Bacterial communities associated to the urethra of healthy gilts and pregnant sows undergoing different reproductive protocols[1]

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ABSTRACT

Nowadays, it is known that the urogenital microbiota plays a key role in the urinary health of mammalians. Despite of the urinary infections affect the health and the welfare of breeding sows, the urethral microbiota of healthy sows remains unknown. Therefore, this work evaluates the urethral bacterial communities of healthy gilts and sows to determine the presence of *Enterobacteriaceae* populations, and the structure of this microbiota in gilts (G) and pregnant sows (P). Samples were collected by scraping the urethral mucosa of G (n = 9) and P, that included natural mating (NM, n = 9) and artificial inseminated (AI, n = 7) sows. Samples were analysed by culture-dependent techniques and 16S-rRNA gene High-Throughput-Sequencing. All females were positive for *Enterobacteriaceae* culture, without significant differences (Kruskal-Wallis) between G and P (median values: 2.78 and 3.09 Log CFU/mL, respectively; P = 0.497). Also, the rate *Enterobactericeae*/total mesophilic microorganisms was individually calculated, without significant differences between G and P (median values: 0.61 and 0.66, respectively; P = 0.497). When analysing the bacterial communities, it was found similar richness in G, NM and AI; however, diversity was lower in P than G (Mann Whitney/Kruskal-Wallis test, P < 0.01). The dominating phyla that constituted a "core microbiome", included *Firmicutes*, *Proteobacteria*, *Cyanobacteria*, *Actinobacteria*, and *Bacteroidetes*; which were common for all the studied females. The relative abundance for phyla, families and genera was estimated and *Firmicutes* was significantly higher in NM than AI sows (P = 0.02, Mann-Whitney/Kruskal Wallis test for univariate statistical comparisons), *Pseudomonadaceae* and *Enterobacteriaceace* were higher in AI than in NM (Mann Whitney/Kruskal-Wallis, P < 0.05). *Lactobacillus* and *Pseudomonas* were among the dominant genera; however, only *Pseudomonas sp.* was significantly higher in AI than NM (Mann Whitney/Kruskal-Wallis, P = 0.006). The results represent the first evidence about the existence of a urethral microbiota that includes

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Enterobacteriaceae, as well as the patterns of this microbiota in G and P sows. The knowledge of this urethral microbiota might allow for future research to develop innovative protocols to restore and/or preserve the healthy ecology of the urinary microbiome to prevent diseases ensuring the welfare of breeding sows.

Keywords: gilts, next generation sequencing, pregnant sows, urethral microbiota.

ABBREVIATIONS

µL: microlitre.

16S: minor subunit component of prokaryotic ribosomes.

AI: pregnant sows by artificial insemination (artificial inseminated).

bp: base pairs.

DNA: deoxyribonucleic acid.

G: gilts.

Log CFU/mL: logarithm of colony forming unit per millilitre.

mL: millilitre.

n: number of animals.

NM: pregnant sows by natural mating (natural inseminated).

OTU: Operational taxonomic unit.

P: pregnant sows.

Past: Paleontology Statistics.

PBS: phosphate-buffered saline solution.

PCoA: principal coordinate analysis.

PCR: polymerase chain reaction.

QIIME: Quantitative Insight into Microbial Ecology.

RNA: ribonucleic acid.

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rRNA: ribosomal RNA.

SPRI: solid phase reversible immobilization.

UT: urinary tract.

UTI: urinary tract infection.

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INTRODUCTION

The microbiome is defined as the genome of all the microorganisms, symbiotic and pathogenic, living in and on all vertebrates (Berg et al., 2020). The microbiota comprises all living members forming the microbiome, which means the living organisms of an ecosystem or a particular area (Berg et al,. 2020). Thus, the mucosal surfaces of humans and animals are colonized by communities of commensal, symbiotic and pathogenic microorganisms (Proctor, 2019). The interactions between this commensal microbiota and the host influence in their physiology, regulating metabolism and immune function, as well as their complex behaviors (Lynch and Hsiao, 2019). Several studies concluded that the structure of the bacterial communities in the urinary tract could have an important participation in the host´s health (Horwitz et al., 2015; Whiteside et al., 2015; Thomas-White et al., 2016; Bao et al., 2017; Brubaker and Wolfe, 2017). However, the urinary microbiome of sows has been unexplored and the patterns of their microbiota in gilts and pregnant sows remain unknown up to date.

The urogenital health of gilts and sows is determinant for the reproductive performance, which is a key factor for productivity in the pig farming (Koketsu et al., 2017). The urinary tract infections are a common problem in breeding sows, reducing animal welfare, decreasing productivity and resulting in a premature culling (Wanyoike and Bilkei, 2006; Stalder et al., 2012; Drolet, 2019). *Escherichia coli* and *Proteus* sp. belong to the *Enterobacteriaceae* family and are recognized pathogens of these urinary infections (Moreno et al., 2018; Drolet, 2019). Nevertheless, it not clear if *Enterobacteriaceae* are part of healthy urinary microbiota and thus, some species could be potential pathogens. Therefore, the aim of this work was to evaluate the microbial communities of the urethral mucosa in healthy gilts and sows, to determine if pregnancy drives changes in the autochthonous microbiota. Thus, the urethral microbial ecology of gilts and pregnant sows was examined by High-Throughput Sequencing

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approach based on Illumina MiSeq sequencing of the V3-V4 16S rRNA and culture-dependent methods focusing the study on the *Enterobacteriaceae* population. The knowledge of the patterns of the urethral microbiota in pregnancy will allow for future research about the urinary microbiome and might promote the development of innovative therapeutic strategies to prevent diseases ensuring the welfare of breeding sows.

MATERIALS AND METHODS

Animals and sampling

Twenty five contemporary healthy females (Duroc × [Landrace × Yorkshire]) were sampled: 9 gilts (G; body weight 121.4 ± 6.3 kg [average ± SD], age 7 ± 1 months [average ± SD]) and 16 pregnant sows (P; average body weight 223.4 ± 12.5 kg [average ± SD], age 18.6 ± 5.6 months [average ± SD], gestation 60 ± 5 days). They were group-housed in pens (250 m^2 per female) according to the category (G or P). Gilts and sows had free access to water and received standard gestation feed: 74% corn, 23% soybean expeller and 3% premix for gestation (Vetifarma S.A., Buenos Aires, Argentina). The gilts expressed two estrous cycles before sampling. Sows: pregnancy have been achieved by natural mating (NM; by hand mating system; n = 9), they were two nulliparous, three primiparous and four multiparous sows (with 3 to 4 previous farrowing). Seven of the pregnant sows were artificial inseminated (AI); all of them were multiparous sows (2 to 3 previous farrowing).

The sampling was conducted at outdoor pig farm located in Leales, Tucumán, Argentina (27°12'54.1"S 65°15'15.8"W) during autumn (May 2018, AI group) and winter (July 2018, G and NM groups).

