal microbiome. This study provides evidence that there are several microbial taxa, including methanogenic archaea, that are shared across the respiratory, gastrointestinal, and reproductive tracts, suggesting the need for a holistic evaluation of the bovine microbiota when considering potential maternal sources for seeding calves with pioneer microbiota.

Keywords: Beef heifers, Core taxa, Maternal utrition, Nasopharyngeal microbiota, Offspring, Ruminal microbiota, Vaginal microbiota.

INTRODUCTION

Host genetic selection has been a primary target for improving animal health and productivity over the last several decades and has resulted in tremendous progress in both dairy and beef cattle production systems. Recently, the microbiota colonizing different mucosal surfaces of cattle have become a new target for manipulation/engineering with great potential to improve animal health and production (Huws et al., 2018; Matthews et al., 2019). Diverse and dynamic microbial communities present in the respiratory, gastrointestinal and reproductive tracts of cattle are vital to health and performance (Galvão et al., 2019; Matthews et al., 2019; Timsit et al., 2020). Among these microbial communities, the ruminal microbiota in cattle, which is the most densely populated and involved in both nutrient metabolism and immune system development, has become the primary target for manipulation/engineering (O'Hara et al., 2020).

Recent developments including the advent of high-throughput sequencing techniques, heritable ruminal microbiota compositional changes that are associated with feed efficiency (Difford et al., 2018; Li et al., 2019) and methane emission phenotypes in cattle (Difford et al., 2018), suggest that the ruminal microbiome and host genetics can be targeted independently to improve feed efficiency and mitigate enteric methane emissions from cattle. One of the challenges associated with manipulation of the ruminal microbiome in mature animals is its resiliency that allows the microbiome to revert to the original state following the cessation of an intervention (Weimer, 2015). To overcome this challenge, early life microbial programming in young ruminants was recommended and has shown some efficacy (Yáñez-Ruiz et al., 2015; Saro et al., 2018; Belanche et al., 2020). For example, Palma-Hidalgo et al. (2021) reported that the direct inoculation of fresh ruminal fluid from adult goats to kids in early life accelerated ruminal microbial community development and improved the weaning process. Early life microbial

94 programming is based on the current dogma that microbial colonization of the rumen starts at birth,
95 and the developing ruminal microbiota within the first 3 weeks of life is less resilient to
96 manipulation (Yáñez-Ruiz et al., 2015). A recent study by Guzman and colleagues (2020),
97 however, provided sequencing and culture-based evidence indicating that the intestine of calf fetus
98 is not sterile and colonization by so-called "pioneer" microbes may occur during gestation. This is
99 further supported by our preliminary data which suggested that colonization of the fetal intestine
100 by archaea and bacteria may take place within the first 12 weeks of gestation in cattle (Amat et al.,
101 unpublished data). These observations highlight the potential and extended role of the maternal
102 microbiome in calf ruminal microbiome development.

103 Although the role of maternal nutrition in programming of the offspring metabolic, immune
104 and nervous system development has been well documented in humans and food-producing
105 animals including cattle (Palmer, 2011; Caton et al., 2019), the potential involvement of the
106 maternal microbiome in the developmental origins of health and disease has recently began to be
107 better appreciated (Stiemsma and Michels, 2018; Calatayud et al., 2019; Codagnone et al., 2019).
108 It was hypothesized that undesired alterations in the maternal microbiota could indirectly influence
109 fetal development, and that these effects may subsequently be transmitted to progeny, resulting in
110 the development of an altered microbiota in offspring (Calatayud et al., 2019). Undesirable
111 outcomes in offspring resulting from changes in the maternal microbiota include increased
112 susceptibility to the development of metabolic disorders and respiratory infections (Calatayud et
113 al., 2019; Yao et al., 2020). Recent evidence from studies in mice demonstrated that the maternal
114 microbiota during pregnancy modulates the programming of fetal metabolic and nervous system
115 development (Kimura et al., 2020; Vuong et al., 2020). Considering the increased evidence
116 showing the importance of the maternal microbiota in developmental programming in rodent

models, and the greater evidence regarding the involvement of the microbiome in defining cattle health and productivity, exploring the role of the maternal microbiota in fetal programming and offspring microbiome development may provide important information for improving cattle health and feed efficiency.

In the present study, we used 16S rRNA gene sequencing to characterize the nasopharyngeal, ruminal and vaginal microbiota of virgin yearling heifers from dams given different nutritional diets during their first trimester of gestation, and in pregnant beef heifers in response to direct feeding of a mineral and vitamin (VTM) supplement during the first 6 months of gestation. Of note, a well-defined positive impact of maternal VTM supplementation exists on offspring health and performance in beef cattle, and the role of VTM on fetal programming assessed during the first trimester of pregnancy have been documented (Mee et al., 1995; Wilde, 2006; Van Emon et al., 2020; Diniz et al., 2021; Menezes et al., 2021). Questions remain, however, pertaining to whether these maternal VTM supplementation-associated positive outcomes are dependent on VTM-induced alterations of ruminal microbiota. To provide a more holistic view of the microbiota residing within the respiratory, gastrointestinal, and reproductive tract of cattle, the similarity of the microbiota within these sites was evaluated, and taxa shared amongst the three microbial habitats were identified. Given the relevance of these microbial communities to respiratory and reproductive health and rumen fermentation/nutrient metabolism, and most importantly, as potential maternal inoculant sources for seeding the fetal and offspring microbiota, a holistic evaluation of bovine microbiota is therefore necessary rather than focusing on only one microbial community.

MATERIALS AND METHODS

Animals used in this study were cared for in accordance with the guidelines set by the Olfert et al. (1993) and all experimental procedures involving cattle were approved by the North Dakota State University Institutional Animal Care and Use Committee (#A20085 and #A20085, for virgin yearling heifers and for pregnant heifers, respectively).

Animal Husbandry and Experimental Design

Virgin yearling heifers:

Deep nasopharyngeal swabs, ruminal fluid, and vaginal swabs were collected from 45 F1 virgin heifers (9-month-old, BW = 688 ± 57 kg) whose dams were assigned to either a low gain treatment (**LG**, targeted average daily gain of 0.28 kg/d, n = 22) or a moderate gain treatment (**MG**, 0.79 kg/d, n = 23) during the first 84 days of gestation. To achieve the LG, dams were maintained on a basal diet consisting of prairie grass hay, corn silage, and dried distillers grains plus solubles. To achieve the MG (0.79 kg/d), heifers were fed the basal diet with the addition of a protein/energy supplement fed at the rate of 0.58% BW as-fed daily. Up to d 84 of gestation dams were housed and individually fed (Insentec; Hokofarm B.V. Repelweg 10, 8316 PV Marknesse, the Netherlands) at the Beef Cattle Research and Extension Center (BCRC) in Fargo, ND. After day 84 of gestation, dams were transported to the Central Grasslands Research Extension Center (CGREC) in Streeter, ND, where they were managed as a single group on common diets until parturition and subsequent weaning of the F1 offspring. Upon weaning, the F1 heifers (approx. 6-months old) were transported to the BCRC where they were housed in two pens and individually fed (Insentec; Hokofarm B.V. Repelweg 10, 8316 PV Marknesse, the Netherlands) a common diet (Table 1).

Pregnant heifeirs:

The nasopharyngeal, ruminal, and vaginal microbiota of the replacement pregnant heifers (1 year 9 months old, BW = 1001 ± 128 kg) during the sixth month of gestation were also evaluated. At breeding, heifers were assigned to one of two treatments: 1) vitamin and mineral supplementation (**VTM**; n = 17) or 2) no vitamin and mineral supplementation (**Control**; n = 15). Heifers were housed at the BCRC and individually fed (Insentec; Hokofarm B.V. Repelweg 10, 8316 PV Marknesse, the Netherlands) a total mixed ration containing triticale hay, corn silage, modified distillers grains plus solubles, ground corn, and, if indicated by treatment, a VTM premix (Table 1). The VTM premix was fed at 0.45 kg/heifer/day to provide macro and trace minerals and vitamins A, D, and E to meet 110% of the daily requirements (NASEM, 2016). The specific ingredients within the VTM supplement are as previously described (Menezes et al., 2021).

Nasopharyngeal swab, ruminal fluid, and vaginal swab sampling

Nasopharyngeal swabs, ruminal fluid and vaginal swabs were collected simultaneously from each of the virgin yearling and pregnant heifers by same personnel on the same day. All sample collection was completed within 4 hours.

Nasopharyngeal sampling: Deep nasopharyngeal swabs were collected as previously described (Holman et al., 2017; Amat et al., 2019). Briefly, prior to sampling, the right nostril of the heifer was wiped clean with 70% ethanol and an extended guarded swab (27 cm) with a rayon bud (MW 128, Medical Wire & Equipment, Corsham, England) was used for sampling. Swab tips were then be cut and placed in a sterile 2 mL centrifuge tube on ice until processing. Upon arrival in the lab, the swab was transferred into 1 mL sterile brain heart infusion (BHI) broth containing 20% glycerol stock.

Rumen fluid sampling: Rumen fluid sample collection was performed using the method currently used in our laboratory which was modified from Paz et al. (2016). Briefly, a rigid metal

speculum was placed into the mouth of the heifer and then a flexible plastic tube with multiple holes at the tip was passed through the speculum and into the esophagus. The speculum was used to ensure that the plastic tube was not damaged by the heifers and that the tube entered the esophagus. Once the tube entered the rumen, and was below the ruminal mat layer, suction pressure was applied to the tube to collect ruminal fluid. Up to 120 mL of ruminal fluid was collected on each sampling day. Separate tubing and containers were used for each heifer to avoid cross-contamination. After thorough mixing, an aliquot of 40 ml of rumen fluid was placed into a 50 mL falcon tube and immediately frozen with dry ice.

*Vaginal sampling***:** For vaginal sampling, the vulva was thoroughly cleaned with 70% ethanol and a paper towel. Then the labia majora of the vulva was held open allowing the passage of a swab (15 cm, sterile cotton tipped applicators with aerated tip protector; Puritan). When the swab tip reached the midpoint of the vaginal cavity, it was placed against the vaginal wall, swirled four times, and then withdrawn carefully to minimize contamination. The vaginal swabs were immediately placed in sterile Whirl Pak bags and transported on ice to the lab. All nasopharyngeal and vaginal swabs as well as rumen fluid were stored at -80^{o}C until DNA extraction.

Metagenomic DNA extraction

Metagenomic DNA was extracted from the nasopharyngeal and vaginal swabs using a Qiagen DNeasy Tissue kit (Qiagen Inc., Germantown, MD, USA) according to the kit manual with minor modifications. Briefly, the cotton tip of the nasopharyngeal swab was removed and placed back into the BHI-glycerol mixture, and then centrifuged at 20,000 × g for 5 min at 4^{o}C to pellet the cotton and microbes. The pellet was then re-suspended in 180 µl of enzymatic buffer. The enzymatic buffer [20 mM Tris.CI (pH 8.0), 2mM sodium EDTA, and 1.2% Triton X-100] contained 300 U/ml mutanolysin and 20 mg/ml lysozyme. The mixture was then vortexed and

incubated for 1 h at 37°C with agitation at 800 rpm. After incubation, 25 µl proteinase K and 200 µl Buffer AL (without ethanol) were added and vortexed, and then incubated at 56°C for 30 min with agitation at 800 rpm. Approximately 400 mg of 0.1 mm zircon/silica beads were added to the tube and subjected to mechanical cell lysis using a FastPrep-24 Classic bead beater (MP Biomedicals, Irvine, CA) at 4.0 m/s for 24 s. The mixture was then centrifuged (13,000 × g for 5min), and the supernatant (approx. 300-400 µl) was removed and placed in a new tube and mixed with an equal volume of 100% ethanol. From this step onward, the procedures were performed as described in the DNeasy Tissue Kit instruction manual.

The procedures for metagenomic DNA extraction from the vaginal swabs were identical to those used on the nasopharyngeal swabs with the following exceptions. First, the cotton swab was removed from applicator and placed in a sterile 2 mL centrifuge tube. Then, 360 µl of enzymatic buffer was added to the tube to ensure complete emersion of the swab in the enzymatic buffer. Metagenomic DNA from the rumen fluid samples was extracted using the Qiagen DNeasy PowerLyzer PowerSoil kit (Qiagen Inc.) according to the instructions of manufacturer. The frozen rumen fluid samples were thawed, and vortexed thoroughly before transfer to a sterile 2 mL microfuge tube. The sample was then centrifuged at 20,000 × *g* for 10 min at 4°C to pellet the microbes in the sample. From this point onwards, the protocol for the PowerLyzer PowerSoil kit was followed as per the instructions of the manufacturer. Negative extraction controls were included for all extraction kits.

16S rRNA gene sequencing and analysis

The V3-V4 hypervariable regions of the 16S rRNA gene were amplified using the 341-F (5'-CCTAYGGGRBGCASCAG-3') and 806-R (5'-GGACTACNNGGGTATCTAAT-3') primers. All PCR steps were carried out using the Phusion High-Fidelity PCR Master Mix (New England

Biolabs). The PCR products were electrophoresed on a 2% agarose gel and stained with SYBR Safe DNA gel stain. The DNA fragment was excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen Inc.,). Sequencing libraries were generated with NEBNext Ultra DNA Library Prep Kit (New England BioLabs, Ipswich, MA, USA) for Illumina, following the recommendations of the manufacturer. The library quality was assessed with a Qubit 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Libraries were then sequenced on a NovaSeq 6000 instrument with a SP flow cell (2 x 250 bp) (Illumina Inc., San Diego, CA, USA).

