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Exploiting *16S* rRNA-based metagenomics to reveal neglected microorganisms associated with infertility in breeding bulls in Spanish extensive herds

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ABSTRACT

Bovine infectious infertility represents a problem due to the high impact on animal production and, in many cases, in public health. A lack of information on the characteristics of the bacterial population of the bovine reproductive system can hamper a comprehensive understanding of reproductive pathologies and the role that the microbiome could play. A metagenomic study based on the V3-V4 hypervariable region of the bacterial *16S* rRNA gene was performed in 1029 preputial samples from bulls raised in an extensive regimen in Spain (944 from herds with low fertility rates -case group-, and 85 samples from reproductively healthy herds -control group-). The most representative phyla as well as the most 10 abundant bacterial families and their abundance did not show significant differences in both case and control groups. Similarly, the (alpha and beta) diversity of the bacterial populations was similar in both type of herds: the Shannon and Simpson indices show a high diversity of species, while the Bray-Curtis dissimilarity index did not show relevant differences in the bacterial communities. A deeper analysis of the operational taxonomic units showed the presence of one genera, *Mycoplasma* spp. significantly associated with fertility problems. Our study highlights the promising potential that the application of sequencing techniques (e.g. *16S* rRNA-based metagenomics) possesses in examining bovine infertility, as they are able to reveal different pathogens that could go unnoticed using diagnostic approaches for only the main known pathogens.

1. Introduction

Bovine infectious infertility represents a serious problem in the livestock sector due to the impact on animal production and, in many cases, in public health (Yoo, 2010). Different types of microorganisms can be associated with bovine infertility; primary or opportunistic pathogens such as *Campylobacter fetus* (Mshelia et al., 2010) or *Trueperella pyogenes*, respectively (Rzewuska et al., 2019), or microorganisms ubiquitous in the environment such as *Proteus* spp. (Drzewiecka, 2016). Many of microorganism associated with cattle infertility can be sexually transmitted through natural breeding and artificial insemination (Givens, 2018); especially problematic being those such as *C. fetus* which produce asymptomatic infections in males that may go unnoticed within herds (Mshelia et al., 2010). In spite of the large number of pathogens associated with bovine infertility, scarce information on the bacterial population of the bovine reproductive bull system is available, which hampers the understanding of the association between microbiome and reproductive pathologies described in other species, e.g., vaginal bacteriosis in humans due to dysbiosis (Ling et al., 2010).

Due to the limitation of some microorganisms to be cultivated, high

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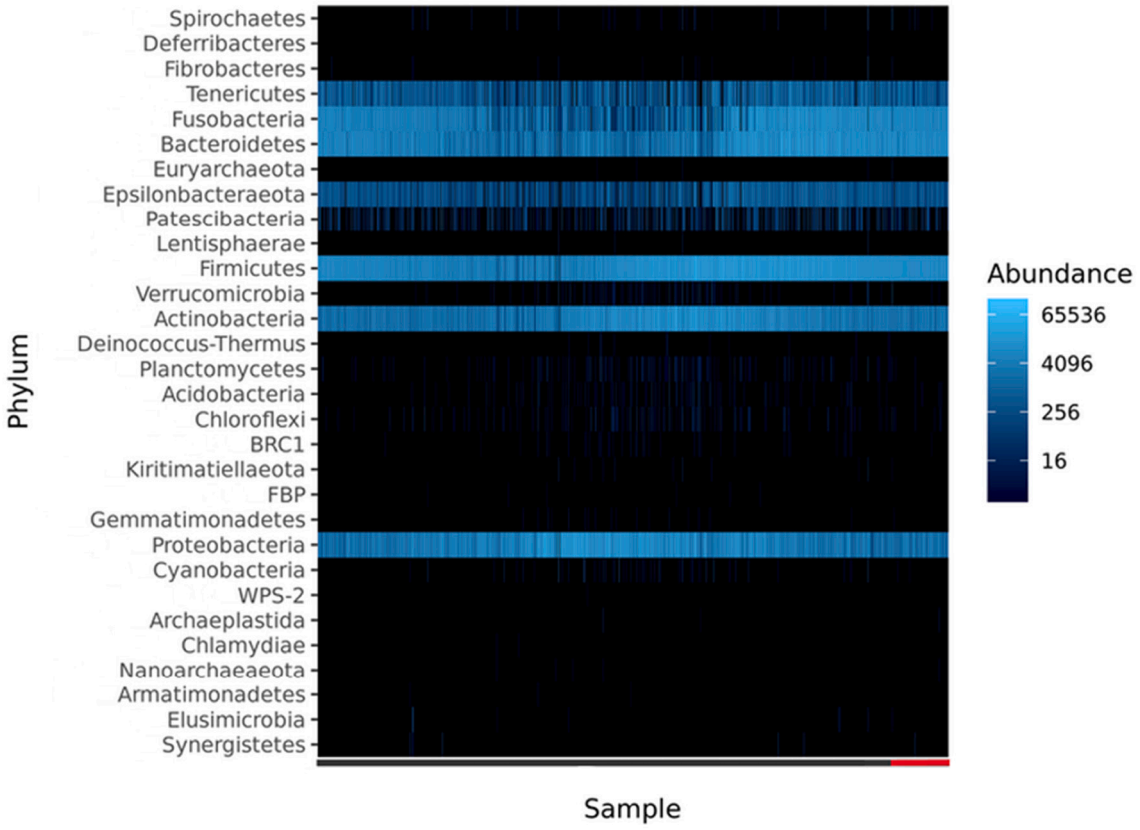