For samples collection, perineum and vulvar areas were washed with sterilized water and dried by using paper towels. Then, stainless steel specula were placed to access to the meatus, and cytobrushes were used to scrape the urethral wall approximately at the internal urethral

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orifice level. Finally, cytobrushes were put in 1 mL phosphate buffered saline solution (PBS)-containing tubes, pH 7.0 and kept at 4°C until processing. All procedures were conducted under the Argentinean Animal Welfare Legislation, Law N°14.346, SENASA-R70/2001 with the approval of the Institutional Committee for the Care and Use of Laboratory Animals of the National University of Tucumán (CICUAL–UNT, Research Protocol N° 030/2019).

Microbial populations: culture-dependent methods.

Enterobacteriaceae population. The tubes with cytobrushes and PBS were vigorously agitated during 2 min to dislodge cells. Then, 50 µL of pure and 0.01 dilution of each sample were inoculated on plates containing LAPTg agar (in g/L: peptone, 15; tryptone, 10; yeast extract, 10; D-glucose, 10, agar, 15) (Raibaud et al. 1963), Columbia agar supplemented with 5% sheep blood (Britania Laboratories, Buenos Aires, Argentina) and MacConkey agar (Britania Laboratories, Buenos Aires, Argentina). Plates were incubated for 24 to 48 h at 37°C in aerobic conditions, with the exception of Columbia agar plates which were incubated in microaerophilic conditions (5% CO_2-enriched chamber). After incubation, the colonies grown on MacConkey plates were evaluated by morphology and Gram staining. The number of viable microorganisms, expressed as Colony Forming Units per mL (CFU / mL) was determined to quantify the cultivable microbial populations of mesophilic microorganisms (LAPTg and Columbia plates) and *Enterobacteriaceae* (MacConkey plates).

Microbial populations: culture-independent techniques

Nucleic acid extraction and amplifications. The DNA of the samples was extracted using QIAGEN kits (QIAamp DNA mini kit, Hilden, Germany) according to the manufacturer's instructions. Quantification and integrity were checked before amplification reactions, and stored at −20°C. The bacterial V3-V4 16S rRNA region was amplified with the primer pairs

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343F (5′-TACGGRAGGCAGCAG-3′) and 802R (5′-TACNVGGGTWTCTAATCC-3′) using Phusion Flash High-Fidelity MasterMix (Thermo Fisher Scientific, Inc. Waltham, MA, USA). A two-step nested-PCR was applied and conditions used for reaction mix and amplification experiments were those described by Vasileiadis et al. (2015). In the second PCR the 343F primer was labeled with a different "barcode" for each sample. The PCR products from all samples were joined in a single pool in equimolar concentrations based on the QuBit quantification data and were concomitantly purified by solid phase reversible immobilization (SPRI) using the Agencourt AMPure XP kit (Beckman Coulter, Milano, Italy). The PCR product pool was sequenced by PTP – Science Park (Parco Tecnologico Padano, Lodi, Italy) using a MiSeq Illumina Reagent Kit v3 (Illumina Inc., San Diego, CA, USA) which generated 300 bp paired-end reads.

Data processing and bioinformatics analysis. Quality check from raw reads was performed using FastQC v0.11.2 (Babraham Bioinformatics, Cambridge, UK). Samples were demultiplexed using ea-utils v.1.1.2-537 fastq-multx (Aronesty, 2013) relying on a metadata file provided by the customer. Illumina raw sequences were trimmed using Trimmomatic v0.32 (Bolger et al., 2014). Minimum base quality 20 (Phred-scale) over a 4 bases sliding window was required. Only sequences above 36 nucleotides in length were included into downstream analysis. For original amplicon reconstruction, overlapping R1 and R2 paired reads were joined using ea-utils v.1.1.2-537 fastq-join tool (Aronesty, 2013). Non overlapping R1 and R2 paired reads were concatenated using one “N” base separator. Amplicons were dereplicated, sorted and clustered at 97% identity using VSEARCH v1.1.3 (Rognes et al., 2016) following standard QIIME (Quantitative Insight into Microbial Ecology, Caporaso et al., 2010) pipeline parameters. For taxonomy-based analyses QIIME formatted Greengenes v.13.8 database was used. Taxonomies were adapted to QIIME

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taxonomy standards uniforming to the 7 main taxa ranks (superkingdom, phylum, class, order, family, genus and species). The operational taxonomic unit (OTU) were identified against reference databases (Greengenes v.13.8 database) using NCBI-Blast v2.2.27 (Basic Local Alignment Search Tool of National Center for Biotechnology Information online website). After counting the abundance of each OTU, a final OTU-table output file was created using custom scripts.

Statistics

Data from the bacterial cultures (logarithmically transformed) were tested for normality and homoscedasticity. Then, a non-parametric test (Kruskal Wallis test) was applied to compare G and P groups. Minitab Statistical Software version 15.1.20.0 (Minitab. LLC. State College, PA, USA) was used for this analysis.

To analyze DNA read mapping two indexes, Chao's wealth and Shannon's diversity and a principal coordinate analysis (PCoA) were performed using QIIME package, version 1.5.0 in the pipeline Microbiome Analyst (http://microbiomeanalyst.ca/faces/home.xhtml). Past (Paleontology Statistics) software version 3.23 (Hammer et al., 2001) was used to perform the Mann-Whitney/Kruskal Wallis test for comparison of the relative abundance of OTU among the groups of females.

RESULTS

Urethral microbiota: Enterobacteriaceae population (studies based on cultures)

The magnitude of the urethral colonization by *Enterobacteriaceae* was assessed by culture-dependent techniques using a selective medium. It is interesting to point out that all females were positive for this culture and there were no significant differences (Kruskal Wallis test) between G and P groups (median values: 2.78 and 3.09 Log CFU/mL, respectively; H = 0.46;

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GL = 1; *P* = 0.497). As an estimation of the overall colonization, the data were also analyzed taking account the total mesophilic microorganisms detected in each sample; thus, the rate *Enterobacteriaceae*/total mesophilic microorganisms (E/M) was individually calculated and no differences were found between G and P groups (median values: 0.61 and 0.66, respectively; H= 0.46; GL= 1; P= 0.497).

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Urethral microbiota: 16S metagenomics approach

The microbial diversity was measured using the Shannon and Chao1 indices, that evaluate abundance (number of different species) and homogeneity (Shannon 1997) and richness, respectively. When comparing the index values of G and P groups, significant differences were observed between Shannon index, but not between the Chao1 index estimated for each group (Mann Whitney/Kruskal-Wallis test, $P < 0.01$) (Fig. 1A). Therefore, the urethral microbiota from P had lower diversity than G, but all urethral samples had a similar richness. Moreover, a PCoA based on the β-diversity/Bray-Curtis was performed to evaluate the differences between the bacterial communities associated to each group. Thus, there was no significant separation or distinct clustering (PERMANOVA, $P > 0.01$) in the taxonomic composition of the urethral microbiota in G and P sows (Fig. 1B).

Considering only the P group and comparing the estimators for the urethral microbiota from AI and NM, not significant differences were detected (Shannon and Chao1 indexes, Mann Whitney/Kruskal-Wallis test, $P > 0.05$) (Fig. 2A). However, when evaluating β-diversity based Bray-Curtis, significant differences were observed (PERMANOVA, $P < 0.02$) between the microbial communities' structures from AI and NM sows, although the data were partially overlaid (Fig. 2B).