The 16S rRNA gene sequences were processed using DADA2 v. 1.18 (Callahan et al., 2016) in R. 4.0.3. Briefly, the forward reads were truncated at 225 bp and the reverse reads at 220 bp. The reads were merged, chimeric sequences removed, and taxonomy assigned to each merged sequence, referred to here as operational taxonomic units (OTUs) at 100 % similarity, using the naïve Bayesian RDP classifier (Wang et al., 2007) and the SILVA SSU database release 138 (Quast et al., 2013). OTUs that were predominantly in the negative extraction control samples and likely to be contaminants were removed prior to analyses as were those OTUs classified as chloroplasts, mitochondria, or eukaryota. The number of OTUs per sample (richness), the Shannon and inverse Simpson's diversity indices, and Bray-Curtis dissimilarities were calculated in R using Phyloseq 1.34.0 (McMurdie and Holmes, 2013) and vegan 2.5-7 (Oksanen et al., 2013). To account for uneven sequence depths, samples were randomly subsampled to 7,100, 73,500, and 10,300 for the nasopharyngeal, ruminal, and vaginal samples respectively, prior to the calculation of Bray-Curtis dissimilarities and diversity measures for the virgin heifers. For the pregnant heifers, these values were 6,200, 74,500, and 8,200.

252 **Statistical Analysis**

253 Permutational multivariate analysis of variance (PERMANOVA; adonis2 function; 10,000
254 permutations) of the Bray-Curtis dissimilarities was performed using vegan to determine the effect
255 of maternal nutrition on the nasopharyngeal, ruminal and vaginal microbial community structure
256 in virgin heifers whose dams were managed to targeted LG or MG during the first 84 days of
257 gestation. The effect of VTM supplementation on the microbial community structure of these three
258 microbiotas in pregnant heifers was also assessed. Differentially abundant genera between
259 treatment groups for both the virgin and pregnant heifers were identified using MaAsLin2 v. 1.5.1
260 in R (Mallick et al., 2021). Only those genera with a relative abundance greater than 0.1% within
261 each sample type were included. Diversity metrics were compared by treatment for both virgin
262 and pregnant heifers using an unpaired *t*-test. The number of OTUs (richness), diversity indices,
263 relative abundance of the most relatively abundant genera between the LG and MG groups of
264 virgin yearling heifers, or between the VTM and Control groups of pregnant heifers, and the
265 relative abundance of *Methanobrevibacter* spp. between the virgin and pregnant heifers were
266 compared using the generalized liner mixed model estimation procedure (PROC GLIMMIX) in
267 SAS (ver. 9.4, SAS Institute Inc. Cary, NC). The means were compared using the LSMEANS
268 statement and significance was declared at $P < 0{\cdot}05$.

269 Spearman's rank-based correlations between *Methanobrevibacter* and the other 24 most
270 relatively abundant genera in the ruminal and vaginal microbiota were calculated using the CORR
271 procedure in SAS with the SPEARMAN option. From these 24 genera, the genera that have
272 significant effect on *Methanobrevibacter* abundance were predicted using a stepwise-selected
273 GLIMMIX model with beta-binomial distribution as described previously (Amat et al., 2019). The
274 model used was: logit $(\hat{Y}) = \ln(\pi/(1-\pi)) = b0 + b1\,(X1) + \ldots + bn\,(Xn)$, where π represents the

relative abundance of the *Methanobrevibacter* genus (0–1) and Xn represents the relative abundance (0–100%) of a bacterial genus n. The stepwise selection method involved backward elimination and forward selection to eliminate any variables in the model that have no significant effect ($p > 0.05$) on the predicted outcome.

RESULTS

Sequencing Results

An average of 66,045 ± 31,588 (SD) 16S rRNA gene sequences per sample (min. = 2,374; max. = 139,012) were obtained from 219 nasopharyngeal, ruminal fluid, and vaginal samples. From these sequences, a total of 81,391 archaeal and bacterial OTUs were identified and classified into 58 unique phyla (8 archaeal and 50 bacterial phyla), and 1,511 unique genera.

Effect of Maternal Restricted Gain During the First Trimester of Gestation on Offspring Microbiota Development

To determine the effect of maternal nutrition during the first trimester of gestation on microbial populations of their offspring, we characterized and compared the nasopharyngeal, ruminal and vaginal microbiota of virgin yearling heifers from two groups of dams that were subjected to diets resulting in either a LG or MG phenotype during the first 84 days of gestation. The microbial community structure of the nasopharynx (PERMANOVA: $R^2 = 0.027$, $p = 0.57$), rumen (PERMANOVA: $R^2 = 0.02$, $p = 0.98$) and vagina (PERMANOVA: $R^2 = 0.028$, $p = 0.37$) in the virgin heifers did not differ between the LG and MG groups (Fig. 1A). Microbial richness and diversity as measured by the number of OTUs, and the Shannon and inverse Simpson's diversity indices of these microbiotas also did not significantly differ by maternal nutrition group

297 (Fig. 1B, C and D; $p > 0.05$). There was, however, a strong tendency ($p = 0.06$) in LG offspring to
298 harbor a richer ruminal microbial community compared to MG offspring (2605 vs. 2515 OTUs).
299 The nasopharyngeal microbiota across all animals was dominated by *Actinobacteriota*
300 (51%), *Firmicutes* (28.2%), *Bacteroidota* (10.8%) and *Proteobacteria* (4.9%). The relative
301 abundance of the eight most relatively abundant phyla did not differ between LG and MG virgin
302 heifers ($p > 0.05$) (Fig. 2A). *Bacteroidota* was the most relatively abundant phylum in the ruminal
303 microbiota (65.5%) followed by *Firmicutes* (24.2%). As with the nasopharyngeal microbiota,
304 none of the eight most relatively abundant phyla in the rumen microbiota differed between the two
305 treatment groups. The most relatively abundant phylum present in the vaginal tract was *Firmicutes*
306 (52%) followed by *Bacteroidota* (23.0%) and *Actinobacteriota* (17.4%). Similar to the rumen and
307 nasopharyngeal microbiota, no difference between treatments was detected in the relative
308 abundance of eight most relatively abundant phyla in the vaginal microbiota ($p > 0.05$).
309 The 25 most relatively abundant genera in the nasopharyngeal, ruminal and vaginal
310 microbiota are listed in Table 2. Overall, the predominant nasopharyngeal genera did not differ in
311 their relative abundance between the LG and MG groups ($p > 0.05$). In the rumen, the relative
312 abundance of only one genus ([*Eubacterium] ruminantium group)* was significantly different
313 between the two groups, being greater in the MG group than in the LG group ($p = 0.029$). Within
314 the vaginal microbial community, *Alistipes*, *Ruminococcus* and *Oscillospiraceae* NK4A214 group
315 were significantly less relatively abundant in the LG group compared to the MG group ($p < 0.05$).
316 The relative abundance of *Romboutsia* ($p = 0.061$) and *Paeniclostridium* ($p = 0.092$) tended to be
317 lower while *Arcanobacterium* ($p = 0.090$) tended to be higher in MG group compared to LG group.
318 **Effect of Vitamin and Mineral Supplementation During the First Six Months of Gestation**
319 **on the Maternal Microbiota**

To investigate whether VTM supplementation during the first 6 months of gestation affects the maternal microbiota, we compared the nasopharyngeal, ruminal and vaginal microbiota between the VTM and Control groups of replacement pregnant heifers. The community structure of the nasopharyngeal (PERMANOVA: $R^2 = 0.038$, $p > 0.05$), ruminal (PERMANOVA: $R^2 = 0.032$, $p > 0.05$) and vaginal (PERMANOVA: $R^2 = 0.049$, $p > 0.05$) microbiota was not affected by VTM supplementation (Fig.2A). Microbial richness and diversity also did not differ by VTM supplementation in any of the three microbial communities ($p > 0.05$) (Fig. 2B, C and D). At the phylum level, the relative abundance of the eight most relatively abundant phyla in the nasopharynx, rumen and vagina did not differ between the control and VTM groups (Fig. 3B). However, the relative abundance of several genera present in the nasopharynx (5 genera) and rumen (3 genera) was affected by VTM supplementation ($p < 0.05$, Table 3).

Mycoplasma, the third most relative abundant genera in the nasopharyngeal microbiota, was enriched in pregnant heifers receiving the VTM supplement (8.95% vs. 2.74%, $p = 0.039$). VTM supplementation also resulted in a reduced relative abundance of *Oscillospiraceae* UCG-005, *Christensenellaceae R-7 group*, *Ruminococcus* and *Ornithinimicrobium* genera ($p < 0.05$). Among the 26 most predominant ruminal genera, statistically significant difference in relative abundance was observed in only three genera (*Oscillospiraceae* NK4A214 group, *Butyrivibrio* and *Ruminococcaceae* CAG-352), and all of which were enriched in the VTM group ($p < 0.05$). The relative abundance of the 27 relatively most abundant genera in the vaginal microbiota did not differ between the VTM and control groups ($p > 0.05$) (Table 3).

Holistic View of Nasopharyngeal, Ruminal and Vaginal Microbiota and Identification of Core Taxa Shared Across These Microbiomes

342 To provide a holistic view of the microbiota residing within the respiratory,
343 gastrointestinal, and reproductive tracts of each animal, we attempted to identify similarities
344 among these microbial communities in virgin and pregnant heifers. To do this, the sequence data
345 from all animal groups and treatments were combined and all samples were randomly subsampled
346 to 6,200 sequences. As expected, each anatomical site had a distinct microbiota (Fig.4). In terms
347 of microbial richness, the rumen had the richest microbiota followed by the vagina and
348 nasopharynx in both virgin (Fig.1B) and pregnant heifers (Fig. 2B). Overall, the ruminal and
349 vaginal microbiota were also more diverse than the nasopharyngeal microbiota in both groups of
350 heifers (Fig. 1C and D and Fig. 2C and D).

351 Many taxa appeared to be highly specific to one of the three microbial habitats as shown
352 in the heatmap (Fig. 5). For example, OTUs classified as *Prevotella*, *Papillibacter*,
353 *Oscillospiraceae NK4A214* group and *Pseudobutyrivibrio* were more exclusively present in the
354 rumen. While the most of the OTUs within the archaeal *Methanobrevibacter* genus were present
355 in all three habitats, the rumen was most predominantly colonized by members of this genera.
356 Some taxa, including *Mycoplasma*, *Filobacterium*, *Streptomyces*, *Nocardioides*, *Marmoricola*,
357 *Arthrobacter* and *Cellulomonas spp.*, were associated with the nasopharynx. Certain
358 *Corynebacterium* OTUs appeared to be specific to the vaginal microbiota.

359 Although the nasopharyngeal, ruminal and vaginal microbiota were clearly distinct, a small
360 number of taxa were present in all three microbial communities. We identified 43 OTUs that were
361 shared by more than 60% of all samples from the virgin yearling heifers (Table 4). From these
362 OTUs, two were classified as *Methanobrevibacter* (OTU8 and OTU23). The remaining shared
363 OTUs were bacterial in origin, with 17% and 80% of them belonging to the *Actinobacteria* and
364 *Firmicutes* phyla, respectively. Of note, three bacterial OTUs [OTU25 (*Eubacterium*

coprostanoligenes group; OTU561(*Colidextribacter*) and OTU29 (*Ruminococcus*)] were shared by more than 90% of the samples. In pregnant heifers, 47 OTUs were present in more than 60% of all samples (Table 5), and most of them were identical to those taxa shared among the virgin yearling heifer samples. One taxon identified as *Romboutsia ilealis* (OTU11) was found in 100% of the samples, and OTU24 (*Paeniclostridium*), OTU25, OTU29 and OTU68 (*Bifidobacterium pseudolongum*) were identified in 95% of the samples.

As listed in Table 7, 41 OTUs were identified in 60% of all virgin yearling and pregnant heifer samples. Nine of these OTUs were also present in more than 80% of the samples. These included OTU23 (*Methanobrevibacter ruminantium*), OTU26 (*Corynebacterium*), three OTUs (OTU25, OTU62 and OTU1688) within the *Lachnospiraceae* NK3A20 group, two *Ruminococcus* OTUs (OTU29 and OTU83), OTU24 and OTU11. OTU68 was also found in 75% of the samples. Regardless of animal group and diet, these OTUs were shared by a high proportion of the nasopharyngeal, ruminal fluid, and vaginal samples, suggesting that these taxa may be so-called "core taxa" among these anatomical locations.

Comparison of Methanogenic Archaeal Relative Abundance Between Virgin Yearling and Pregnant Heifers, and Identification of Bacterial Genera Associated with *Methanobrevibacter*

Methanogenic archaea, and in particular members of the *Methanobrevibacter* genus, have been reported to colonize the intestine of 84-day-old (Amat et al., unpublished), and 5- to 7-month-old calf fetuses (Guzman et al., 2020), as well as newborn calves (Guzman et al., 2015; Alipour et al., 2018). In addition, we identified here two *Methanobrevibacter* OTUs (OTU8 and OTU23) that were shared by a relatively high portion (≥ 65%) of all samples collected from both virgin yearling and pregnant heifers (Table 4). Therefore, we assessed whether the relative abundance of

388 *Methanobrevibacter* changed in response to pregnancy. For this, we compared the overall relative
389 abundance of *Methanobrevibacter* spp. within each sample type between virgin yearling (non-
390 pregnant) and pregnant heifers. Overall, the mean relative abundance of *Methanobrevibacter* in
391 the nasopharynx, rumen fluid and vagina was 0.17%, 5.67%, and 0.47%, respectively (Fig.6A).
392 The relative abundance of *Methanobrevibacter* in the rumen was greater in pregnant heifers
393 compared to yearling heifers ($p < 0.0001$), but similar in the other two microbial habitats ($p > 0.05$)
394 (Fig. 6B and D).

