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Next generation sequencing and bio-informatics analysis for the investigation of the vaginal bacterial microflora of clinically healthy mares

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ABSTRACT. This study was focused on the investigation of the constitution of the vaginal bacterial microflora of mares, using 16Sr-RNA next generation sequencing and bio-informatics analysis. Samples were collected from the inner vaginal wall of 30 clinically healthy adult mares from various locations in central Greece. Successful reads were retrieved from 28 samples resulting to the presumptive identification of 192 bacterial species belonging to 87 genera. None of the bacterial genera or species that were detected was present in all the test samples, which indicates that the vaginal bacterial microflora of the study population is diverse. The bacterial genera detected the most in the test samples were *Staphylococcus* (89%) and *Acinetobacter* (54%). The bio-informatics analysis produced evidence of a much richer microbiota for the Greek native, compared to the Arabian and other breeds, and indicated variations in its constitution associated with the reproduction and vaccination records.

Keywords: Mares; vaginal bacterial microflora; NGS; *Staphylococcus*; *Acinetobacter*.

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Introduction

The normal microbial microflora of the vagina is a physiological parameter that contributes significantly to immune regulation and development in the newborn (Jašarević et al., 2021; Klein-Jöbstl et al., 2019), and prevention of infection of the urogenital tract in adult animals (Adnane & Chapwanya, 2022; Appiah et al., 2020). In spite of its significance, this subject has not been adequately addressed in the mare. This study was focused on the determination of the bacterial species present in the vagina of mares and the investigation for similarity or discriminating traits with regards to breed, reproduction record, and vaccination. The last parameter was included in this investigation because vaccination constitutes a stimulus with strong immunological effect that can be protective and modulatory. Thereof, the outcome of vaccination, which to the best of our knowledge has not been investigated before in connection with the study subject, could affect the immunological balance between the host and its microbial microflora (*Ciabattini et al.,* 2019; Jamieson, 2015; Lynn et al., 2018; Zimmermann & Curtis, 2018).

Materials and methods

Samples were collected from 30 clinically healthy, adult mares (4-32 years old), from various equestrian groups and horse clubs of the Attika and Boeotia Prefectures, in central Greece, between May and September 2020. The following information was recorded through personal interview with their owners or the veterinarians who supervised them: age, breed, breeding location and origin, kinship to other study animals, vaccination, and management, including nutrition and breeding records. The study animals had not been

used for breeding for at least one year before sampling, during which, none of them present behavioral or clinical evidence of estrus. All animals of the study population were housed throughout the year with controlled access to fenced pastures, and were fed with meadow grass, clover, and oat hay. Age, breed, breeding site, location, breeding and vaccination records, of the animal under study, are reported in Table 1.

Table 1. The samples and the information recorded for the study population. Samples collected from the same site correspond to animals housed in the same breeding establishment.

No of sample	Background information					
	Age	Breed	Sampling site	Location	Reproduction record	Vaccination (annually)
1	32	Native Greek	A	Attika Prefecture	Never mated or inseminated	Never vaccinated
2	9	Native Greek	A	Attika Prefecture	Never mated or inseminated	Never vaccinated
3	13	Native Greek	A	Attika Prefecture	Never mated or inseminated	Never vaccinated
4	7	Native Greek	A	Attika Prefecture	Never mated or inseminated	Never vaccinated
5	12	Native Greek	A	Attika Prefecture	Never mated or inseminated	Influenza-Tetanus
6	20	Irish sport horse	A	Attika Prefecture	AI* once - Did not conceive	Influenza-Tetanus/ Herpesvirus
7	10	Bulgarian sport horse	A	Attika Prefecture	Never mated or inseminated	Influenza-Tetanus
8	18	KWPN (Holland)	A	Attika Prefecture	Never mated or inseminated	Influenza-Tetanus/ Herpesvirus
9	6	Native Greek	A	Attika Prefecture	Never mated or inseminated	Influenza-Tetanus
10	10	British sport horse	A	Attika Prefecture	Never mated or inseminated	Influenza-Tetanus/ Herpesvirus
11	4	Native Greek	A	Attika Prefecture	Never mated or inseminated	Never vaccinated
12	7	Native Greek	A	Attika Prefecture	AI once – 1 successful** gestation	Never vaccinated
13	20	Native Greek	A	Attika Prefecture	AI once - Did not conceive	Influenza-Tetanus
14	11	Bulgarian horse	A	Attika Prefecture	Never mated or inseminated	Influenza-Tetanus
15	11	Native Greek	A	Attika Prefecture	Never mated or inseminated	Influenza-Tetanus
16	25	Native Greek	A	Attika Prefecture	Never mated or inseminated	Never vaccinated
17	15	Native Greek	A	Attika Prefecture	Never mated or inseminated	Never vaccinated
18	16	Native Greek	A	Attika Prefecture	Never mated or inseminated	Never vaccinated
19	16	Native Greek	A	Attika Prefecture	Never mated or inseminated	Never vaccinated
20	26	Arabian	B	Boeotia Prefecture	Never mated or inseminated	Influenza-Tetanus
21	12	Arabian	B	Boeotia Prefecture	AI once/gestation – 5 successful gestations	Influenza-Tetanus
22	5	Arabian	B	Boeotia Prefecture	AI once - Did not conceive	Influenza-Tetanus
23	16	Arabian	B	Boeotia Prefecture	Never mated or inseminated	Influenza-Tetanus
24	24	Arabian	B	Boeotia Prefecture	Never mated or inseminated	Influenza-Tetanus
25	3	Arabian	B	Boeotia Prefecture	Never mated or inseminated	Influenza-Tetanus
26	18	Native Greek	C	Attika Prefecture	Never mated or inseminated	Never vaccinated
27	24	Native Greek	C	Attika Prefecture	Never mated or inseminated	Influenza-Tetanus
28	24	Haflinger	C	Attika Prefecture	Never mated or inseminated	Influenza-Tetanus
29	20	Native Greek	C	Attika Prefecture	AI once/gestation – 2 successful gestations	Influenza-Tetanus
30	12	Native Greek	C	Attika Prefecture	Never mated or inseminated	Never vaccinated