Structure of the microbial communities

Nineteen phyla were found in the porcine urethral microbiota. The bacterial taxa with the highest relative abundances were *Firmicutes* (37%), *Proteobacteria* (26%), *Actinobacteria* (12%), *Cyanobacteria* (8%), *Fusobacteria* (8%), *Bacteroidetes* (6%), *Acidobacteria* (1%), and *Thermi* (1%) (Fig. 3A). The remaining 11 phyla were represented by less than 1% of the total sequence reads.

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The urethral core microbiome defined as the group of phyla present in 90% of the samples (Lorenzen et al., 2015) was constituted by *Firmicutes*, *Proteobacteria*, *Cyanobacteria*, *Actinobacteria*, and *Bacteroidetes*. Moreover, sequences from *Fusobacteria*, *Acidobacteria*, and *Thermi (Deinococcus–Thermus)* were detected in 17, 16 and 14 from a total of 25 samples, respectively. The remaining 11 phyla defined in our pooled urethral sequence dataset were present in ≤ 10 samples; among them, *Verrucomicrobia*, *Gemmatimonadetes*, *Spirochaetes*, *Nitrospirae*, and *Planctomycetes* were detected in 10/25, 6/25, 6/25, 5/25, and 5/25 samples, respectively.

Taxonomical assignment at the bacterial order level resulted in 52 taxa; however, only 14 showed a relative abundance > 1%, with *Clostridiales*, *Actinomycetales*, *Lactobacillales*, *Fusobacteriales*, *Enterobacteriales*, and *Pseudomonadales*, being the most abundant (≥ 7%) (Fig. 3B). One hundred and thirty one OTU families were identified; those with ≥ 0.5% relative abundance and present in half of the animals at least in one group (G or P) or in half of the total animals were included in the Table 1. From this group, the most prevalent (> 5%) were *Tissierellaceae*, *Fusobacteriaceae*, *Clostridiaceae*, *Enterobacteriaceae*, and *Streptococcaceae*. The sequences that could be assigned at genus level and that were present in > 90% of the samples were identified as *Lactobacillus*, *Pseudomonas*, *Rhodoplanes*, *Enterococcus*, and unclassified OTUs derived from *Clostridiaceae*, *Micrococcaceae*, and *Bradyhizobiaceae* families (Supplementary Fig. S1).

Comparison of the urethral microbiota in gilts and pregnant sows (AI and NM)

Overall, no significant differences were found for bacterial relative abundance with respect to the dominant phyla between G and P sows (Mann-Whitney/Kruskal-Wallis, $P > 0.05$), with the exception of *Thermi*, that was significantly higher in P than G sows (Mann

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Whitney/Kruskal-Wallis, $P < 0.05$) (Fig. 4A). Considering the most abundant families (relative abundance ≥ 0.5%), no differences were found between G and P groups (Fig. 4B).

The relative abundances of phyla present in both AI and NM pregnant sows are shown in Fig. 5A. *Proteobacteria* and *Firmicutes* were the most abundant in both groups; the relative abundances of *Proteobacteria* were 51 and 25% for AI and NM, respectively; however, no significant differences were detected. Conversely, the relative abundance of *Firmicutes* was significantly higher in NM (39%) than AI (17%) ($P = 0.02$, Mann-Whitney/Kruskal Wallis test for univariate statistical comparisons). Among the remaining phyla, only *Cyanobacteria* showed significant difference between both groups, being higher in AI sows ($P = 0.04$) (Fig. 5A).

At family level, the relative abundance of *Jonesiaceae*, *Streptococcaceae*, *Flavobacteriaceae*, and *Peptostreptococcaceae* was significantly higher in NM than in AI sows; while *Pseudomonaceae and Enterobacteriaceace* were significantly higher in AI than NM (Mann Whitney/Kruskal-Wallis, $P < 0.05$) (Fig. 5B).

When analyzing the genera with at least 0.5% relative abundance, *Pseudomonas* was significantly (Mann Whitney/Kruskal-Wallis, $P = 0.006$) higher in AI (0.176 ± 0.06) than NM sows (0.04 ± 0.04), while *Streptococcus* was most abundant (Mann Whitney/Kruskal-Wallis, $P = 0.02$) in NM than AI sows (0.08 ± 0.02 and 0.007 ± 0.003, respectively) (Fig. 5C).

DISCUSSION

For the first time, the present study provides evidences of the existence of a urethral microbiota in healthy gilts and sows. Also, through culture-dependent techniques, it was possible to demonstrate that this microbiota includes *Enterobacteriaceae* populations. Moreover, next generation sequencing allowed to describe the patterns of this microbiota for

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gilts (G) and pregnant sows (P), to define an urinary "core microbiome" and to describe the bacterial communities at family/genus levels in NM and AI pregnant sows.

A carefully sampling of the mucosal surfaces (by scraping), instead of a microbial recovery from urine (Gusmara et al., 2011; Moreno et al., 2018), ensured that the collected microorganisms were those colonizing the urethra and free of faecal contamination.

It is worth highlighting that all sows and gilts were positive for the *Enterobacteriaceae* culture. Several genera belonging to this taxon, represent recognized pathogens for urinary infections in sows (Gusmara et al., 2011; Moreno et al., 2018; Drolet, 2019); therefore, their presence in the microbiota of healthy female could mean that some of them have a potential pathogenic role.

The metagenomic approaches allowed us to identify the complex microbial communities colonizing the urethral mucosa of G and P sows, the richness of these communities being similar in both groups, regardless how pregnancy was achieved. However, the Shannon predictor indicated a low diversity in P sows. Probably, physiological conditions induce a loss of diversity that naturally characterizes the porcine urethral microbiota as observed in the G group. The changes in the native urethral microbiota during pregnancy must be further studied to determine if they are responsible for dysbiosis and increased susceptibility to infections during the gestation and after farrowing (Fangman and Carlson Shannon, 2007; Baricco, 2011). In this sense, it was demonstrated that a loss of diversity in the urinary microbiota in women predisposed to developing urinary tract infections (Brubaker et al., 2014; Brubaker and Wolfe, 2017).

Likewise, we evaluated and compared the structure of urethral microbial communities by β-diversity analysis between G and P (NM and AI) groups. This analysis indicated compositional differences between AI and NM groups. Further research with a higher

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number of animals, must be carried out to evaluate these differences and to identify if populations of potential urogenital pathogens are implicated.

The analysis of the taxonomic distribution of the bacterial communities indicated that *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* were the most prevalent phyla associated to the porcine urethral microbiota. Regarding the porcine urogenital tract, only the vaginal microbiota has been previously described (Wang et al., 2017) and these same phyla were present as the main constituents in both healthy and endometritic adult sows. In our study, the occurrence and dominance of these three phyla together with *Cyanobacteria* and *Fusobacterium*, could indicate that they constitute the microbial nucleus that colonize the urethra of G and P sows (without significant differences between both groups); therefore, the dynamics of colonization under physiological conditions, would probably take place among the populations of these taxonomic groups. These key issues were also addressed in studies of the urinary microbiota in pregnant (Ollberding et al., 2016) and no-pregnant women (Siddiqui et al., 2011; Wolfe et al., 2012; Lewis et al., 2013).