395 There is increased research interest in the mitigation of enteric methane emissions from
396 ruminant livestock via the manipulation of the rumen microbiota, primarily targeting commensal
397 bacterial species involved in the supply or consumption of methanogenic substrates. Therefore, we
398 assessed correlations between *Methanobrevibacter* and the other 24 most relatively abundant
399 genera present in vaginal and ruminal microbiota of virgin yearling and pregnant heifers. Of note,
400 the relative abundance of *Methanobrevibacter* in the nasopharynx was relatively low compared to
401 the vagina and rumen and therefore, only genera in the vaginal and ruminal microbiota were
402 included for this correlation analysis. The Spearman correlation analysis revealed that 15 out of
403 these 24 genera in the rumen of virgin yearling heifers exhibited significant ($p > 0.05$) correlations
404 with *Methanobrevibacter*. Among which, the following 10 genera were positively correlated:
405 *Christensenellaceae* R-7 group, *Ruminococcus*, *Oscillospiraceae* NK4A214 group, *Papillibacter*,
406 *Pseudobutyrivibrio*, *Prevotellaceae* NK3B31 group, *Lachnospiraceae* NK3A20 group,
407 *Lachnospiraceae* XPB1014 group, *Eubacterium hallii group*, *Butyrivibrio* and *Olsenella*.
408 Whereas genera within the *Prevotellaceae* family (*Prevotella*, *Prevotellaceae* UCG-003 and
409 *Prevotellaceae* UCG-001) and *Anaeroplasma* were strongly and inversely associated with
410 *Methanobrevibacter* (Fig.7A). Varying degrees of positive or negative associations among the

411 *Methanobrevibacter*-associated 15 genera and between other genera were also identified (Fig.7A).
412 Within the vaginal microbial community of virgin yearling heifers, there were only three genera
413 (*Monoglobus*, *Akkermansia* and *Rikenellaceae* dgA-11 gut group) that displayed significant
414 correlations with *Methanobrevibacter* ($p < 0.05$) and these were positive correlations (Fig. 7B).
415 In pregnant heifers, there were similar correlation patterns between *Methanobrevibacter*
416 and other ruminal genera in the yearling heifers, with 14 genera significantly ($P < 0.05$) and
417 positively (n = 8) or negatively (n = 6) correlated with this genus (Fig. 8A). In contrast to the
418 vaginal tract of virgin yearling heifers, there were 10 genera in the vaginal microbiota of pregnant
419 heifers that were significantly associated with *Methanobrevibacter*, nine of them positively
420 correlated. Interestingly, although *Prevotella* and *Prevotellaceae UCG-003* were inversely
421 correlated with *Methanobrevibacter* in the rumen of both virgin yearling and pregnant heifers, they
422 were strongly and positively correlated with this genus in the vagina microbiota. Only the inverse
423 correlations between *Methanobrevibacter* and *Corynebacterium* were significant ($p < 0.05$) (Fig.
424 8B).
425 Next, we applied a stepwise-selected generalized linear mixed model to further identify
426 genera that have a significant effect on the relative abundance of *Methanobrevibacter* spp. in the
427 rumen and vagina. In the virgin yearling heifers, *Prevotella* and the *Christensenellaceae* R-7
428 group were predicted to have a significant effect (p <.0001) on *Methanobrevibacter*
429 [1/(*Methanobrevibacter*$^{\wedge 2}$) = 0.02956 + (0.002514 × *Prevotella*) + (-0.00875 ×
430 *Christensenellaceae* R-7 group)]. As for pregnant heifers, *Methanobrevibacter* abundance was
431 predicted to be negatively affected by *Prevotellaceae UCG 003* ($p = 0.037$), and positively by
432 *Christensenellaceae* R-7 group ($p = 0.0326$) [1/(*Methanobrevibacter*$^{\wedge 2}$) = 0.03793 + (-0.00602 ×
433 *Christensenellaceae* R-7 group) + (0.007088 × *Prevotellaceae* UCG-003)]. Within the vaginal

434 microbial community of pregnant heifers, *Ruminococcus* (p = 0.0098), *Prevotellaceae* UCG-003
435 (p = 0.0005) and *Prevotella* (p <.0001) were predicted to have a significant negative effect on
436 *Methanobrevibacter* [1/(*Methanobrevibacter*$^{\wedge 2}$) = -0.3780 + (0.1501 × *Ruminococcus*) + (0.2875
437 × *Prevotella* UCG-003) + (0.2898 × *Prevotella*)]. Among the 24 most relatively abundant genera
438 in the vaginal microbiota of yearling heifers, only *Oscillospiraceae* UCG-005 (p = 0.0138) was
439 predicted to have a negative impact on *Methanobrevibacter* [1/(*Methanobrevibacter*$^{\wedge 2}$) = 0.05975
440 + (0.04037× *Oscillospiraceae* UCG-005).

441 **DISCUSSION**

442 New evidence from our laboratory (Amat et al., unpublished data) and Guzman and
443 colleagues (Guzman et al., 2020) indicate that microbial colonization of the calf gastrointestinal
444 tract may take place before birth. These observations suggest that the maternal microbiome may
445 have a role in shaping the development of the offspring microbiome in cattle. In addition, it is
446 believed that undesirable alterations of the maternal microbiota may indirectly influence fetal
447 development, and that these effects may be transmitted to progeny, resulting in a dysbiotic
448 microbiota (Calatayud et al., 2019) and increased offspring susceptibility to the development of
449 metabolic disorders and respiratory infections (Calatayud et al., 2019; Yao et al., 2020). Recent
450 evidence from mouse studies has demonstrated that the maternal microbiota during pregnancy
451 modulates the programming of fetal metabolic and nervous system development (Kimura et al.,
452 2020; Vuong et al., 2020).

453 Although the role of maternal nutrition in developmental programming in cattle has been
454 relatively well appreciated (McLean et al., 2017; Caton et al., 2019; Crouse et al., 2019; Menezes
455 et al., 2021), the potential involvement of the maternal microbiota in fetal programming and
456 offspring microbiome development remains largely unexplored. Considering the current evidence,

it is important to explore whether bovine maternal nutrition/microbiome during pregnancy influences feto-maternal crosstalk, subsequently influencing offspring microbiome development. Maternal vitamin and mineral supplementation before calving has been well documented to be associated with improved fetal programming and offspring health and productivity in cattle (Mee et al., 1995; Wilde, 2006; Van Emon et al., 2020; Diniz et al., 2021; Menezes et al., 2021). However, whether VTM supplementation-associated positive outcomes observed pre- and post-calving are dependent on alterations in the ruminal microbiota remains unexplored. In the present study, we evaluated whether differences in maternal weight gain during the first trimester of gestation affected the postnatal nasopharyngeal, ruminal, and vagina microbial communities of virgin heifers at 9 months of age. We also characterized and compared these three microbiota in pregnant heifers to evaluate the impact of VTM supplementation during the first six month of gestation on the maternal microbiome. Finally, we identified core taxa that are shared within the respiratory, gastrointestinal, and reproductive tract microbiota of cattle.

Effect of Maternal Restricted Gain During the First Trimester of Gestation on Offspring Microbiota Development

The virgin yearling heifers born from the dams that were subjected to LG (0.29 kg/d) during the first 84 days of gestation harbored a similar nasopharyngeal, ruminal and vaginal microbiota to those born from MG (0.79 kg/d) dams. The LG dams had a reduced average daily gain ($p < 0.01$) were 40 kg lighter than MG dams at calving ($p < 0.01$), and their calves had a lower birth weight than those from MG dams (28.6 vs. 30.8 kg, $p = 0.03$) (Baumgaertner, 2020). As previously reported, fetuses harvested from a subset of the LG and MG dams at 84 days of gestation exhibited distinct fetal metabolic programming (Menezes et al., 2021), including altered amino acid profiles in the fetal fluids (Menezes et al., 2021). In addition, we identified the presence

of an archaeal and bacterial microbiota in intestinal and fluid samples from these 84-day-old calf fetuses (Amat et al., unpublished data). Therefore, we hypothesized that the divergent microbiome may be detected in virgin heifers that were exposed to divergent in utero nutrition (i.e. LG or MG) during their first trimester of gestation. However, no significant effect of maternal nutrition was found on the microbial community structure in the offspring nasopharynx, rumen, or vagina. There may be many reasons for this finding, including the timing of sample collection. For example, samples were collected when the offspring heifers were at 9 months of age, which was about 15 months after fetal exposure to the restrictive maternal diets. This may simply be too late in their development to detect microbial community alterations in the offspring as a result of maternal nutrition. Therefore, future studies investigating the impact of maternal nutrient and microbiome on offspring microbiome development should include a more robust profile of early life microbiome measurements.

Effect of Vitamin and Mineral Supplementation During the First 6 Months of Gestation on Maternal Microbiota

VTM supplementation during the first 6 months of gestation did not induce significant alterations in community structure and diversity of the nasopharyngeal, rumen or vaginal microbiota. While there is limited information on the effect of mineral and vitamin supplementation on the gut microbiota of ruminant animals, the impact of dietary mineral and vitamin intake on potentially beneficial or pathogenic gut microbes in humans and rodent animals have been relatively well documented (Yang et al., 2020). For example, calcium and phosphorus supplementation increased the relative abundance of *Clostridium*, *Ruminococcus* and *Lactobacillus* spp. while reducing *Bifidobacterium* spp. in healthy men or mice (Nadeem Aslam et al., 2016; Trautvetter et al., 2018; Li et al., 2019). The impact of dietary supplementation with

504 selenium, magnesium, iron or zinc on certain gut commensal or pathogenic microbes was also
505 reported in children and mice (Yang et al., 2020). Of note, a significant effect of mineral
506 supplementation on the gut microbiota was observed but only at the microbial taxa level and not
507 on the microbial community structure and diversity (Yang et al., 2020).

508 The results from a limited number of studies performed on cattle also indicate that mineral
509 supplementation may influence ruminal microbiota composition. Clay mineral supplementation
510 increased the relative abundance of *Butyrivibrio* while reducing the relative abundance of
511 *Lactobacillus*, *Fusobacterium*, and *Treponema* genera in the rumen of non-lactating Holstein cows
512 (Neubauer et al., 2019); however, it did not alter the rumen microbial community structure or
513 diversity. Similarly, we observed that VTM supplementation increased the relative abundance of
514 *Butyrivibrio* in the rumen ($p < 0.05$). *Butyrivibrio* spp. are considered commensal members of the
515 rumen microbiota, producing butyrate through degradation of otherwise indigestible plant
516 polysaccharides (Kelly et al., 2010). The *Oscillospiraceae* NK4A214 group and
517 *Succinivibrionaceae* CAG-352 were the only other ruminal genera that responded to VTM
518 supplementation in the present study. These are uncultured taxa and their role in the rumen is
519 largely unknown. In contrast to our findings and those of Neubauer et al. (2019), Liu and others
520 (2017) observed noticeable alterations in microbial richness and diversity of ruminal microbiota
521 in both lactating Holstein cows (3-4 years old) and yearling heifers (10-months old) in response to
522 feeding mineral salt bricks containing Mg, Co, Cu, Fe, Mn, Se, Zn, I and Na for one month.

523 Compared to the rumen microbiota, the effect of mineral supplementation on the bovine
524 respiratory and reproductive microbiota ha been less characterized. Feeding beef calves with
525 selenium-biofortified alfalfa hay has been reported to alter the nasopharyngeal microbiota (Hall et
526 al., 2017; Hall et al., 2020). In the present study, although no significant changes were detected in

527 the microbial community structure and diversity in the nasopharynx following VTM
528 supplementation, changes were detected in relative abundance of five relatively abundant genera
529 (*Mycoplasma*, *Oscillospiraceae* UCG-005, *Christensenellaceae* R-7 group, *Ruminococcus* and
530 *Ornithinimicrobium*). Among these genera, *Mycoplasma*, which includes a bovine respiratory
531 disease (BRD)-associated pathogen, *Mycoplasma bovis*, was enriched in pregnant heifers that
532 receiving VTM supplementation. BRD is not a significant health concern among adult and
533 pregnant cattle but it is the number one health problem affecting newly weaning calves arriving in
534 the feedlot (Johnson and Pendell, 2017). The positive association between VTM supplementation
535 and nasopharyngeal *Mycoplasma* observed here poses the question of whether maternal VTM
536 supplementation influences colonization of the offspring respiratory tract by *Mycoplasma* spp. No
537 information has been reported regarding the impact of mineral supplementation on reproductive
538 microbiota in cattle.

539 Vitamins A, D_3 and E were included in the VTM supplement given to the pregnant heifers.
540 Thus, it is impossible to discern whether the subtle changes observed at the taxa level in both the
541 nasopharyngeal and ruminal microbiota are due to the minerals and vitamins supplemented.
542 Evidence from human, rodent and pig studies suggest that the gut microbiota responds to vitamin
543 supplementation (Yang et al., 2020). Gastrointestinal-associated *Bifidobacterium* (vitamin A, C)
544 *Akkermansia* (vitamin A), and *Lactobacillus* spp. (vitamin C) were more relatively abundant while
545 *E. coli* (vitamin C) and *Clostridium* (vitamin D) spp. decreased in relative abundance after vitamin
546 supplementation (Xu et al., 2014; Talsness et al., 2017; Huda et al., 2019; Yang et al., 2020). Our
547 results indicate that vitamin supplementation has less impact on the ruminal microbiota. Overall,
548 VTM supplementation for first 6 months of gestation did not affect the maternal microbiota. There
549 could be due to several factors. Considering that mineral salt intake was reported to alter the

ruminal microbiota in 3-4-year-old lactating cows (Liu et al., 2017), the resilience and robust of the mature ruminal microbiota can likely be ruled out as a reason for the absence of any VTM effect on the ruminal microbiota in pregnant heifers (1 year 9 months-old).