Fig. 1. Heatmap of the most abundant taxa detected in the bovine preputial samples of the study. Black bar (at the bottom) represents the case group samples and red bar the control group samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

throughput sequencing has become a fundamental molecular tool for the study of the microbial populations and their dynamics (Alves et al., 2018; Weinstock, 2012), and *16S* rRNA gene deep sequencing used as standard for microbial typing (Bharti and Grimm, 2021).

The present study aimed at performing a characterization of bacterial populations in bull preputial samples from Spanish extensive production herds with low fertility rates and healthy herds via the deep sequencing of the V3-V4 hypervariable region of the bacterial *16S* rRNA gene, and we correlated the operational taxonomic units (OTUs) obtained to previously describe primary and/or opportunistic pathogens associated with bull infertility.

2. Material and methods

2.1. Preputial samples recovered from bulls

A total of 1029 preputial samples from bulls (one sample per bull) from spanish herdswere selected and included in the present study: 944 samples from herds diagnosed with low fertility rates (case group, fertility rates under 80%), and 85 samples from reproductive healthy herds (control group). Bulls belongs to herds from central Spain, with mediterranean-continental climate, raised in extensive regimen based in grazing areas acording to "dehesa" system ("dehesa" is defined as a ecosystem featuring scattered holm and cork oak trees throughout the landscape), most of them with all-year breeding season, with 100 cows average herd size and mainly composed by crossbreed animals (Spanish breeds as Avileña and other beef breeds as Limonusin and Charolais). Samples had been submitted for routine diagnostics for *C. fetus* and *Tritrichomonas foetus*. The samples consisted of 15 mL of preputial washings in PBS subjected to centrifugation at 1512 $\times g$ for 10 min. The pellets were stored at −80 °C until analysis.

2.2. Total DNA extraction, 16S rRNA gene amplicon library preparation and sequencing

Total DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany). A first cellular lysis buffer [20 mM of Tris-Cl (pH 8), 2 Mm of sodium EDTA, 1.2% Triton X-100 and 20 mg / mL of lysozyme in a 360 µL of final volume] was added to the pellet and incubated at 37 °C for 1 h, and afterwards subsequent steps were performed according to manufacturer's instructions. The DNA concentration was determined using a Qubit fluorimeter 4 (Invitrogen). Microbial diversity was studied by sequencing the amplified V3-V4 region of the *16S* rRNA gene using primers and PCR conditions previously reported (Klindworth et al., 2013). Sample multiplexing, library purification, and sequencing were carried out as described in the "*16S* Metagenomic Sequencing Library Preparation" guide by Illumina. Libraries were sequenced on a MiSeq Illumina platform at the Universidad de Burgos (UBU, Burgos, Spain), leading to 460 bp, paired-end reads.

2.3. Bioinformatics and data analysis

>100,000,000 raw reads were analysed using different bioinformatics tools. Raw demultiplexed sequence data was processed using QIIMEReporter pipeline (https://github.com/dabadgarcia/qiimereporter). This straightforward pipeline for the analysis of amplicon sequences integrates basic QIIME2 commands (Bolyen et al., 2019) with R programming language. In brief, the DADA2 package (Callahan et al., 2016) was used to quality filter reads, merge paired ends, remove chimeras and assign amplicon sequence variants (ASV). ASVs rely on single nucleotide differences between sequences and can be considered as Operational Taxonomic Units (OTUs) clustered at 100% identity threshold. A pre-trained Naïve Bayes classifier (Wang et al., 2007) was used to obtain the taxonomic assignment of the ASVs, using the SILVA