In this work, the occurrence of *Enterobacteriaceae* as commensal microorganisms of the porcine urethra was detected by using both culture dependent and independent techniques. To our knowledge, there are few reports describing *Enterobacteriaceae* as members of the urethral microbiota in healthy female; in this sense, Ollberding et al. (2016) reported *Serratia* sp. in urine from pregnant women.

Our analysis showed that the largest numbers of sequence reads belonged to the *Enterobacteriaceae* in AI sows. This subject should be further studied since the gestation implies high susceptibility to infections; particularly, the colonization by *E. coli*, *Proteus* spp. or *Klebsiella* spp. must be taken into consideration because they were related to urogenital tract infections in sows during the postpartum period (Fangman and Carlson Shannon, 2007; Baricco, 2011; Gusmara et al., 2011; Moreno et al., 2018; Drolet, 2019).

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Among the predominant OTUs, *Pseudomonas* stands out as one of the most prevalent (> 90% of the urethral samples) and associated to P group. Its presence should be troubling, since this genus has been described as agent of endometritic and vaginal discharges in sows (Torremorrell, 2007). If the urethral microbiota is part of "a urogenital microbiome" (Burton et al., 2017), we could hypothesize that the increase of urethral population of *Pseudomonas* among the pregnant sows, could represent a risk for the genital health.

The *Firmicutes* members, *Lactobacillus* and *Streptococcus*, detected in this study may have a putative antagonistic role in the urinary tract. To our knowledge, this is the first report on the presence of urethral *Lactobacillus* in healthy sows; previously, they have been described in the porcine vagina (Lorenzen et al., 2015; Wang et al., 2017). This genus of lactic acid bacteria was widely studied because of their benefic effects on the human urinary tract (Siddiqui et al., 2011; Hilt et al., 2014; Jacobs et al., 2017). Therefore, we can assume that the presence of these bacterial populations in the anterior urethra of sows would play a protective role against potential pathogenic microorganisms, such as members of *Enterobacteriaceae*. Further research is necessary to determine the potential protective role of urethral *Lactobacillus*; especially after farrowing, a stress condition that increases the infection risks in sows (Fangman and Amass, 2007; Falceto et al,. 2012).

In this study *Streptococcus* appears mostly associated to urethra of P sows. This finding should be considered in futures studies, because they might to access at the vagina from urethra and colonize the birth canal; this would represent a risk since some *Streptococcus* species are pathogens in newborn piglets (Gottschalk and Segura, 2019).

The results presented in this work contribute to the knowledge regarding the urinary ecosystem and their bacterial communities in healthy gilts and pregnant sows.

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CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest.

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Table 1. Distribution of most prevalent family OTUs in the urethral microbiota of sows

Family OTU[1]	mean abundance %	N° samples/25
Tissierellaceae	10.13	22
Fusobacteriaceae	8.23	22
Clostridiaceae	7.83	24
Enterobacteriaceae	6.59	24
Streptococcaceae	5.33	20
Lachnospiraceae	4.05	24
Micrococcaceae	3.92	25
Ruminococcaceae	3.79	24
Pasteurellaceae	3.44	21
Moraxellaceae	3.39	22
Lactobacillaceae	2.36	24
Sphingomonadaceae	1.96	22
Pseudomonadaceae	1.87	24
Peptostreptococcaceae	1.72	18
Flavobacteriaceae	1.6	10
Campylobacteraceae	1.49	17
Bacillaceae	1.42	22
Jonesiaceae	1.36	11
Actinomycetaceae	1.31	18
Hyphomicrobiaceae	1.24	24
Bradyrhizobiaceae	1.14	23
Cellulomonadaceae	1.08	14
Porphyromonadaceae	0.98	22
Intrasporangiaceae	0.95	19
Veillonellaceae	0.94	20
Aeromonadaceae	0.94	14
Bacteroidales[2]	0.88	19
Aerococcaceae	0.79	15
Burkholderiaceae	0.79	17
Nocardioidaceae	0.74	21
Bacteroidaceae	0.67	15
Enterococcaceae	0.64	24
Deinococcaceae	0.61	19
Rhodobacteraceae	0.61	15
Caulobacteraceae	0.6	21
Rhizobiaceae	0.59	20
Rhodospirillaceae	0.58	17
Comamonadaceae	0.55	22
Staphylococcaceae	0.51	18
Gaiellaceae	0.5	21

[1]OTUs with ≥ 0.5% relative abundance.

[2]Family not identified from order *Bacteroidales*.

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FIGURES

Figure 1.A Box-plot showing α-diversity in samples using Chao1 and Shannon index in samples from urethra of gilts (**G**) and pregnant (**P**) sows. Mean (♦), median and quartile range are shown. *Indicates significant differences between G and P groups ($P < 0.01$; Mann-Whitney/Kruskal Wallis test). **B** Principal coordinate analysis (PCoA) based on Bray Curtis ß-diversity showed no clear distinct clustering of the gilts (pink) and pregnant sows (blue).

Figure 2.A Box-plot showing α-diversity in samples using Chao1 and Shannon index in samples from urethra of pregnant sows by natural mating (NM) or artificial insemination (AI). Mean (♦), median and quartile range are shown. **B.** Principal coordinate analysis (PCoA) based on Bray Curtis ß-diversity showed partially overlaid distinct clustering of the pregnant sows: AI (red) and NM (blue). (PERMANOVA, $P < 0.02$).

Figure 3. Composition of the bacterial urethral microbiota of sows. Contribution of the most abundant phyla (A) and orders (B).

Figure 4. Urethral microbiota of healthy sows. **A**. Relative abundance of major bacterial phyla found in the sequence pool of urethral samples from gilts (G) and pregnant sows (P). "Other" represent minor groups. **B**. Relative abundance of the most prevalent families found in urethral samples from G and P. *Indicates significant differences between G and P groups ($P < 0.05$; Mann-Whitney/Kruskal Wallis test).

Figure 5. Urethral microbiota in pregnant sows. Relative abundance profiles at phylum (**A)**, family (**B)** and **(C)** genus (or the lowest common taxon) levels obtained from the sequence classification of the 16S rRNA gene. AI: artificial insemination NM: natural mating. *Indicates significant differences between AI and NM groups ($P < 0.05$; Mann-Whitney/Kruskal Wallis test).

Supplementary Figure S1. Stacked bar charts showing relative abundance of microbial DNA detected via 16S rRNA amplicon sequencing and annotated to the genus or the lowest common taxon, in samples from healthy gilts (G) and pregnant sows (AI: artificial insemination; NM: natural mating).