Pregnancy status rather than age, however, could be associated with the non-responsiveness of the ruminal microbiota to VTM supplementation. In rodent studies, the maternal gut microbiota undergoes profound changes over the course of pregnancy (Collado et al., 2008; Koren et al., 2012; Nuriel-Ohayon et al., 2016; Smid et al., 2018). As pregnancy progresses from the 1st to 3rd trimester, the maternal gut microbiota becomes less diverse (Koren et al., 2012) but with a higher microbial density, which may result in a microbiota that is more robust and resilient to perturbations. Hence, future studies are warranted to investigate the impact of VTM supplementation and other dietary interventions on the maternal microbiota of cattle using a non-pregnant control cohort.

Holistic View of Microbial Communities Across Respiratory, Gastrointestinal and Reproductive Tract and the Core Taxa Shared Across These Habitats

As expected, the overall microbial structure, diversity and composition were noticeably different among the nasopharyngeal, ruminal and vaginal microbiota in both virgin yearling and pregnant heifers. The ruminal microbiota was dominated by the anaerobic phylum *Bacteroidota* (66%), while the nasopharyngeal and vaginal microbiota the majority of 16S rRNA gene sequences were classified as *Actinobacteriota* (51%) and *Firmicutes* (52%), respectively. Various factors including niche-specific physiological factors (temperature, pH, oxygen and nutrient availability), dietary, and environmental factors are involved in shaping the microbiota of the bovine respiratory tract (Zeineldin et al., 2019; Timsit et al., 2020), rumen (O'Hara et al., 2020; Cholewińska et al., 2021) and reproductive tract (Galvão et al., 2019). Subtle physiological and

573 anatomical differences in the mucosal surfaces of the bovine respiratory tract have been shown to
574 significantly influence the microbial distribution along the respiratory tract (McMullen et al.,
575 2020).

576 In the present study, although the nasopharynx, rumen and vagina have drastically
577 different physiological and anatomical properties, we identified 41 OTUs that were shared by a
578 high portion (60%) of all samples from both virgin yearling and pregnant heifers. This indicates
579 that these core taxa can colonize and inhabit the respiratory, gastrointestinal and reproductive tracts
580 regardless of the drastic differences in physiological conditions in these locations. The majority
581 (80%) of these core taxa are members of the *Firmicutes*, which is one of the most ubiquitous and
582 relatively abundant bacterial phyla in the respiratory, gastrointestinal and reproductive tract-
583 (vagina, uterus) (Galvão et al., 2019), mammary gland- (Derakhshani et al., 2018), ocular-
584 (Bartenslager et al., 2021) and hoof- (Zinicola et al., 2015) -associated microbiota in cattle,
585 demonstrating the adaptability of members of this phylum. Nine taxa within the *Actinobacteria*
586 phylum including *Bifidobacterium pseudolongum* and several *Corynebacterium* spp. were among
587 these core taxa. *B. pseudolongum* is widely found in the mammalian gut (Lugli et al., 2019) and
588 has long been noted for its probiotic properties in human, cattle and pigs (Abe et al., 1995; Kissels
589 et al., 2017). Given the known beneficial effects of this species on the host, and as a core taxon
590 present in the respiratory, gut and reproductive tracts of cattle, *B. pseudolongum* may have the
591 potential to enhance cattle health and productivity, as may some of the other core taxa identified
592 in this study. Species and strain level resolution of these core taxa using shotgun metagenomic
593 sequencing and characterization of their functional features using culturing should be the focus of
594 future studies.

595 Two *Methanobrevibacter* OTUs were also identified among the core taxa. Although
596 members of this methanogenic genus are well known for their involvement in ruminal methane
597 production (Hook et al., 2010; Danielsson et al., 2017; Greening et al., 2019), and are frequently
598 observed in the vaginal microbiota (Laguardia-Nascimento et al., 2015) in cattle, it is interesting
599 to note that this genus is also found in the bovine respiratory tract. The presence of these
600 *Methanobrevibacter* OTUs within the respiratory, gastrointestinal, and reproductive tracts has
601 important implications for the identification of maternal seeding of the calf microbiota with
602 pioneer methanogens before and during birth. *Methanobrevibacter* spp. are predominant in 5- to
603 7-month-old calf fetuses (Guzman et al., 2020) as well as newborn calves (Guzman et al., 2015;
604 Alipour et al., 2018). Our results indicate that the respiratory microbiota may also seed the calf
605 gastrointestinal tract with *Methanobrevibacter* spp. perinatally. This highlights the necessity of
606 holistic assessment of respiratory, gastrointestinal and reproductive tract microbiota to trace the
607 origin of pioneer calf microbiota. To our best of knowledge, this is the first study to evaluate
608 nasopharyngeal, ruminal and vaginal microbiota in an individual ruminant animal, and to identify
609 the core taxa shared amongst these microbial ecologies.

610 **Ruminal *Methanobrevibacter* Enriched in Pregnant Heifers and Associations of**
611 ***Methanobrevibacter* with Predominant Ruminal and Vaginal Bacterial Genera**

612 Given that lowering methane emissions in cattle benefits both environment and cattle
613 production (Beauchemin et al., 2020), and increasing evidence suggesting that the ruminal
614 microbiome and host genetics can be targeted independently to improve feed efficiency and
615 mitigate enteric methane emissions from cattle (Difford et al., 2018; Li et al., 2019), we therefore
616 focused on this methanogenic archaeal genus, *Methanobrevibacter*. We identified that pregnant
617 heifers harbored a greater relative abundance of ruminal *Methanobrevibacter* compared to non-

618 pregnant virgin yearling heifers. Confounding factors associated with dietary (11 % more hay fed
619 to virgin heifers than pregnant heifers) and age differences makes it difficult to associate pregnancy
620 with the colonization of the rumen with methanogenic archaea. However, the impact of pregnancy
621 and mitigation of maternal ruminal methanogens on offspring enteric methane emissions warrants
622 further investigation.

623 Our correlation analysis revealed that in comparison to vaginal *Methanobrevibacter*, the
624 relative abundance of ruminal *Methanobrevibacter* is highly influenced by many other commensal
625 genera in the rumen. For example, in the rumen microbiota many genera within the *Prevotellaceae*
626 family were inversely associated with the relative abundance of *Methanobrevibacter*.
627 Interestingly, the opposite was found in the vaginal microbiota, suggesting that the nature of the
628 interaction between *Methanobrevibacter* and *Prevotella* and *Prevotellaceae* UCG-003 may be
629 niche specific and that *Prevotella* spp. in the rumen may become pro-methanogenic if they present
630 in reproductive microbial ecosystem.

631 The stepwise-selected GLM model identified *Prevotella* and *Prevotellaceae UCG-003* as
632 having a significant and negative effect on the relative abundance of *Methanobrevibacter* in rumen
633 of both virgin and pregnant heifers and vaginal tract of pregnant heifers. This is in agreement with
634 previous studies reporting that microbial communities with highly-abundant lactate-consuming
635 bacteria (*Prevotella bryantii*) and high H_2-consuming (e.g. certain *Prevotella* spp.) has been
636 associated with lower ruminal methane production (Denman et al., 2015; Danielsson et al., 2017;
637 Tapio et al., 2017; Granja-Salcedo et al., 2019). Thus, members of the *Prevotella* and
638 *Prevotellaceae UCG-003* in the bovine rumen and vagina may have anti-methanogenic potential
639 to mitigate methane emissions in cattle. The *Christensenellaceae R*-7 group was identified as the
640 genus that can have significant positive effect on *Methanobrevibacter* in the present study. This

may suggest that some species within this genus may be involved in producing methanogenic substrates such as H_2 and acetate. Future *in vitro* studies are needed to confirm the anti-methanogenic properties of *Prevotella* and *Prevotellaceae UCG-003* and pro-methanogenic activity of *Christensenellaceae R-7* group spp. originating from the rumen of cattle.

In conclusion, no noticeable difference was observed in α and β-diversity in any of the nasopharyngeal, ruminal and vaginal microbiota between virgin heifers raised from dams exposed to divergent rates of gain during the first trimester of pregnancy, or between pregnant heifers consuming control and VTM diets. Only in the vaginal microbiota were there relatively abundant genera that were affected by maternal rate of gain during early gestation. Maternal VTM supplementation resulted in subtle compositional alterations in the nasopharyngeal and ruminal microbiota. A total of 41 archaeal and bacterial OTUs were shared by over 60% of all samples from both virgin and pregnant heifers. Two taxa within the *Methanobrevibacter* genus were among these taxa this genus was more relatively abundant in pregnant compared to virgin heifers. Compared to the vaginal *Methanobrevibacter*, *Methanobrevibacter* in the rumen was predicted to be highly interactive with other commensal members.

Among the 25 most relatively abundant genera, *Prevotella* and *Prevotella* UCG-003 (negative) and *Christensenellaceae R-7* group (positive) were predicted to have a significant effect on the relative abundance of ruminal *Methanobrevibacter* spp. Overall, the results of this study suggest that there is little impact of maternal gestational nutrition during the first trimester on the calf microbiota assessed at 9 months of age, and that VTM supplementation during pregnancy may not alter the maternal microbiota. This study provides evidence that there are several microbial taxa, including methanogenic archaea, that are shared across the respiratory, gastrointestinal, and reproductive tracts. Therefore, this suggests that there is a need for a holistic evaluation of the

664 bovine microbiota when considering potential maternal sources for seeding calves with pioneer
665 microbiota, and when targeting the maternal microbiome to enhance offspring health and
666 development.
667
668 **DATA AVAILABILITY**
669 The datasets generated for this study can be found in the Sequences that were submitted to
670 the NCBI sequence read archive under BioProject accession PRJNA721423.
671
672 **ETHICS STATEMENT**
673 All animals used in this study were cared for in accordance with the guidelines set by the
674 Olfert et al. (1993) and all experimental procedures involving cattle were approved by the North
675 Dakota State University Institutional Animal Care and Use Committee (#A20085 and A20047).
676
677 **AUTHOR CONTRIBUTIONS**
678 Conceiving the idea, designing the study, and providing supervision—S.A. and C.R.D.;
679 Cattle management— A.C.B.M., F.B., C.R.D. and K.K.S.; Animal care and sample collections—
680 S.A., C.R.D. K.S., A.C.B.M., T.W., J. D. K., F.B.; Sample processing—K.S. and S.A.;
681 Bioinformatics analysis— D.B.H., T.L. and S.A.; Data processing and statistical analysis—T.D.S.,
682 D.B.H, and S.A.; Manuscript writing—S.A.; Manuscript review and editing—S.A., D.B.H.,
683 A.C.B.M and C.R.D. All authors have read and agreed to the published version of the manuscript.
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685
686

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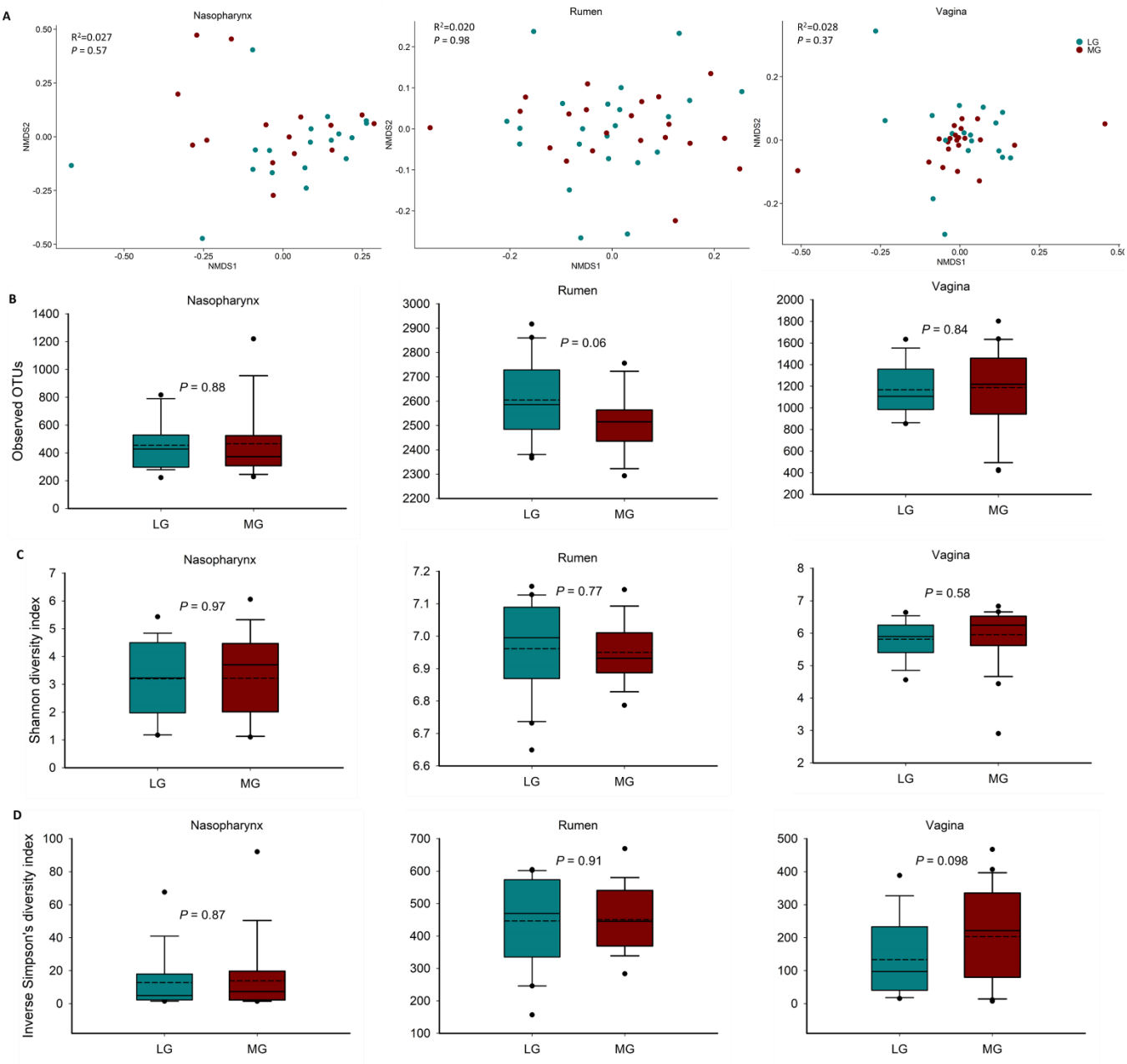