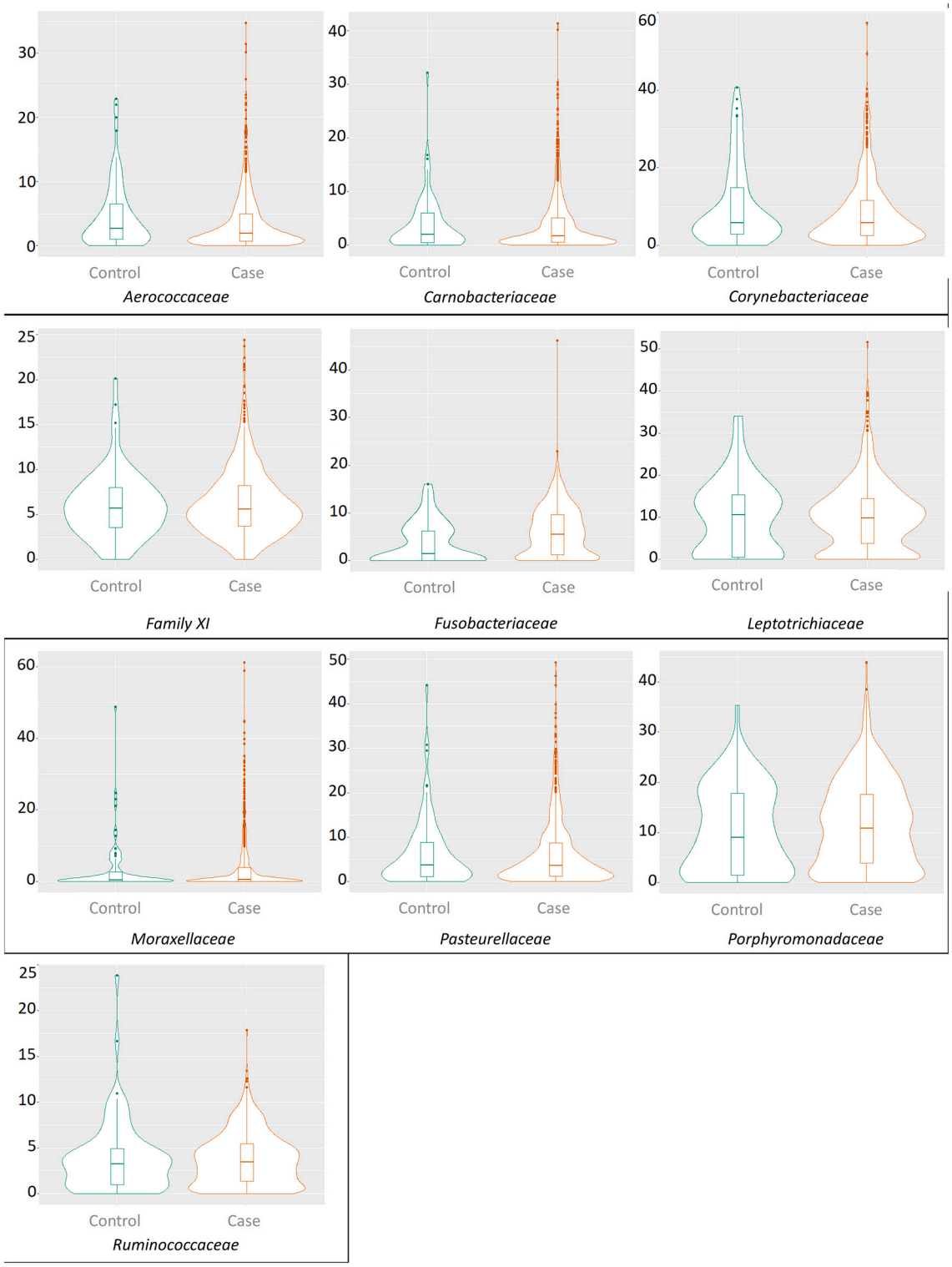

Fig. 2. The 10 most abundant bacterial families detected in the bovine preputial samples of the study. The Y axis corresponds to the abundance (relativized between 0 and 100 values). In the X axis is indicated the control and case samples.

database version 132 (Quast et al., 2013) as reference, which resulted in a table containing the microbial composition for each of the samples. Alpha- and beta-diversity were analysed by using diversity (https://docs.qiime2.org/2018.4/plugins/available/diversity/) and taxa (https://docs.qiime2.org/2018.4/plugins/available/taxa/) plugins. For beta-diversity studies, samples were rarefied to 23,761 reads per sample, in order to avoid biases due to different sequencing depths, and weighted UniFrac distances (Lozupone and Knight, 2005) were calculated. Plotting was carried out in R (R Core Team, 2021) using packages *dplyr* (Wickham et al., 2021), *ggplot2* (Wickham, 2016), *made4* (Culhane et al., 2005) and *reshape2* (Wickham, 2007) packages.