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Fig 1A

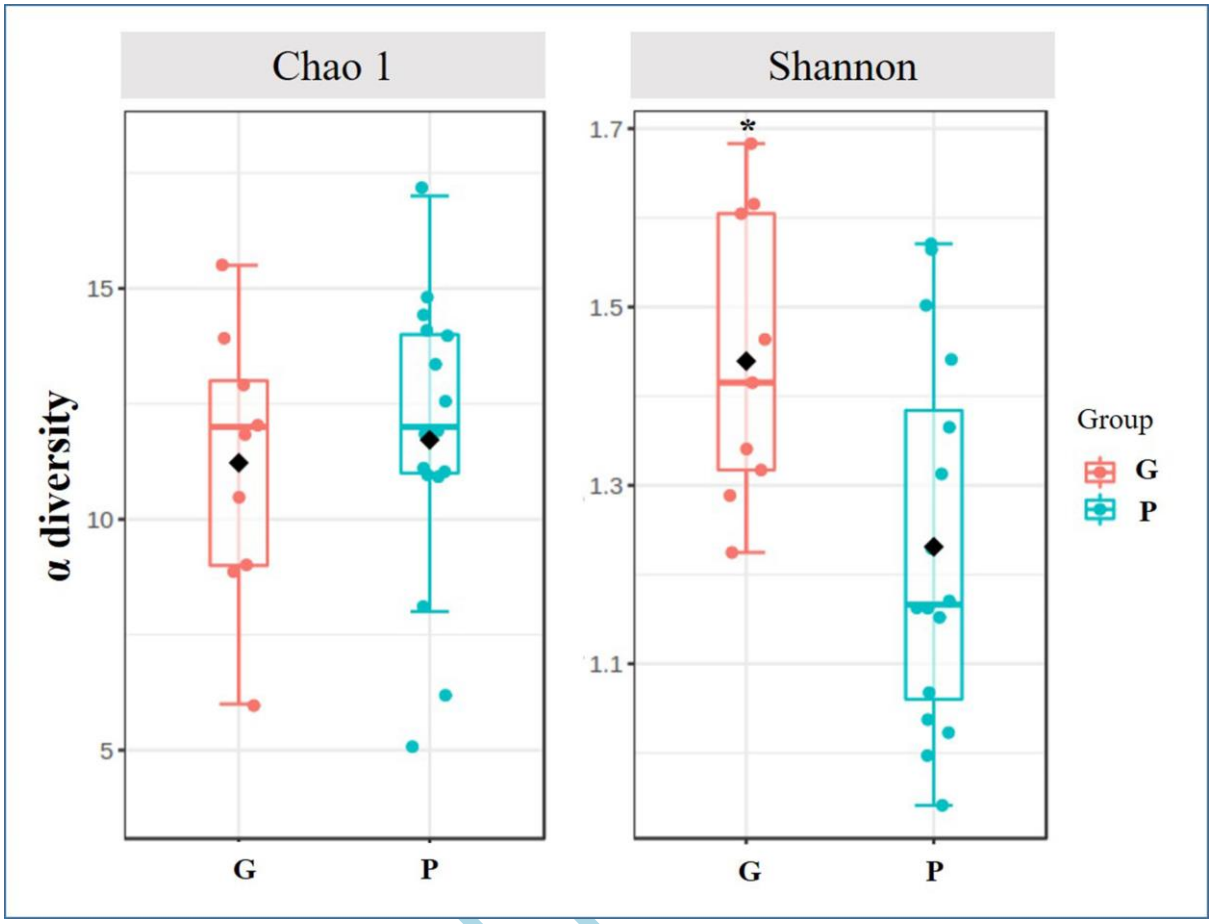

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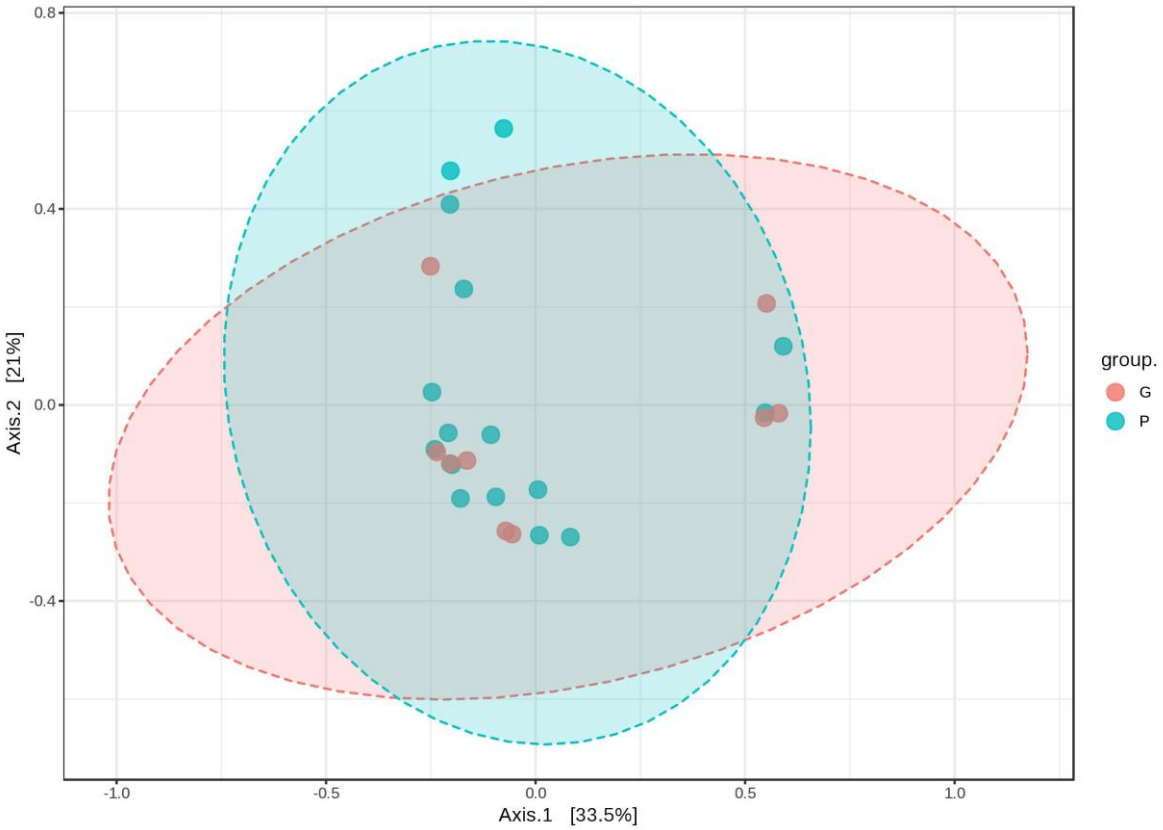
Fig 1B