706

707 **Figure 1**. Beta and alpha diversity of the nasopharyngeal, ruminal and vaginal microbiota of virgin
708 yearling heifers from low gain (LG) or medium gain (MG) dams as determined during the first
709 trimester of gestation. (**A**) Non-metric multidimensional scaling (NMDS) plots of the Bray-Curtis
710 dissimilarities, (**B**) number of operational taxanomic units (OTUs), and Shannon (**C**) and inverse
711 Simpson's diversity index (**D**) of each microbial community.
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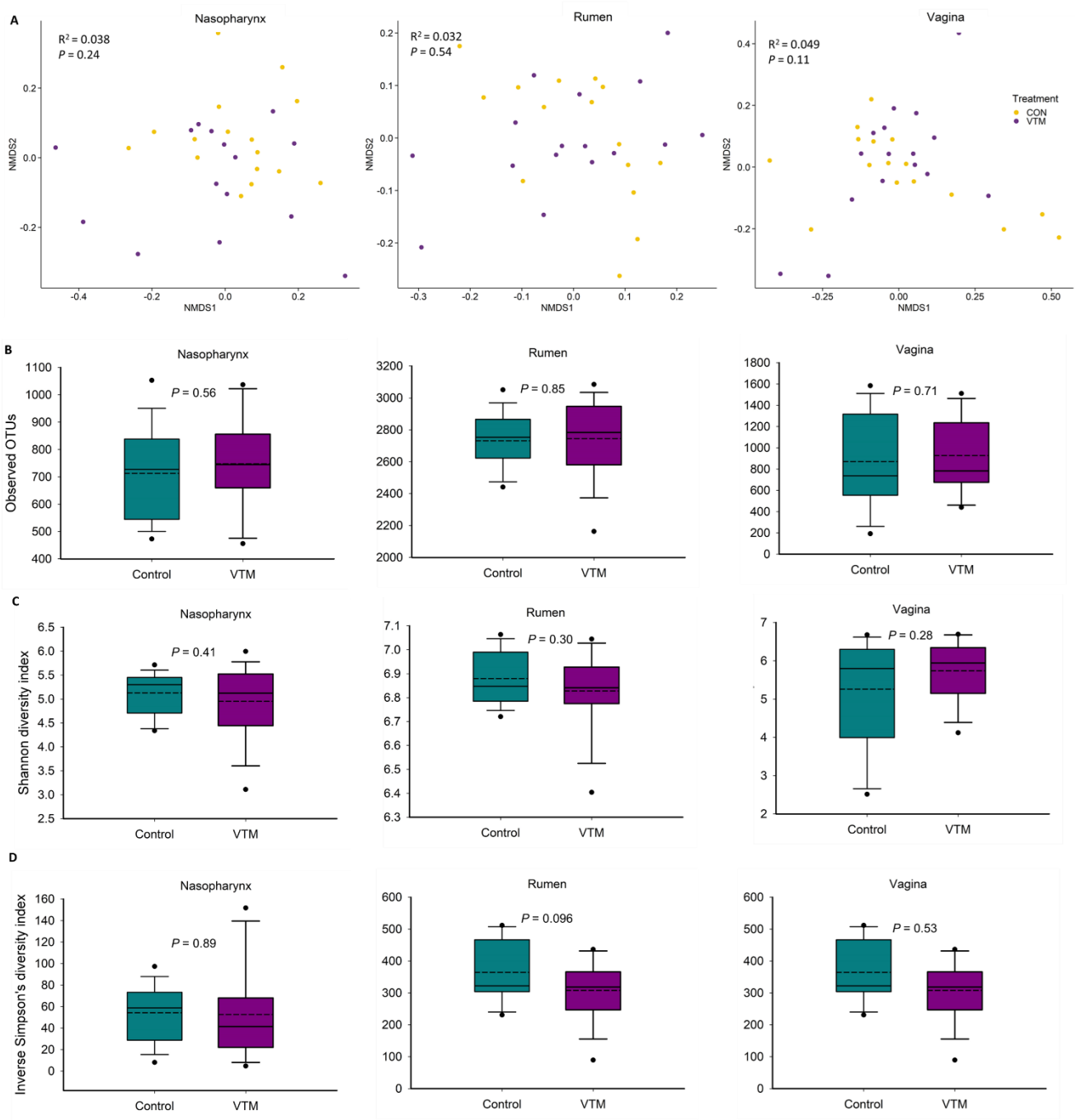

Figure 2. Beta and alpha diversity of the nasopharyngeal, ruminal and vaginal microbiota of pregnant heifers that received a vitamin and mineral supplement (VTM) or a control diet (Control) during the first six months of gestation. **A**) Non-metric multidimensional scaling (NMDS) plots of the Bray-Curtis dissimilarities, **B**) number of operational taxanomic units (OTUs), and Shannon (**C**) and inverse Simpson's diversity index (**D**) of each microbial community.

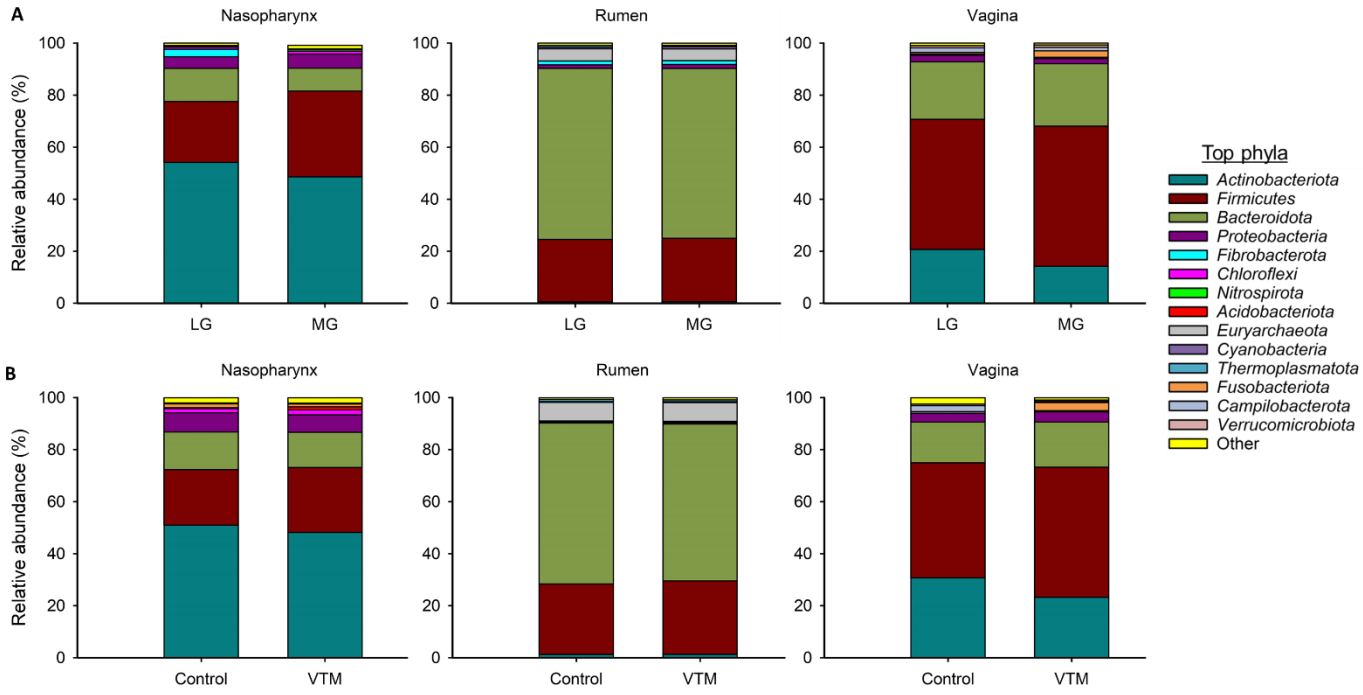

Figure 3. Percent relative abundance of the eight most relatively abundant phyla in the nasopharyngeal, ruminal and vaginal microbiota of (**A**) virgin yearling heifers from low gain (LG) or medium gain (MG) dams and (**B**) pregnant heifers that received a vitamin and mineral supplement (VTM) or a control diet (Control) during the first 6 six months of gestation

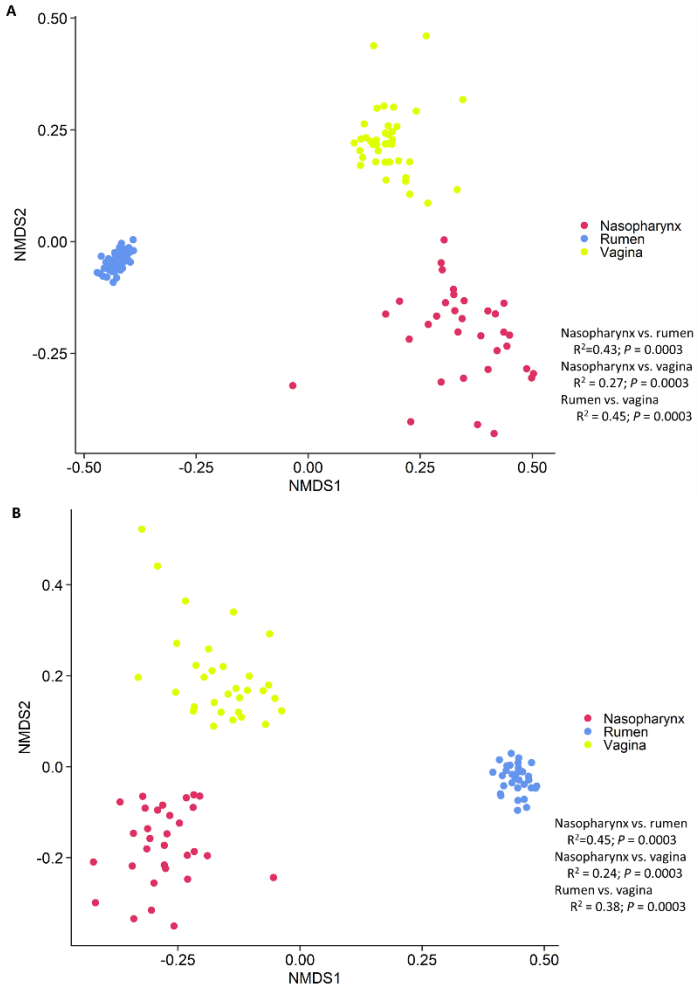

Figure 4. Non-metric multidimensional scaling (NMDS) plots of the Bray-Curtis dissimilarities of the nasopharyngeal, ruminal and vaginal microbiota of (**A**) virgin yearling and (**B**) pregnant heifers.

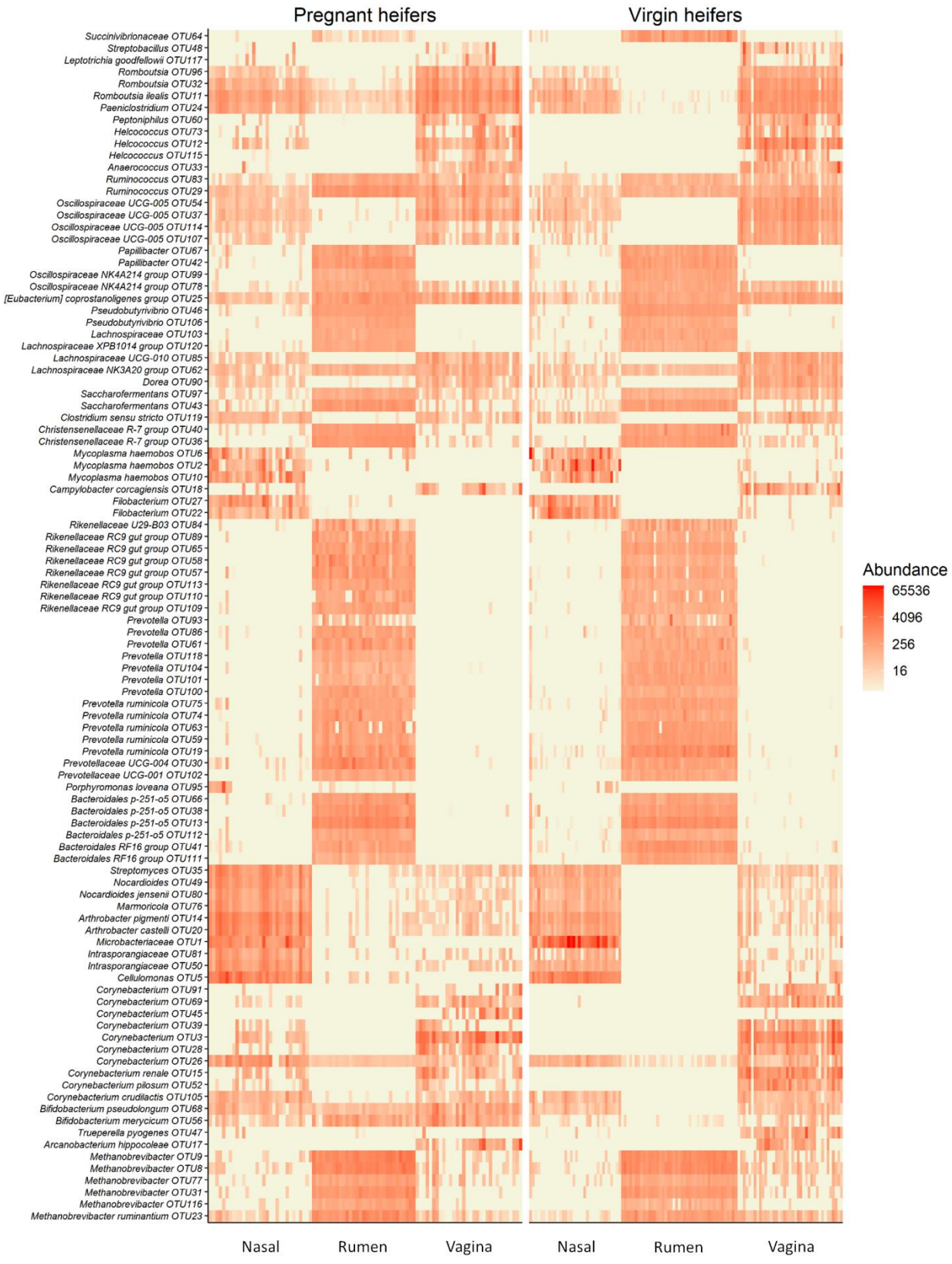

Figure 5. Heat map showing the 100 most abundant OTUs (log_4) overall by sample type within each animal group (Pregnant and virgin heifers).