In order to establish the most abundant phyla in the total samples, a heatmap is included in the report resulting from the QIIMEreporter pipeline performed which provided information about the alpha

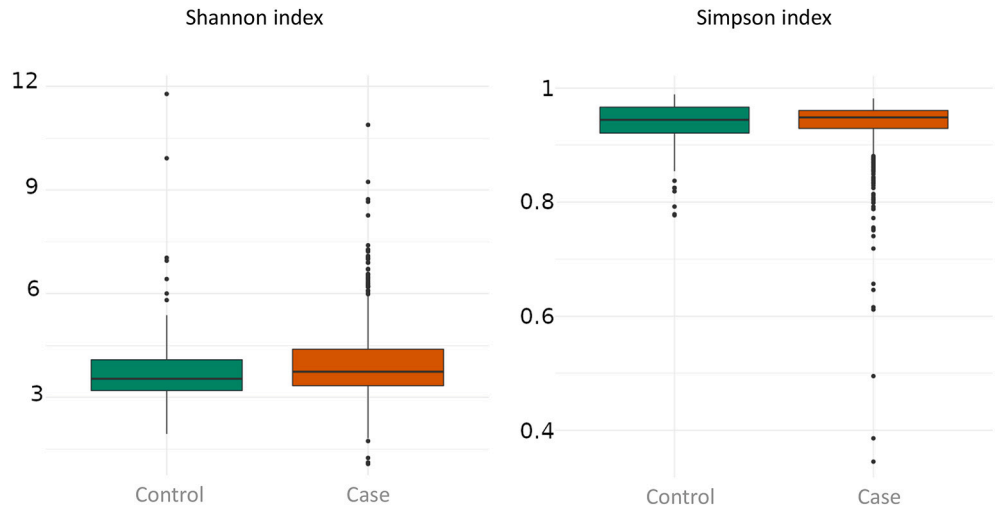

Fig. 3. Alpha biodiversity in the samples tested. Representation of Shannon and Simpson index values (Y axis) for the control and case samples (X axis) where no significant differences in means were noted (p-value = 0.745 and 0.403 respectively).

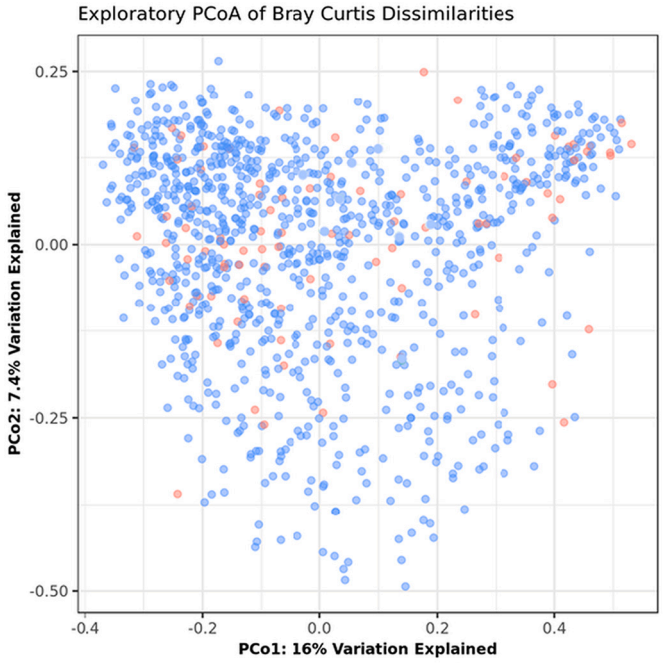

Fig. 4. Beta biodiversity in the samples tested. Representation of Bray-Curtis Dissimilarities where the blue circles correspond to animals from infertile herds (case group) and the pink circles correspond to bulls from healthy herds (control group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

diversity measured using the Simpson and Shannon index metrics, and beta diversity estimated by computing Bray-Curtis distances abundance profiles. Bray-Curtis distances were used for Principal Coordinates Analysis (PCoA), and the significance of separation was tested by permutational multivariate analysis of variance using the function "Adonis" that consist in an analysis of variance using distance matrices based on the work of Anderson (2001). On the other hand, the ten most abundant families are analysed through the OTU tables on R implemented with the R package MicrobeR (https://github.com/jbisanz/MicrobeR) and Bioconductor (https://www.bioconductor.org/).