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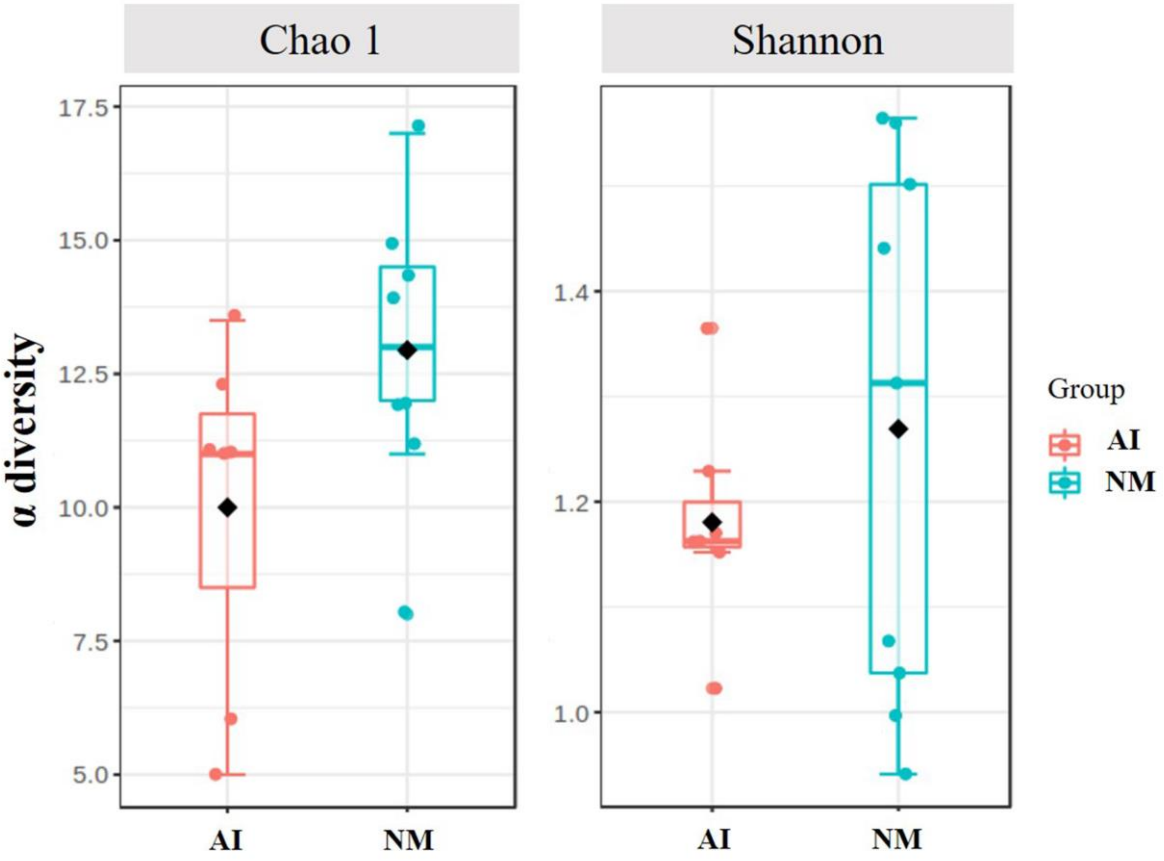