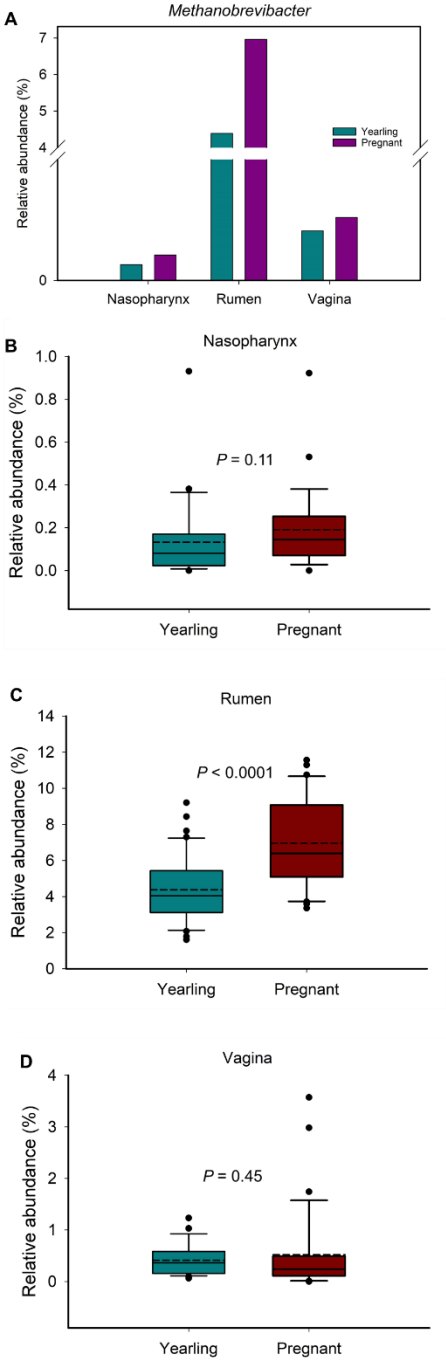

Figure 6. Overall relative abundance of *Methanobrevibacter* in nasopharyngeal, ruminal and vaginal microbiota by sample types (A) and by animal groups (B, C and D).

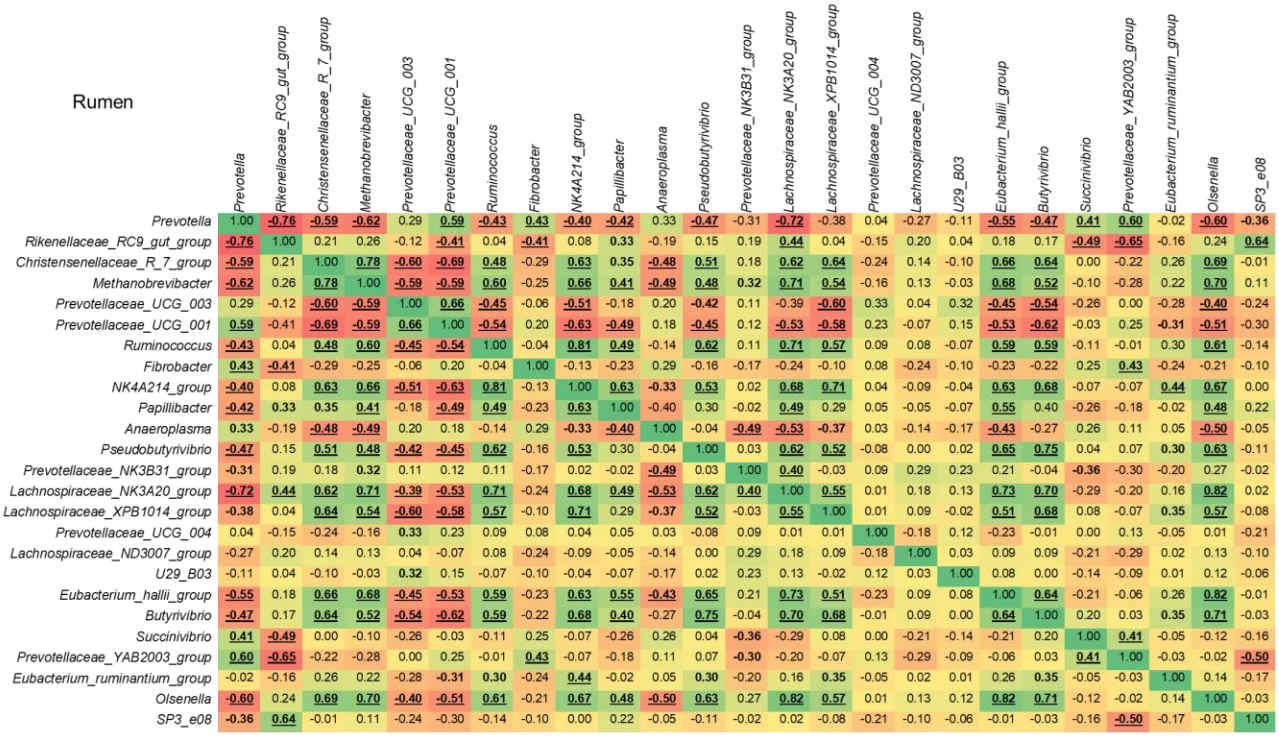

Figure 7. Correlations between the 25 most relatively abundant genera in the ruminal and vaginal microbiota of virgin yearling heifers. Spearman's rank correlation coefficients (*r*). Bold correlation coefficients with $0.01 \leq p < 0.05$, and underlined bold correlation coefficients with $p < 0.01$.

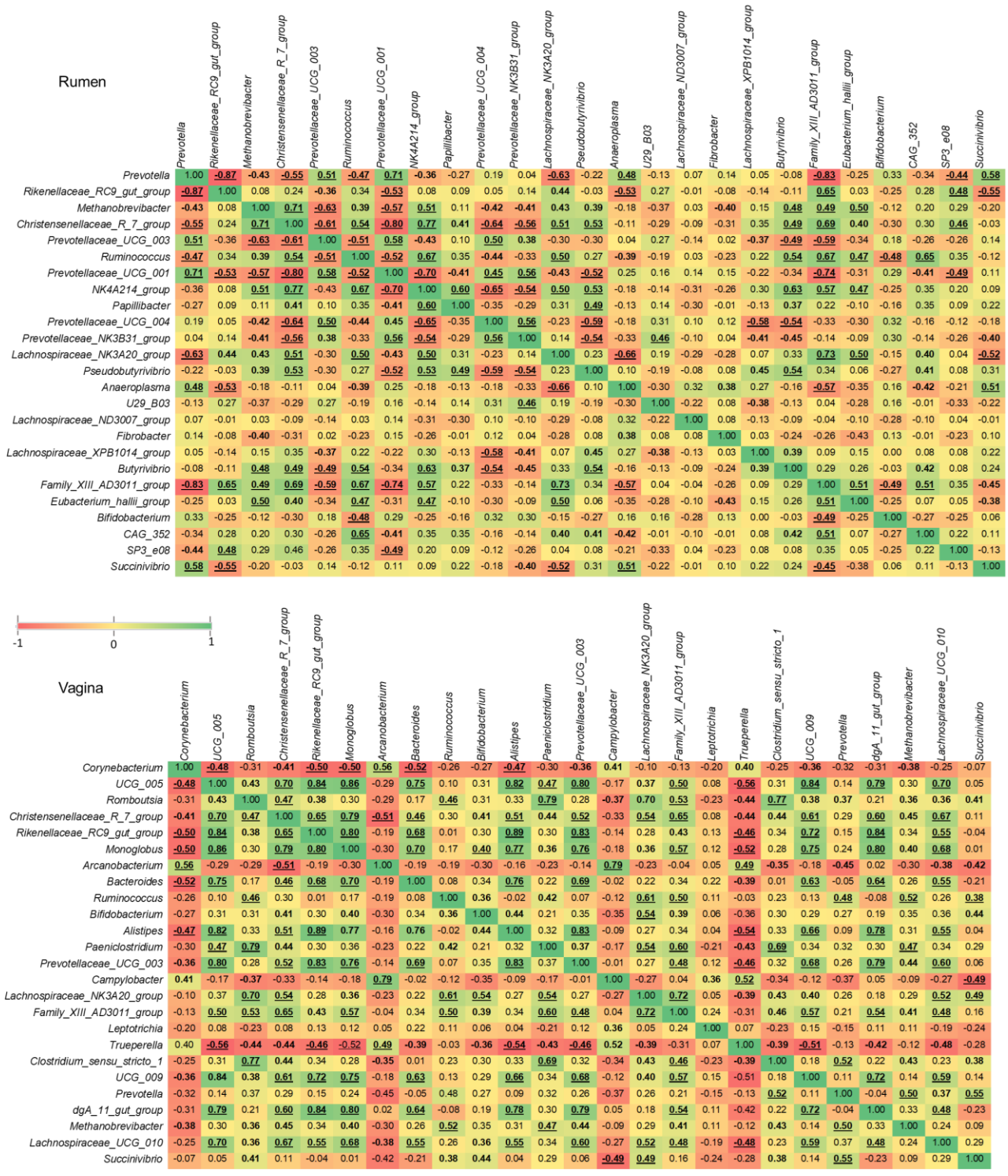

Figure 8. Correlations between the 25 most relatively abundant genera in the ruminal and vaginal microbiota of pregnant heifers. Spearman's rank correlation coefficients (r). Bold correlation coefficients with $0.01 \leq p < 0.05$, and underlined bold correlation coefficients with $p < 0.01$.

Table 1. Nutrient composition of the diets fed to the dams of virgin yearling heifers during the first 84 days of gestation, and virgin yearling heifers and pregnant heifers at the time of sample collection.

Diet composition, % DM	Dams of virgin yearling		Virgin yearling	Pregnant heifers	
	LG[1]	MG[2]		Control	VTM[3]
Corn silage	37	29	20	30	30
Prairie hay	53	41	70	59	59
Dried distillers grains plus solubles	10	5	5	6	6
Premix	-	-	5	5	5
Energy and protein supplement[1]	-	25	-	-	-

[1]Basal total mixed rations (TMR) contained a commercially available mineral supplement (Purina® Wind & Rain® Storm® All-Season 7.5 Complete Mineral, Land O'Lakes Inc., Arden Hills, Minn.) fed at a rate of 113.4 gram per head per day, targeting gain of 0.28 kg/d.
[2]The supplement fed was an energy/protein supplement formulated with a blend of ground corn, DDGS, wheat midds, fish oil and urea, targeting gain of 0.79 kg/d.
[3]VTM: Vitamin mineral supplement was a pelleted product fed at 0.45 kg/head/day (consisting of 113 g of a vitamin and mineral supplement [Purina Wind & Rain Storm All-Season 7.5 Complete, Land O'Lakes Inc., Arden Hills, Minn.] and 337 g of a carrier) (Menezes et al., 2021).