*AI: Artificial insemination; **Gestation that resulted in delivery of a viable foetus

Sampling was conducted aseptically from the inner (cranial) vaginal wall (caudal and ventral to the cervix), using speculum and sterile cotton swabs attached to probes. After sample collection, the swabs were immersed into 2.0 mL Dulbecco's modified Eagle's medium (DMEM) and stored in dry ice, until transportation to the laboratory. There, they were centrifuged (10,000g, 3 min.) and the supernatants (1 mL) were divided into two aliquots that were stored at -20ºC (test sample) and -80ºC (backup sample).

The samples (200 µL) were processed for DNA isolation using the DNeasy PowerSoil PRO kit (Qiagen S.p.A., Milan, Italy) and the products were analyzed with the polymerase chain reaction (PCR), using the TaKaRa LA Taq™ (Takara Bio Europe S.A.S., Saint-Germain-en-Laye, France) mix with the universal primers 27F and 1492R targeting the complete sequence (1,500 base pairs) of the *16S-rRNA* gene. The results were incorporated into the construction of a DNA library using the 16S barcoding kit SQK-RAB204 (Oxford Nanopore Technologies, UK). After determining DNA concentration, the samples were submitted to sequencing using MinION-Mk1C (Oxford Nanopore Technologies), fitted with a Flongle adapter (sequencing time adjusted to 24h). FASTQ files of successful reads were uploaded to the EPI2ME platform (https://epi2me.nanoporetech.com/) using the workflow Fastq 16S 2021.09.09 (Metrichor Agent, Oxford

Nanopore Technologies) with quality score 10, minimum length filter 1,500 and BLAST E-value 0.01. The results were submitted to bio-informatics analysis.

Results and discussion

Successful reads were retrieved from 28 of the 30 samples (93.5%), which resulted to the presumptive identification of 192 bacterial species (Supplementary Table S1)[1] belonging to 87 genera (Supplementary Table S2). The two most frequently detected bacterial genera were *Staphylococcus* spp. 89% (25 of 28) and *Acinetobacter* spp. 54% (15 of 28). The following genera were detected in at least 25% of the test samples: *Staphylococcus* (89%), *Acinetobacter* (54%), *Klebsiella* (43%), *Pseudomonas* (36%), *Psychrobacter* (36%), *Bacillus* (32%), *Enterococcus* (25%), *Providencia* (25%) and *Sphingomonas* (25%) (Table 2).

Table 2. The bacterial genera detected in at least 25% of the test samples.

Bacterial genus	Positive samples (%)
Acinetobacter	54%
Bacillus	32%
Enterococcus	25%
Klebsiella	43%
Providencia	25%
Pseudomonas	36%
Psychrobacter	36%
Sphingomonas	25%
Staphylococcus	89%

The relevant findings with regards to the detected bacterial species are recorded in Table 3.

Table 3. The bacterial species detected in at least 25% of the test samples.

Bacterial species	Positive samples (%)
Acinetobacter bouvetii	29%
Acinetobacter equi	32%
Acinetobacter lwoffii	25%
Acinetobacter variabilis	25%
Klebsiella oxytoca	46%
Psychrobacter faecalis	25%
Psychrobacter pulmonis	25%
Staphylococcus cohnii	25%
Staphylococcus equorum	68%
Staphylococcus hominis	25%
Staphylococcus saprophyticus	46%
Staphylococcus succinus	29%

Regarding the number of NGS reads per bacterial genus, scores ranged between 7 and 326,500. The highest scores were recorded for *Enterobacter* (326,500), *Porphyromonas* (102,705), *Frigoribacterium* (54,957), and *Gracilibacter* (50,674), whereas the lowest for *Bacillus* (7), *Ruminococcus* (10), *Staphylococcus* (12), *Acinetobacter* (13), and *Buttiauxella* (16) (Supplementary Figure S1).