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Fig 2B

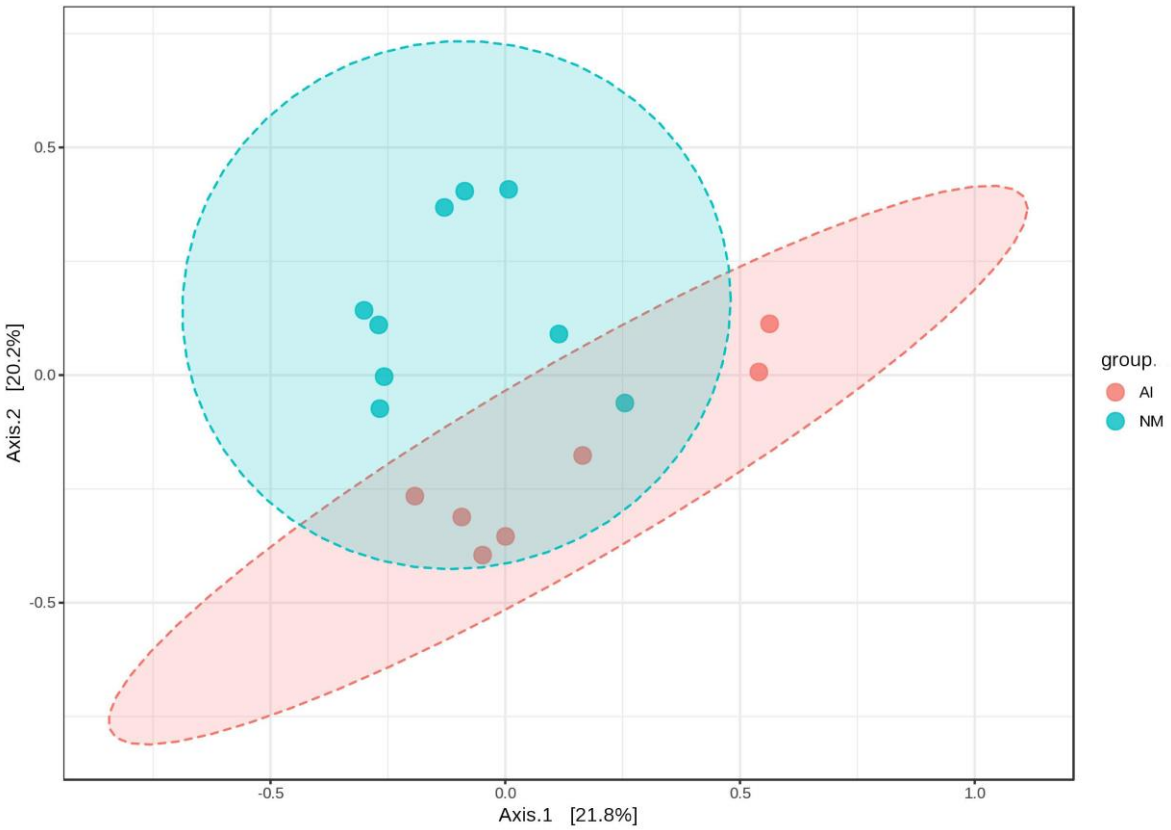

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Fig 3A

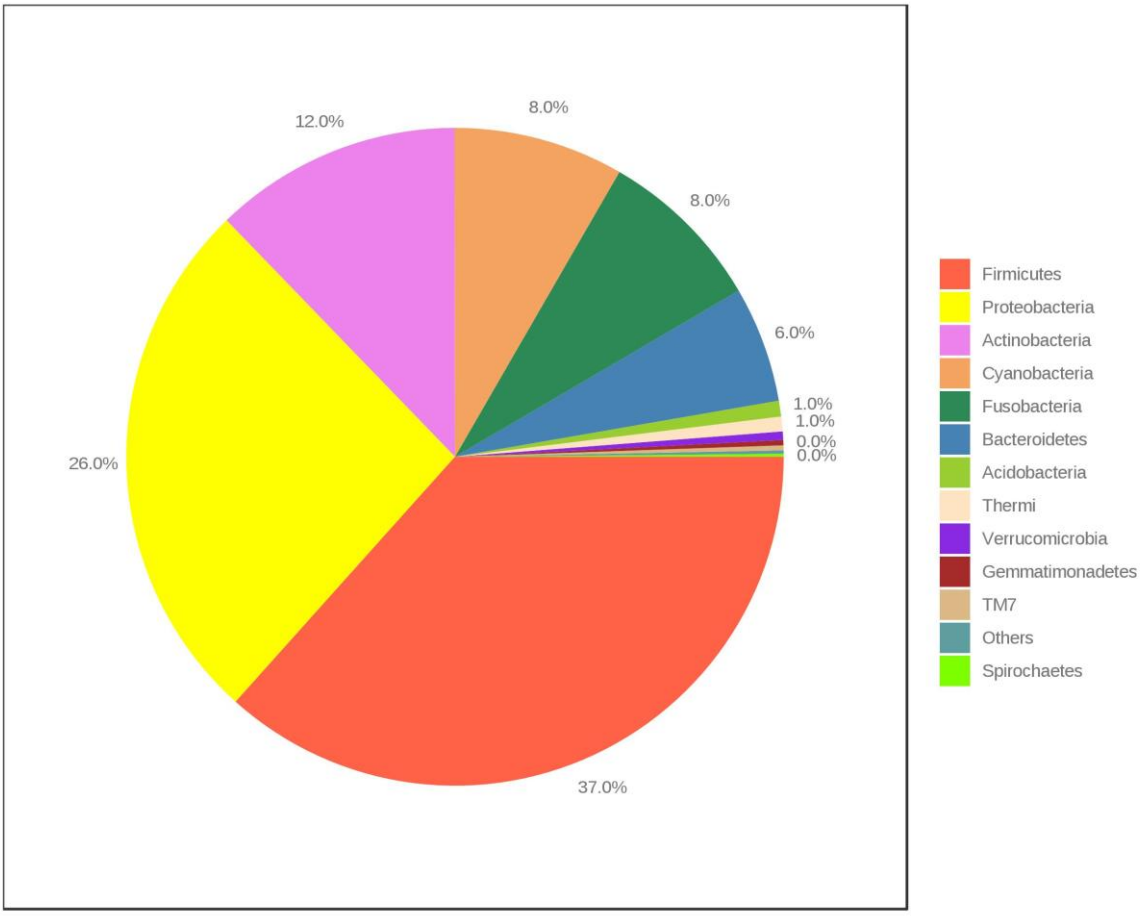

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Fig 3B

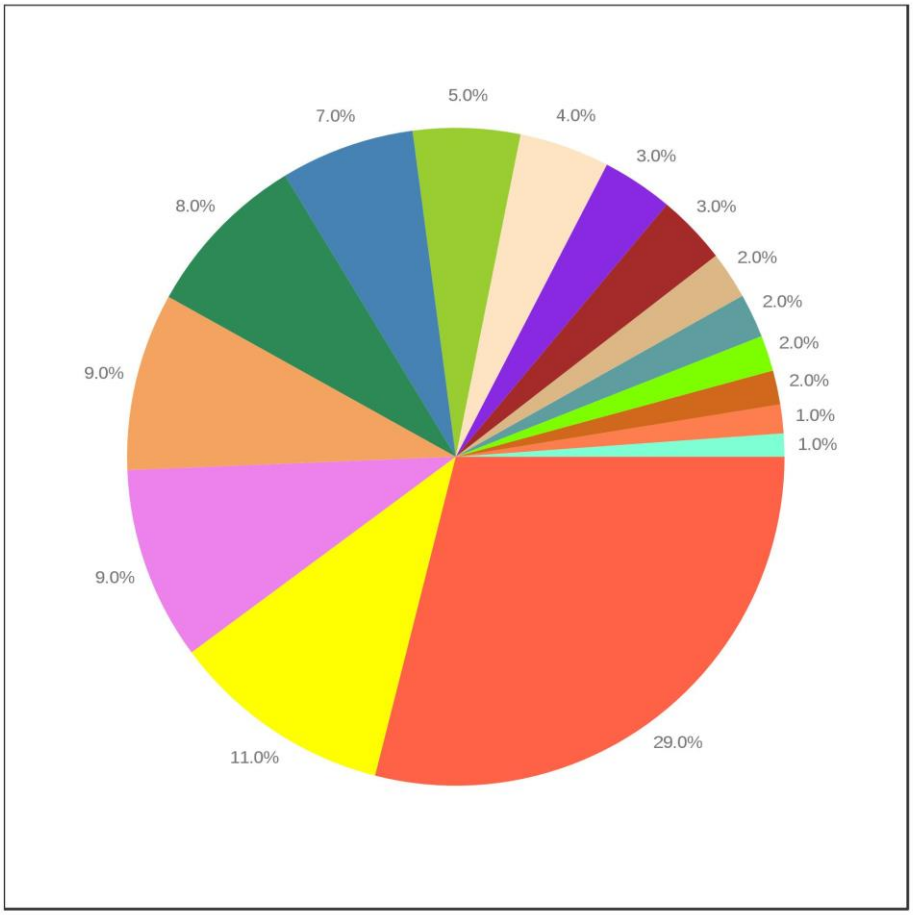

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Fig 4A

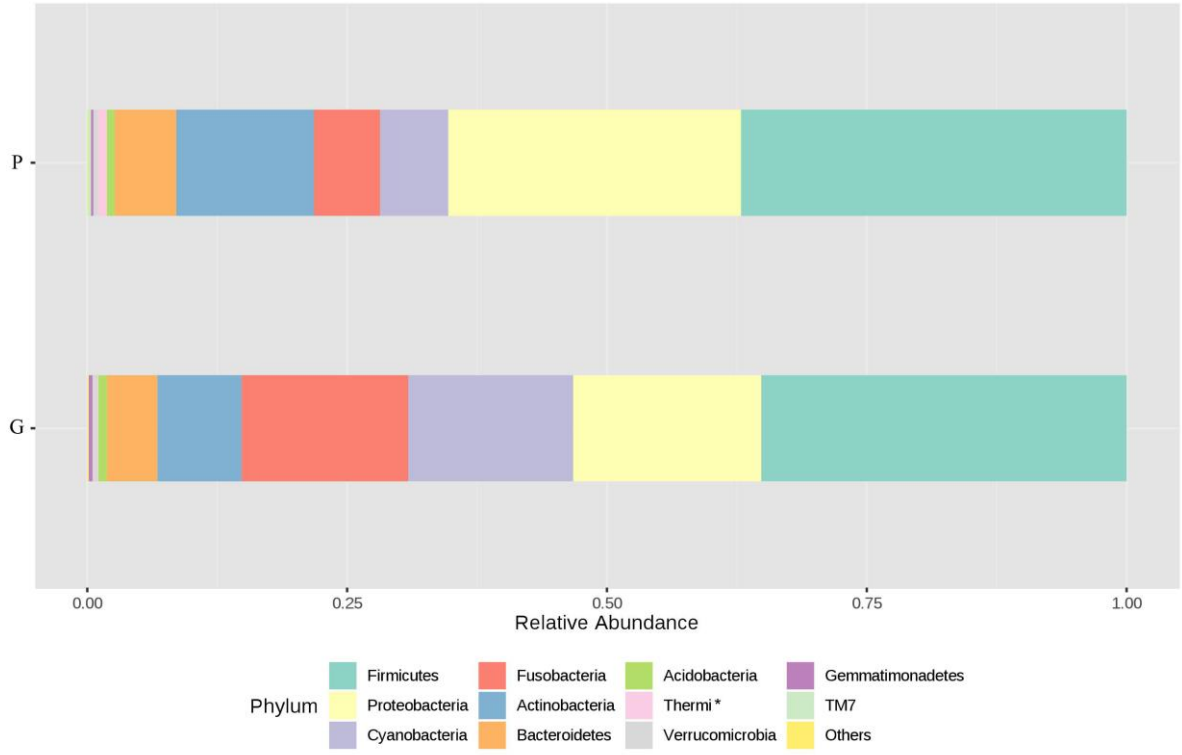