Table 2. Percent relative abundance of the most relatively abundant genera in nasopharyngeal (n = 26), ruminal (n= 24) and vaginal (n = 27) microbiota of virgin yearling heifers that were born from the dams received a basal diet to achieve a moderate gain (MG) or to achieve a low gain (LG) during the first 84 days of gestation[1]

Nasopharynx						Rumen						Vagina					
Genus	**Rank**	**MG**	**LG**	**SEM**	***P*-value**	**Genus**	**Rank**	**MG**	**LG**	**SEM**	***P*-value**	**Genus**	**Rank**	**MG**	**LG**	**SEM**	***P*-value**
Mycoplasma	2	23.8	12.3	9.16	0.218	*Prevotella*	1	31.4	30.2	1.85	0.505	*Corynebacterium*	1	8.75	12.00	2.54	0.209
Cellulomonas	3	5.41	6.40	1.92	0.608	*Rikenellaceae RC9 gut group*	2	8.89	9.14	0.84	0.769	*Oscillospiraceae UCG-005*	2	9.52	7.86	0.91	0.076
Filobacterium	4	2.62	8.03	3.00	0.081	*Christensenellaceae R-7 group*	4	4.70	4.81	0.48	0.804	*Rikenellaceae RC9 gut group*	4	4.30	3.87	0.51	0.406
Arthrobacter	5	2.36	2.05	0.51	0.546	*Methanobrevibacter*	5	4.30	4.47	0.55	0.756	*Bacteroides*	5	4.15	3.95	0.62	0.757
Corynebacterium	6	1.60	1.81	0.45	0.636	*Prevotellaceae UCG-003*	7	3.82	3.77	0.19	0.803	*Christensenellaceae R-7 group*	8	3.25	3.22	0.37	0.952
Nocardioides	7	1.77	1.65	0.60	0.842	*Prevotellaceae UCG-001*	8	2.77	2.85	0.19	0.660	*Alistipes*	9	3.55	2.55	0.40	0.017
Oscillospiraceae UCG-005	9	1.35	1.36	0.58	0.975	*Ruminococcus*	9	2.55	2.36	0.18	0.305	*Monoglobus*	10	2.83	2.44	0.29	0.182
Streptomyces	13	1.06	0.76	0.34	0.389	*Fibrobacter*	12	1.36	1.45	0.30	0.773	*Romboutsia*	11	2.44	1.89	0.28	0.061
Romboutsia	15	0.85	0.80	0.22	0.809	*Oscillospiraceae* NK4A214 group	14	1.42	1.34	0.10	0.444	*Prevotellaceae UCG-003*	13	1.71	1.79	0.29	0.771
Christensenellaceae R-7 group	16	0.83	0.71	0.31	0.702	*Papillibacter*	18	1.11	1.17	0.13	0.668	*Campylobacter*	16	1.24	1.69	1.03	0.668
Ornithinimicrobium	18	0.55	0.72	0.31	0.600	*Anaeroplasma*	21	0.96	0.90	0.10	0.551	*Trueperella*	17	0.66	2.30	1.14	0.160
Rikenellaceae RC9 gut group	19	0.49	0.56	0.18	0.697	*Pseudobutyrivibrio*	22	0.82	0.81	0.05	0.856	*Ruminococcus*	18	1.64	1.20	0.19	0.025
Bacteroides	21	0.52	0.49	0.16	0.865	*Prevotellaceae NK3B31 group*	26	0.69	0.79	0.08	0.193	*Lachnospiraceae NK3A20 group*	21	1.07	0.90	0.17	0.307
Marmoricola	22	0.58	0.41	0.13	0.187	*Lachnospiraceae NK3A20 group*	30	0.47	0.45	0.05	0.599	*Paeniclostridium*	22	1.13	0.79	0.19	0.092
Ruminococcus	24	0.43	0.50	0.18	0.658	*Lachnospiraceae XPB1014 group*	31	0.44	0.44	0.05	0.957	*Arcanobacterium*	24	0.49	1.35	0.50	0.090
Lachnospiraceae NK3A20 group	25	0.53	0.31	0.14	0.110	*Prevotellaceae UCG-004*	32	0.44	0.41	0.05	0.547	*Family XIII AD3011 group*	25	0.97	0.81	0.10	0.116
Prevotella	26	0.58	0.24	0.26	0.196	*Lachnospiraceae ND3007 group*	33	0.39	0.44	0.06	0.429	*Streptobacillus*	26	1.58	0.07	1.00	0.139
Ornithinicoccus	29	0.33	0.42	0.13	0.527	*Rikenellaceae U29-B03*	34	0.40	0.41	0.07	0.997	*Akkermansia*	27	0.85	0.79	0.20	0.754
Clostridium sensu stricto 1	30	0.41	0.34	0.14	0.615	*[Eubacterium] hallii group*	36	0.40	0.38	0.03	0.573	*Porphyromonas*	28	0.34	1.23	1.01	0.382
Olsenella	31	0.32	0.39	0.14	0.618	*Butyrivibrio*	38	0.38	0.33	0.05	0.295	*Butyricicoccaceae UCG-009*	30	0.74	0.66	0.09	0.395
Bifidobacterium	32	0.33	0.37	0.17	0.819	*Succinivibrio*	39	0.32	0.36	0.04	0.402	*Oscillospiraceae NK4A214 group*	31	0.81	0.56	0.09	0.010
Paeniclostridium	33	0.40	0.31	0.11	0.393	*Prevotellaceae YAB2003 group*	40	0.35	0.32	0.04	0.435	*Rikenellaceae dgA-11 gut group*	33	0.71	0.59	0.08	0.149
Prevotellaceae UCG-003	35	0.43	0.22	0.13	0.111	*[Eubacterium] ruminantium group*	41	0.32	0.27	0.02	0.029	*Prevotellaceae UCG-004*	37	0.68	0.54	0.11	0.209
Monoglobus	36	0.30	0.32	0.13	0.859	*Olsenella*	42	0.27	0.26	0.04	0.754	*Lachnospiraceae UCG-010*	38	0.62	0.59	0.08	0.683
Rhodococcus	37	0.28	0.31	0.12	0.778							*Clostridium sensu stricto 1*	39	0.54	0.68	0.21	0.514
Brachybacterium	38	0.31	0.27	0.12	0.777							*Olsenella*	40	0.30	0.92	0.55	0.263
*Mannheimia**		0.01	0.00									*Alloprevotella*	42	0.57	0.42	0.13	0.246
*Pasteurella**		0.02	0.07			*Fusobacterium**		0.01	0.02								
*Histophilus**		0.00	0.01			*Trueperella**		0.00	0.00			*Fusobacterium**		0.00	0.00		

[1]The genera whose relative abundance was ranked within the top 42 are listed in this table and any ones within top 42 rank that were unclassified at genus level were excluded.
*These genera included in this table because of their relevance to bovine respiratory disease and liver abscesses in cattle.

Table 3. Percent relative abundance of the 42 most relatively abundant genera in nasopharyngeal (n = 29), ruminal (n= 26) and vaginal (n = 27) microbiota of received diets without (CON) and with vitamin and mineral supplementation (VTM) during the first 6 months of gestation[1]

Nasopharynx						Rumen						Vagina					
Genus	**Rank**	**CON**	**VTM**	**SEM**	***P*-value**	**Genus**	**Rank**	**CON**	**VTM**	**SEM**	***P*-value**	**Genus**	**Rank**	**CON**	**VTM**	**SEM**	***P*-value**
Cellulomonas	1	9.28	6.03	2.87	0.265	*Prevotella*	1	20.69	20.67	2.59	0.994	*Corynebacterium*	1	18.68	12.25	5.22	0.227
Arthrobacter	2	6.87	7.20	1.40	0.820	*Rikenellaceae* RC9 gut group	2	12.90	12.93	1.48	0.985	*Oscillospiraceae UCG-005*	2	5.08	6.42	1.00	0.190
Mycoplasma	3	2.74	8.95	2.88	0.039	*Methanobrevibacter*	3	6.95	6.97	0.84	0.985	*Romboutsia*	4	4.39	3.62	0.77	0.330
Corynebacterium	5	5.23	4.26	1.01	0.342	*Christensenellaceae* R-7 group	5	5.62	5.98	0.38	0.352	*Christensenellaceae R-7 group*	5	3.16	3.86	0.60	0.244
Nocardioides	6	4.18	3.87	0.67	0.646	*Prevotellaceae* UCG-003	7	3.22	3.00	0.23	0.348	*Rikenellaceae RC9 gut group*	7	3.09	2.97	0.75	0.875
Filobacterium	8	3.29	1.70	1.32	0.238	*Ruminococcus*	9	2.38	2.58	0.18	0.283	*Monoglobus*	8	2.51	3.08	0.56	0.316
Streptomyces	9	2.24	2.24	0.40	0.995	*Prevotellaceae* UCG-001	10	2.52	2.28	0.29	0.414	*Arcanobacterium*	9	4.09	1.01	2.38	0.203
Oscillospiraceae UCG-005	10	2.09	1.16	0.38	0.023	*Oscillospiraceae* NK4A214 group	11	1.41	1.73	0.13	0.024	*Bacteroides*	10	2.10	2.75	0.46	0.166
Romboutsia	11	1.56	1.30	0.19	0.194	*Papillibacter*	15	1.09	1.37	0.16	0.102	*Ruminococcus*	11	2.41	2.22	0.56	0.732
Bacteroides	12	1.45	1.20	0.54	0.644	*Prevotellaceae UCG-004*	19	0.91	1.16	0.35	0.484	*Bifidobacterium*	13	2.27	1.93	0.54	0.538
Porphyromonas	13	1.65	0.77	1.29	0.498	*Prevotellaceae NK3B31 group*	21	1.06	0.95	0.16	0.515	*Alistipes*	14	1.89	2.06	0.45	0.709
Marmoricola	14	1.18	1.10	0.22	0.719	*Lachnospiraceae NK3A20 group*	23	0.86	0.84	0.10	0.841	*Paeniclostridium*	16	1.54	1.28	0.34	0.451
Salinimicrobium	16	0.72	1.48	0.40	0.068	*Pseudobutyrivibrio*	25	0.75	0.75	0.06	0.980	*Prevotellaceae UCG-003*	17	1.52	1.20	0.42	0.460
Brachybacterium	19	1.08	0.93	0.21	0.485	*Anaeroplasma*	26	0.68	0.59	0.08	0.303	*Campylobacter*	18	2.17	0.42	1.43	0.231
Ornithinicoccus	21	0.92	0.88	0.17	0.835	*Rikenellaceae U29-B03*	29	0.41	0.45	0.11	0.761	*Lachnospiraceae NK3A20 group*	20	0.98	1.20	0.28	0.451
Rhodococcus	22	0.84	0.78	0.16	0.683	*Lachnospiraceae ND3007 group*	30	0.46	0.40	0.09	0.520	*Family XIII AD3011 group*	21	0.95	1.09	0.19	0.477
Rikenellaceae RC9 gut group	24	0.86	0.59	0.17	0.114	*Fibrobacter*	31	0.38	0.46	0.08	0.296	*Leptotrichia*	26	0.01	1.70	1.20	0.171
Christensenellaceae R-7 group	25	0.84	0.56	0.12	0.033	*Lachnospiraceae* XPB1014 group	32	0.38	0.40	0.03	0.665	*Trueperella*	27	0.91	0.63	0.58	0.641
Bifidobacterium	26	0.53	0.81	0.19	0.146	*Butyrivibrio*	33	0.36	0.42	0.03	0.046	*Clostridium sensu stricto*	28	0.86	0.51	0.22	0.117
Saccharopolyspora	27	0.69	0.63	0.15	0.698	*Family XIII AD3011 group*	34	0.34	0.40	0.06	0.336	*Butyricicoccaceae UCG-009*	30	0.54	0.73	0.13	0.178
Alistipes	28	0.71	0.61	0.16	0.510	*[Eubacterium] hallii group*	36	0.36	0.35	0.03	0.791	*Prevotella*	34	0.27	0.84	0.67	0.400
Paeniclostridium	29	0.75	0.55	0.10	0.061	*Bifidobacterium*	37	0.35	0.34	0.10	0.918	*Rikenellaceae dgA-11 gut group*	35	0.55	0.54	0.16	0.950
Ruminococcus	30	0.78	0.49	0.12	0.024	*Ruminococcaceae CAG-352*	38	0.28	0.39	0.05	0.021	*Methanobrevibacter*	37	0.59	0.45	0.29	0.618
Clostridium sensu stricto 1	31	0.58	0.58	0.15	0.998	*Rikenellaceae SP3-e08*	40	0.29	0.34	0.08	0.537	*Lachnospiraceae UCG-010*	38	0.43	0.56	0.08	0.114
Lachnospiraceae NK3A20 group	33	0.57	0.53	0.12	0.754	*Anaerovorax*	41	0.32	0.31	0.03	0.721	*Streptobacillus*	39	0.03	0.93	0.74	0.229
Ornithinimicrobium	34	0.65	0.44	0.10	0.049	*Monoglobus*	42	0.33	0.27	0.04	0.149	*Oscillospiraceae NK4A214 group*	41	0.47	0.48	0.10	0.932
Altererythrobacter	35	0.60	0.45	0.13	0.240							*Akkermansia*	42	0.43	0.51	0.19	0.667
Microlunatus	36	0.58	0.45	0.10	0.191												
Monoglobus	37	0.61	0.42	0.12	0.122												
*Mannheimia**		0.01	0.02														
*Pasteurella**		0.00	0.00			*Fusobacterium**		0.17	0.07								
*Histophilus**		0.00	0.00			*Trueperella**		0.00	0.00			*Fusobacterium**		0.004	0.005		

[1]The genera whose relative abundance was ranked within the top 42 are listed in this table and any ones within the top 42 rank that were unclassified at genus level were excluded.
*These genera included in this table because of their relevance to bovine respiratory disease and liver abscesses in cattle

Table 4. OTUs identified in the nasopharyngeal, ruminal and vaginal microbiota of at least 60% of samples from virgin yearling heifers.