The OTU table was analysed at the species or at least genus level using R by *dplyr* package, showing the presence/absence of bacterial species and genus previously associated from literature with bovine infertility in the preputial samples included in the study (in both problem and control samples). Association between the detection of each bacterial species and infertility in the herd of origin of the sampled animal was assessed using chi-square tests corrected for multiple comparisons using Holm's method in R (Holm, 1979). Significance level is set to 0.05 throughout the paper.

3. Results and discussion

3.1. Bacterial population in preputial samples in infertility-related and healthy herds

The most representative phyla and their abundance in the total of the samples are shown in Fig. 1. Our data suggests a similar abundance in both infertility-related and healthy herds: the abundance of each representative phylum was homogeneous (Fig. 1), and when the 10 most abundant bacterial families were analysed with R, a similar distribution and median was also observed, with only slight differences (Fig. 2). The alpha diversity (Fig. 3) and beta diversity (Fig. 4) were also evaluated in both case and control groups. While the alpha diversity values (Shannon and Simpson indices) were similar in both groups where no significant differences in means were noted according a t-test performed for Shannon (p-value = 0.745) and Simpson (p-value = 0.403) indices (Fig. 3), our data suggest a high diversity of the samples regardless of the group (Shannon value was between 3 and 4, and Simpson value was close to 1). Similarly, the results of the beta diversity using Bray-Curtis dissimilarity index did not show substantial differences in the bacterial species composition as the samples were not grouped into specific groups according to the type of herds (infertility-related or healthy) (Fig. 4). The Principal Coordinates Analysis (PCoA) explained only the 23.4% total variance of the dissimilarities between samples and the function "Adonis" indicated that only the 0.8% (R^2 = 0.00829) of the variation in distances was explained by the grouping tested (p-value = 0.001). Similar results were observed in a recent study where no significant differences in the composition of the bacterial populations of preputial bull samples were observed according to e.g. the type of breed and feeding, the use of antibiotics or the breeding history (Wickware et al., 2020).

3.2. Infertility-associated bacteria present in preputial samples

When a deeper analysis of the OTUs obtained was performed, we observed the presence of specific bacterial genera and species associated

with infertility within cattle herds, among which we found both primary and opportunistic pathogens. The results from the chi-square tests showed the presence of one genera significantly associated with bull infertility (p-value <0.001): *Mycoplasma* spp. (720 bulls were positive within the case group and 37 bulls were positive within the control group). Species from *Mycoplasma* spp. have been already reported in cattle reproductive pathologies, such as *M. bovis* or *M. bovigenitalium* (Reichel et al., 2018; Kirkbride, 1987). Interestingly, the remaining bacterial species previously associated to bovine infertility did not show a statistically significant correlation with fertility problems. However, *C. fetus* species, whose correlation with fertility problems showed a p-value of 1, was only found in the case group samples (26 bull samples – 2.75%). The species *C. fetus* is a primary pathogen associated to cattle infertility and is particularly troublesome in countries where natural breeding and extensive production is practised (Mshelia et al., 2010). On the other hand, *U. diversum,* detected in a high percentage in both groups, was present in bulls from case group (798 positives) in a higher percentage than bulls from the control group (66 positives). Even though there is not a significant association with bull infertility. However, it is interesting to taking into account that *U. diversum* is an opportunistic pathogen previously associated with bovine infertility (Díaz et al., 2019; Hobson et al., 2013), that is able to colonize the foreskin and urethra in bulls, and can be transmitted to females even through artificial insemination (Buzinhani et al., 2011). Thus, it would be interesting to develop more studies about the true role of *U. diversum* in cattle infertility.

4. Conclusions

In conclusion, we performed the first report on the type and distribution of the bacterial populations in extensive bovine herds with records on low fertility in Spain. While the abundance and diversity of the bacterial population was similar in both infertility-related and healthy herds, we observed a relevant association of one bacterial taxa with the infertility-related herds: *Mycoplasma* spp., while the remaining bacterial species previously associated with bovine infertility did not show a statistically significant correlation with fertility problems, e.g. *C. fetus* or *Ureaplasma diversum*.

Our study highlights the promising potential that the application of sequencing techniques (e.g. *16S* rRNA-based metagenomics) possesses in examining bovine infertility, as they are able to reveal different pathogens that could go unnoticed using diagnostic approaches for only the main known pathogens. Likewise, our research opens a new path in which new and previously neglected bacterial genera and species, that may play a relevant role in bovine infertility associated with bulls, are revealed.

Declaration of Competing Interest

None.

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