Deciphering the role of vaginal and sperm metagenome in the success of artificial insemination in sheep

M.M. Serrano[1], F. Freire[2], J.F. Martinez-Blanch[3], C. González[1], L. Reyes[2], M.C. Solaz-Fuster[3], J.H. Calvo[4],E. Climent[3], M.A. Jiménez[1] & F.M. Codoñer[3]

[1]INIA Dpto. Mejora Genética Animal, Ctra. de la Coruña km 7.5, 28040 Madrid, Spain
[2]OVIGEN, Granja Florencia s/n, 49800 Toro, Zamora, Spain
[3]Lifesequencing SL, Parc Científic Universitat de València, 46980 Paterna, Valencia, Spain
[4]Unidad de Tecnología en Producción Animal CITA, 59059 Zaragoza, Spain. ARAID, 50004 Zaragoza, Spain
fcodoner@lifesequencing.com (Corresponding Author)

Summary

For the first time the vaginal and sperm microbiota of ewes and rams relative to artificial insemination (AI) success have been studied. Hypervariable regions V3 and V4 of the *16s ribosomal* gene have been sequenced with a MiSeq Illumina platform in 50 ewes from 5 different herds and 5 AI rams. Microbiota differences at the phylum, family, genera and species were tested for antibiotic treatment in progesterone releasing intravaginal devices (PRIDs), pregnancy rate, AI ram and herd. Results showed that antibiotic treatment only affects the presence of Streptobacillus which was 21 times more abundant (padj=0.0002) in ewes carrying PRIDs without antibiotic. One species of this genus, *Streptobacillus notomytis*, was 19 times more abundant in non-pregnant that in pregnant ewes, which reveals the efficiency of framycetin in the PRIDs to increase AI fertility. Mageebacillus, Histophilus, Actinobacilllus and Sneathia genera were 70, 25, 22 and 13 (p_adjusted<0.05) times more abundant in non-pregnant than in pregnant ewes. These genera are also highly overrepresented in two of the three herds with the lowest AI success. Mageeibacillus genus belongs to the family Ruminococcaceae and has been recovered from the genital tract of women with bacterial vaginitis. Sneathia has been associated with bacterial vaginosis, spontaneous abortion and other invasive infections in woman. Actinobacillus and Histophilus belong to the HAP group (Haemophilus-Actinobacillus-Pasteurella), which includes great number pathogens resistant to most cellular defence mechanisms, and are related with epididymitis, vaginitis, placentitis, infertility, abortions in the ovine species. However, Actinobacillus and Histophilus were not detected from the sperm samples of the AI rams here analysed. The high presence of these genera in non-pregnant ewes and herds with low AI success could be due to the venereal transmission of these microorganisms by natural mating with rams exiting in the herds.

Keywords: metagenomics, fertility, reproductive tract, sheep

Introduction

Artificial insemination (AI) is an essential tool in genetic breeding programs of milk ruminants, since allows to develop the sires' progeny test and to connect herds, which is necessary to compare estimated breeding values among animals of the whole population. Among ruminants, sheep has one of the lower AI pregnancy rates (30% to 70%) depending on breeds, production systems and season (Granleese et al., 2015). This fact has a high

negative economic impact in milk sheep breeding programs since generational intervals are so long, delaying genetic response, and because many males must be tested each year to provide enough progenies. Many known factors contribute to a greater or lesser extent on the low AI fertility obtained in sheep (sinuous reproductive tract, use of fresh semen, unknown step of the ovulatory cycle, management constraints, etc.), but also, still not well-known factors such as the microbiota existing in the reproductive tracts of both, rams and ewes.

Metagenomics has relied on 16S rRNA gene sequencing of microorganisms, specifically the hypervariable regions within the gene, which serves as a molecular fingerprint down to the genus and species level (Hyman et al., 2005). In livestock species, Swartz and colleagues (Swartz et al. 2014) sequenced the variable regions V3 and V4 of the 16S rRNA genes from vaginal samples of ewes and cows with different pregnancy status and age, showing that microbiota of unmated ewes did not differ from those recently mated, mated and pregnant. In rams there are some former studies focused in the characterization of the microbiota related to some diseases and infertility. *Actinobacillus seminis* and *Histophilus somni* were found in rams' sperm (Foster et al., 1999; De la Puente et al., 2000) and linked with epididymitis in rams and abortion in ewes.