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Fig 4B

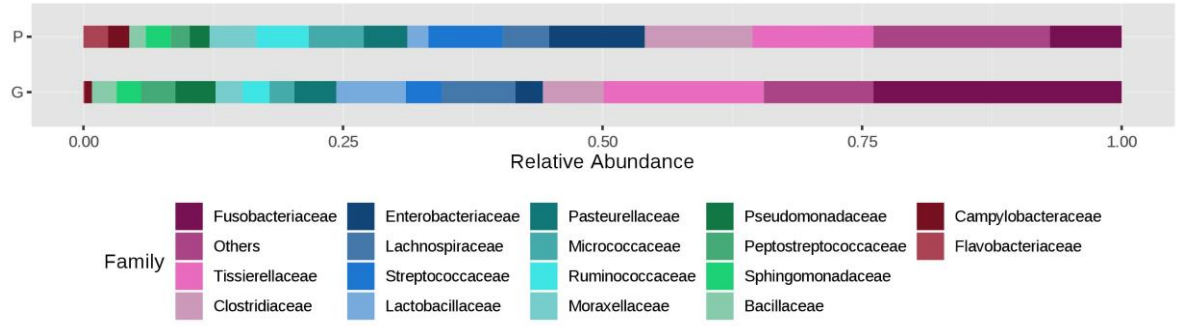

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Fig 5A

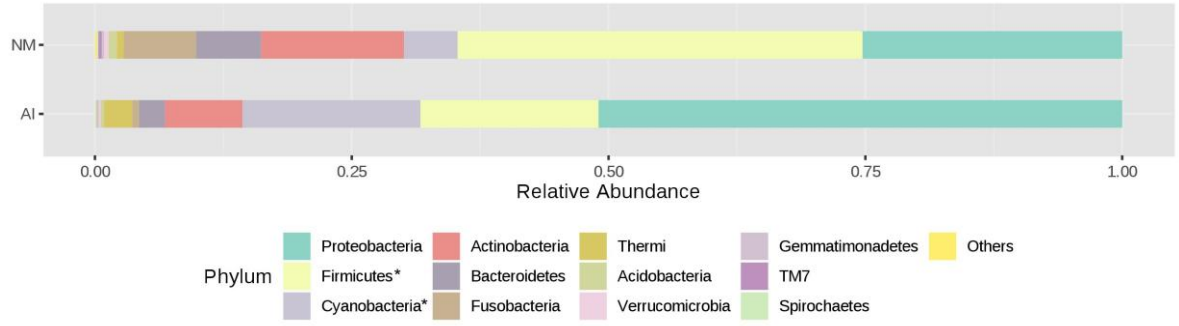

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Fig 5B

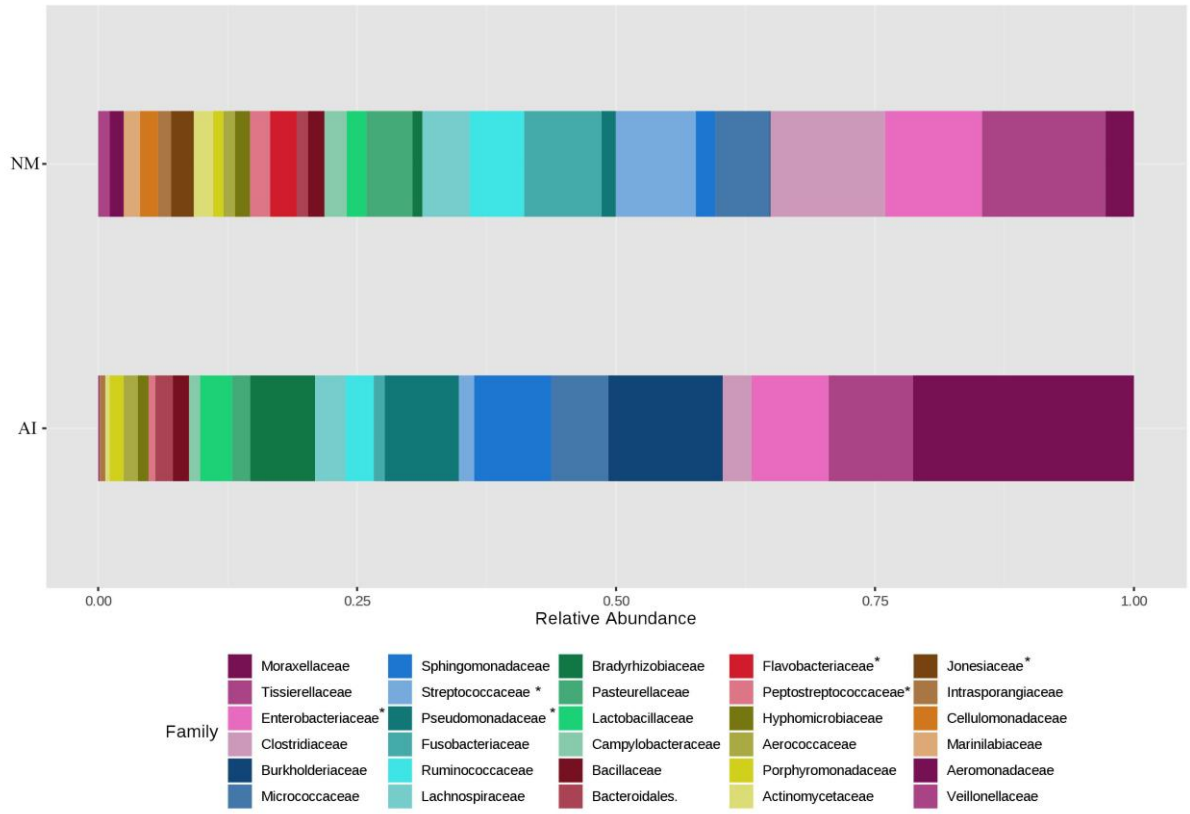

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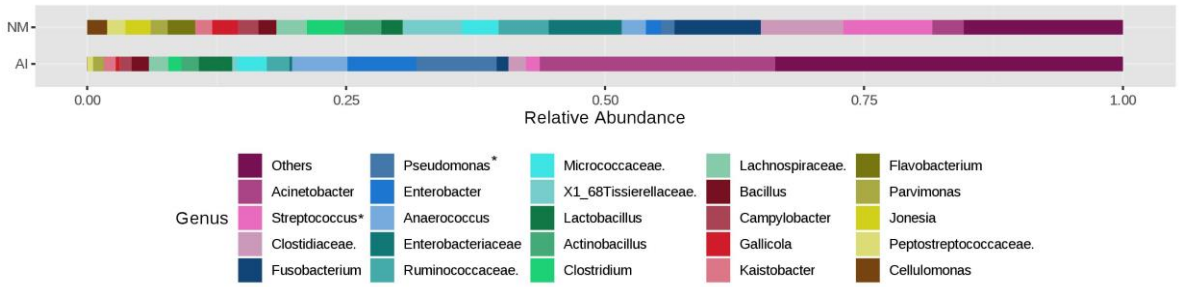
Fig 5C



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