OUT	Taxa	60%	65%	70%	75%	80%	85%	90%	95%	100%
OTU8	[k__Archaea, p__Euryarchaeota, c__Methanobacteria, o__Methanobacteriales, f__Methanobacteriaceae, g__Methanobrevibacter, s__NA]	■	■	■						
OTU23	[k__Archaea, p__Euryarchaeota', c__Methanobacteria, o__Methanobacteriales, f__Methanobacteriaceae, g__Methanobrevibacter, s__ruminantium]	■	■	■	■	■				
OTU68	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Bifidobacteriales, f__Bifidobacteriaceae, g__Bifidobacterium, s__pseudolongum]	■								
OTU147	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Corynebacteriales, f__Corynebacteriaceae, g__Corynebacterium, s__marinum]	■								
OTU26	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Corynebacteriales, f__Corynebacteriaceae, g__Corynebacterium, s__NA]	■	■	■	■	■				
OTU160	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Micrococcales, f__Intrasporangiaceae, g__Ornithinimicrobium, s__NA]	■								
OTU35	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Streptomycetales, f__Streptomycetaceae, g__Streptomyces, s__NA]	■								
OTU351	[k__Bacteria, p__Actinobacteriota, c__Coriobacteriia, o__Coriobacteriales, f__Atopobiaceae, g__Atopobium, s__NA]	■								
OTU368	[k__Bacteria, p__Actinobacteriota, c__Coriobacteriia, o__Coriobacteriales, f__Eggerthellaceae, g__DNF00809, s__NA]	■								
OTU392	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Christensenellales, f__Christensenellaceae, g__Christensenellaceae R-7 group, s__NA]	■								
OTU537	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Christensenellales, f__Christensenellaceae, g__Christensenellaceae R-7 group, s__NA]	■								
OTU927	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Clostridia UCG-014, f__NA, g__NA, s__NA]	■								
OTU97	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Hungateiclostridiaceae, f__Saccharofermentans, g__NA, s__NA]	■	■	■	■					
OTU133	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__[Eubacterium] hallii group, s__NA]	■	■	■	■	■				
OTU1335	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__[Eubacterium] hallii group, s__NA]	■								
OTU158	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__[Eubacterium] hallii group, s__NA]	■	■							
OTU3351	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__[Eubacterium] hallii group, s__NA]	■	■							
OTU489	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__[Eubacterium] hallii group, s__NA]	■								
OTU1111	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Coprococcus, s__NA]	■		■						
OTU1688	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Lachnospiraceae NK3A20 group, s__NA]	■	■	■	■	■				
OTU1742	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Lachnospiraceae NK3A20 group, s__NA]	■	■	■						
OTU373	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Lachnospiraceae NK3A20 group, s__NA]	■	■	■						
OTU62	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Lachnospiraceae NK3A20 group, s__NA]	■	■	■	■					
OTU657	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Lachnospiraceae NK3A20 group, s__NA]	■	■							
OTU758	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Lachnospiraceae NK3A20 group, s__NA]	■								
OTU882	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__NA, s__NA]	■								
OTU25	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__[Eubacterium] coprostanoligenes group, g__NA, s__NA]	■	■	■	■	■	■	■	■	
OTU561	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Oscillospiraceae, g__Colidextribacter, s__NA]	■	■	■	■	■	■	■		
OTU78	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Oscillospiraceae, g__NK4A214 group, s__NA]	■	■	■	■					
OTU37	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Oscillospiraceae, g__UCG-005, s__NA]	■								
OTU188	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__Ruminococcus, s__NA]	■								
OTU201	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__Ruminococcus, s__NA]	■	■							
OTU29	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__Ruminococcus, s__NA]	■	■	■	■	■	■	■	■	
OTU307	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__Ruminococcus, s__NA]	■								
OTU441	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__Ruminococcus, s__NA]	■								
OTU83	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__Ruminococcus, s__NA]	■	■		■	■				
OTU244	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__UCG-001, s__NA]	■	■							
OTU243	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Anaerovoracaceae, g__Family XIII AD3011 group, s__NA]	■	■	■						
OTU518	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Anaerovoracaceae, g__Family XIII AD3011 group, s__NA]	■	■							
OTU372	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Anaerovoracaceae, g__Mogibacterium, s__NA]	■	■	■						
OTU24	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Peptostreptococcaceae, g__Paeniclostridium, s__NA]	■	■							
OTU11	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Peptostreptococcaceae, g__Romboutsia, s__ilealis]	■	■	■						
OTU1655	[k__Bacteria, p__Proteobacteria, c__Alphaproteobacteria, o__Acetobacterales, f__Acetobacteraceae, g__Acetobacter, s__pasteurianus]	■								

Table 5. OTUs identified in the nasopharyngeal, ruminal, and vaginal microbiota of at least 60% of samples from pregnant heifers.

OUT	Taxa	60%	65%	70%	75%	80%	85%	90%	95%	100%
OTU23	[k__Archaea, p__Euryarchaeota, c__Methanobacteria, o__Methanobacteriales, f__Methanobacteriaceae, g__Methanobrevibacter, s__ruminantium]	■	■	■	■	■				
OTU56	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Bifidobacteriales, f__Bifidobacteriaceae, g__Bifidobacterium, s__merycicum]	■	■	■	■	■	■			
OTU68	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Bifidobacteriales, f__Bifidobacteriaceae, g__Bifidobacterium, s__pseudolongum]	■	■	■	■	■	■	■	■	
OTU105	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Corynebacteriales, f__Corynebacteriaceae, g__Corynebacterium, s__crudilactis]	■	■							
OTU147	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Corynebacteriales, f__Corynebacteriaceae, g__Corynebacterium, s__marinum]	■	■	■	■					
OTU26	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Corynebacteriales, f__Corynebacteriaceae, g__Corynebacterium, s__NA]	■	■	■	■	■	■	■		
OTU3812	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Corynebacteriales, f__Corynebacteriaceae, g__Corynebacterium,s__NA]	■	■							
OTU272	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Corynebacteriales, f__Corynebacteriaceae, g__Corynebacterium, s__provencense]	■	■	■						
OTU160	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Micrococcales, f__Intrasporangiaceae, g__Ornithinimicrobium, s__NA]	■								
OTU20	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Micrococcales, f__Micrococcaceae, g__Arthrobacter, s__castelli]	■	■							
OTU14	[k__Bacteri, p__Actinobacteriota, c__Actinobacteria, o__Micrococcales, f__Micrococcaceae, g__Arthrobacter, s__pigmenti]	■								
OTU352	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Micrococcales, f__Micrococcaceae, g__Paeniglutamicibacter, s__NA]	■								
OTU76	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Propionibacteriales, f__Nocardioidaceae, g__Marmoricola, s__NA]	■								
OTU80	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Propionibacteriales, f__Nocardioidaceae, g__Nocardioides, s__jensenii]	■								
OTU377	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Propionibacteriales, f__Propionibacteriaceae, g__Cutibacterium, s__acnes]	■	■							
OTU35	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Streptomycetales, f__Streptomycetaceae, g__Streptomyces, s__NA]	■	■	■						
OTU370	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Clostridia UCG-014, f__NA, g__NA, s__NA]	■	■							
OTU927	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Clostridia UCG-014, f__NA, g__NA, s__NA]	■								
OTU119	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Clostridiales, f__Clostridiaceae, g__Clostridium sensu stricto 1, s__NA]	■								
OTU43	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Hungateiclostridiaceae, f__Saccharofermentans, g__NA, s__NA]	■								
OTU97	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Hungateiclostridiaceae, f__Saccharofermentans, g__NA, s__NA]	■	■	■						
OTU489	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__[Eubacterium] hallii group, s__NA]	■		■						
OTU90	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Dorea, s__NA]	■								
OTU62	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Lachnospiraceae NK3A20 group, s__NA]	■	■	■	■	■	■	■		
OTU295	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Lachnospiraceae NK3A20 group, s__NA]	■	■	■						
OTU373	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Lachnospiraceae NK3A20 group, s__NA]	■	■	■	■					
OTU986	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Lachnospiraceae NK3A20 group, s__NA]	■								
OTU1688	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Lachnospiraceae NK3A20 group, s__NA]	■	■	■	■	■	■			
OTU897	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Syntrophococcus, s__NA]	■								
OTU25	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__[Eubacterium] coprostanoligenes group, g__NA, s__NA]	■	■	■	■	■	■	■	■	
OTU78	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Oscillospiraceae, g__NK4A214 group, s__NA]	■								
OTU37	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Oscillospiraceae, g__UCG-005, s__NA]	■	■	■						
OTU54	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Oscillospiraceae, g__UCG-005, s__NA]	■	■							
OTU360	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__NA, s__NA]	■	■	■						
OTU29	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__Ruminococcus, s__NA]	■	■	■	■	■	■	■	■	
OTU83	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__Ruminococcus, s__NA]	■	■	■	■	■				
OTU188	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__Ruminococcus, s__NA]	■	■	■	■	■				
OTU201	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__Ruminococcus, s__NA]	■	■							
OTU244	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__UCG-001, s__NA]	■								
OTU243	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Anaerovoracaceae, g__Family XIII AD3011 group, s__NA]	■	■	■	■					

OTU518	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Anaerovoracaceae, g__Family XIII AD3011 group, s__NA]	
OTU372	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Anaerovoracaceae, g__Mogibacterium, s__NA]	
OTU24	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Peptostreptococcaceae, g__Paeniclostridium, s__NA]	
OTU11	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Peptostreptococcaceae, g__Romboutsia, s__ilealis]	
OTU32	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Peptostreptococcaceae, g__Romboutsia, s__NA]	
OTU96	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Peptostreptococcaceae, g__Romboutsia, s__NA]	
OTU1655	[k__Bacteria, p__Proteobacteria, c__Alphaproteobacteria, o__Acetobacterales, f__Acetobacteraceae, g__Acetobacter, s__pasteurianus]	

Table 6. OTUs identified in the nasopharyngeal, ruminal and vaginal microbiota of 60% of samples from yearling and pregnant heifers.

OUT	Taxa	60%	65%	70%	75%	80%	85%	90%	95%	100%
OTU8	[k__Archaea, p__Euryarchaeota, c__Methanobacteria, o__Methanobacteriales, f__Methanobacteriaceae, g__Methanobrevibacter, s__NA]	■	■							
OTU23	[k__Archaea, p__Euryarchaeota, c__Methanobacteria, o__Methanobacteriales, f__Methanobacteriaceae, g__Methanobrevibacter, s__ruminantium]	■	■	■	■	■				
OTU56	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Bifidobacteriales, f__Bifidobacteriaceae, g__Bifidobacterium, s__merycicum]	■								
OTU68	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Bifidobacteriales, f__Bifidobacteriaceae, g__Bifidobacterium, s__pseudolongum]	■	■	■	■					
OTU105	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Corynebacteriales, f__Corynebacteriaceae, g__Corynebacterium, s__crudilactis]	■								
OTU147	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Corynebacteriales, f__Corynebacteriaceae, g__Corynebacterium, s__marinum]	■	■							
OTU26	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Corynebacteriales, f__Corynebacteriaceae, g__Corynebacterium, s__NA]	■	■	■	■	■	■			
OTU272	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Corynebacteriales, f__Corynebacteriaceae, g__Corynebacterium, s__provencense]	■								
OTU160	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Micrococcales, f__Intrasporangiaceae, g__Ornithinimicrobium, s__NA]	■								
OTU35	[k__Bacteria, p__Actinobacteriota, c__Actinobacteria, o__Streptomycetales, f__Streptomycetaceae, g__Streptomyces, s__NA]	■	■							
OTU351	[k__Bacteria, p__Actinobacteriota, c__Coriobacteriia, o__Coriobacteriales, f__Atopobiaceae, g__Atopobium, s__NA]	■								
OTU537	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Christensenellales, f__Christensenellaceae, g__Christensenellaceae R-7 group, s__NA]	■								
OTU370	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Clostridia UCG-014, f__NA, g__NA, s__NA]	■								
OTU927	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Clostridia UCG-014, f__NA, g__NA, s__NA]	■								
OTU43	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Hungateiclostridiaceae, f__Saccharofermentans, g__NA, s__NA]	■								
OTU97	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Hungateiclostridiaceae, f__Saccharofermentans, g__NA, s__NA]	■	■	■	■					
OTU133	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__[Eubacterium] hallii group, s__NA]	■	■	■						
OTU158	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__[Eubacterium] hallii group, s__NA]	■								
OTU489	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__[Eubacterium] hallii group, s__NA]	■	■							
OTU62	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Lachnospiraceae NK3A20 group, s__NA]	■	■	■	■	■	■	■		
OTU373	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Lachnospiraceae NK3A20 group, s__NA]	■	■	■						
OTU657	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Lachnospiraceae NK3A20 group, s__NA]	■								
OTU1688	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Lachnospirales, f__Lachnospiraceae, g__Lachnospiraceae NK3A20 group, s__NA]	■	■	■	■	■				
OTU25	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__[Eubacterium] coprostanoligenes group, g__NA, s__NA]	■	■	■	■	■	■	■	■	
OTU78	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Oscillospiraceae, g__NK4A214 group, s__NA]	■	■	■						
OTU37	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Oscillospiraceae, g__UCG-005, s__NA]	■	■							
OTU54	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Oscillospiraceae, g__UCG-005, s__NA]	■								
OTU360	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__NA, s__NA]	■								
OTU29	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__Ruminococcus, s__NA]	■	■	■	■	■	■	■	■	
OTU83	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__Ruminococcus, s__NA]	■	■	■	■	■				
OTU188	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__Ruminococcus, s__NA]	■	■	■						
OTU201	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__Ruminococcus, s__NA]	■	■							
OTU307	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__Ruminococcus, s__NA]	■								
OTU244	k__Bacteria, p__Firmicutes, c__Clostridia, o__Oscillospirales, f__Ruminococcaceae, g__UCG-001, s__NA]	■								
OTU243	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Anaerovoracaceae, g__Family XIII AD3011 group, s__NA]	■	■	■						
OTU518	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Anaerovoracaceae, g__Family XIII AD3011 group, s__NA]	■	■							
OTU372	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Anaerovoracaceae, g__Mogibacterium, s__NA]	■	■	■						
OTU24	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Peptostreptococcaceae, g__Paeniclostridium, s__NA]	■	■	■	■	■				
OTU11	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Peptostreptococcaceae, g__Romboutsia, s__ilealis]	■	■	■	■	■				

OTU32	[k__Bacteria, p__Firmicutes, c__Clostridia, o__Peptostreptococcales-Tissierellales, f__Peptostreptococcaceae, g__Romboutsia, s__NA]		
OTU1655	[k__Bacteria, p__Proteobacteria, c__Alphaproteobacteria, o__Acetobacterales, f__Acetobacteraceae, g__Acetobacter, s__pasteurianus]		

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