For the bio-informatics analysis, the individuals of the study population were assigned to groups based on breed, breeding record, and vaccination, to identify similarity or discriminating traits, with regards to the detected bacterial genera (Supplementary Figure S2, S3, S4). Factors potentially influencing the study parameter, such as the stage of the estrus cycle, nutrition, housing, and management, for which the test population presented little or no variation, were not included in the analysis.

None of the detected bacterial genera or species that were present in all of the samples, indicating that the vaginal bacterial microflora of the study population was diverse, despite its relevant homogeneity regarding location, nutrition, disease, and reproduction record. However, based on the percentage of detection of bacterial genera in the tested samples (> 25%) and the score of the NGS reads (> 150,000), it can be stated that

[1] Supplementary material available at https://acrobat.adobe.com/id/urn:aaid:sc:EU:12170cdb-67e2-4441-9606-e8ffad7bccad

the bacterial genera that are likely to constitute the main elements of the vaginal microflora of the test population are the following: *Staphylococcus* (89%), *Acinetobacter* (54%), *Klebsiella* (43%), *Pseudomonas* (36%), *Psychrobacter* (36%), *Bacillus* (32%), *Enterococcus* (25%), *Providencia* (25%) and *Sphingomonas* (25%).

To the best of our knowledge, the investigation of the constitution of the vaginal bacterial microflora of mares with NGS has been the subject of only three other studies: Barba et al., (2020), Husso et al., (2020) and Malaluang et al., (2022). A comparative assessment of the results of these studies does not provide grounds for safe assumptions about the consistency of their findings (Table 4).

Table 4. Comparison of the results recorded in studies focused on the investigation of the constitution of the vaginal microflora in healthy mares using NGS, in connection with the bacteria more commonly detected.

Bacterial genus detected	Reference			
	Mataragka et al., 2023	Malaluang et al., 2022	Barba et al., 2020	Husso et al., 2020
Acinetobacter	+			
Akkermansia			+	
Arcanobacterium			+	
Bacillus	+			
Campylobacter			+	+
Corynebacterium			+	+
Enterococcus	+			
Escherichia coli		+		
Fusobacterium			+	
Helcococcus				+
Klebsiella	+			
Porphyromonas			+	+
Providencia	+			
Pseudomonas	+			
Psychrobacter	+			
Sphingomonas	+			
Staphylococcus	+	+*		
Streptococcus		+**	+	
uncultured *Kiritimatiaellae*			+	

**Staphylococcus capitis*; ***Streptococcus equisimilis*, *S. thoraltensis*, *S. zooepidemicus*

This could be associated with the dynamic interaction between the constitution of the vaginal microbiota and several environmental or physiological parameters, as well as methodological factors (Barba et al., 2020; Uchihashi et al., 2015; Yildirim et al., 2014). In this regard, it is perhaps worth noting that in the study of Malaluang et al., (2022), the bacterial species more commonly detected was *Escherichia coli*, which is consistent with the close proximity of the vagina to the anus. Interestingly, *E. coli* was not among those most commonly detected in the samples of other two NGS studies, which could be associated with the method or the anatomical site of sample collection. As already mentioned, the swabs analyzed in the study presented here were taken from the inner vaginal wall, whereas those of the other NGS studies are reported to be the vestibule-caudal vagina (Barba et al., 2020), the ventral side of the vaginal vestibulum (Husso et al., 2020), and the vaginal fornix of the cranial vagina (Malaluang et al., 2022).

Conclusion

The bio-informatics analysis conducted with regards to breed, produced evidence of a much richer microbiota for the Greek native (60 bacterial genera), compared to the Arabian (37) and other breeds (23). With regards to the Arabian breed, which was included in the study population of Barba et al., (2020), the number of the bacterial genera detected was 16, and 6 of them were also detected in our study: *Peptoniphilus*, *Campylobacter*, *Ruminococcus*, *Streptococcus*, *Helcococcus* and *Porphyromonas* (Table 4). Notably, eight (8) bacterial genera were detected across breeds. With the exception of *Clostridium* spp., these bacteria were the same with those reported above to be more commonly detected in the study population at high NGS score-reads, which is consistent with the certain bacteria being, as already mentioned, the main elements of the microbiota of the test population (Supplementary Figure S2). The relevant results recorded with regards to reproduction record (Supplementary Figure S3) and vaccination (Supplementary Figure S4), indicate that these parameters may also be associated with variations of the vaginal bacterial microflora in the study animals.

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