The aim of this study is to investigate for the first time, the vaginal and sperm metagenome of ewes and rams to elucidate its role in the AI success by sequencing the hypervariable regions V3 and V4 of the 16S rRNA using next generation sequencing (NGS) technology.

Material and methods

Animals and experimental design

Fifty ewes from five different herds (10 ewes/herd) ageing from 1.5 to 9 years and synchronized for AI with progesterone releasing intravaginal devices (PRIDs) with antibiotic (AB) -framycetin in power 0.6 gr/PRID- and without antibiotic (NAB) were used in this experiment. All ewes had at least one lambing before the AI here conducted. Fresh semen doses of five rams from the AI centre were used to inseminate the 50 ewes. Males aged from 4 to 9 years. Sperm were prepared with fresh semen at a concentration of 400 million of spermatozoids/ml using as diluent INRA96® plus antibiotics (streptomycin 50mg and penicillin 50,000 IU per ml of diluent) and packed in 0.25 ml straws. Two sperm doses of each male were used to perform paired inseminations of one ewe with AB-PRID and one ewe with NAB-PRID in each herd, to avoid biases due to the ram effect. Pregnancy was detected by ultrasound 42 days after AI. Before AI, rectal temperature were measured in all ewes.

Microbiota samples and ADN extraction

Before AI, one sample from the vaginal exudate of each ewe was collected with sterile swab (BBL™ CultureSwab™ Liquid Amies, Single Swab for throat, vaginal, skin and wound specimens BD). Swabs were immediately frozen in carbonic snow and subsequently stored at -20ºC. Rams microbiota samples were obtained from fresh semen straws prepared for the AI. DNA was extracted from swabs and straws by using the DNeasy Blood and Tissue kit (Qiagen). DNA obtained was cleaned and concentrated to obtain adequate values of quality and concentration with the QiAmp Micro kit (Qiagen).

Sequencing microbiota

Libraries were prepared following Illumina protocol for 16s rRNA capture and amplification based on dual PCR as implemented in Klindworth and co-workers (Klindworth et al. 2013). Sequencing was made with a MiSeq Illumina platform yielding 300 nt pair-end reads. Sequences R1 and R2 were paired by PEAR V.0.9.1 (http://www.exelixis-lab.org/web/software/pear) with the overlapping parameter for the sequences of extremes fixed in 70 nt. Sequence extremes with low quality (<Q20) and sequences with quality low than Q20 were removed. CUTADAPT v1.8.1 software (http://cutadapt.readthedocs.org) was used to remove adapters and select sequences with length higher than 200 nt. The software UCHIME (Edgar et al., 2011) was used to remove chimeric sequences obtained in the amplification process. Previously to the BLAST step, sequences were compared against the sheep genome (Oar_v3.1, INSDC Assembly GCA_000298735.1, Aug 2012) and those having high similitude were removed from the analysis. To reduce the annotation process complexity sequences sharing a 97% of similitude were grouped in a unique sequence with the CD-HIT software (http://weizhongli-lab.org/jcvi). Finally, to associate sequences with each taxonomic group these were compared with an rRNA database (RefSeq from NCBI) with the BLAST local alignment strategy.

Bioinformatics

Differences in microorganisms abundance between PRIDs treatment (NAB vs AB), rectal temperatures (in ranks of 0.5^{o}C), herds (5 herds), AI rams and pregnancy status (pregnant P vs non-pregnant NP) at the phylum, family, genera and species level, were tested by using the DESeq2 R package (Anders & Huber, 2010) which utilizes a negative binomial distribution. Pair wise comparisons were performed calculating the $\log_2$fold change of the normalized average counts ratio. To test significance a T test was performed over the $\log_2$FoldChange and its standard error ratio (stat). Statistical p_values were adjusted (padj) to take into account for multiple tests (Benjamini & Hochberg).

RESULTS AND DISCUSSION

AI results.

Table 1 shows percentages of AI fertility (pregnancy rate) of the 50 ewes inseminated and the 5 AI males. Ultrasound diagnoses 42 days after AI yielded 20 ewes pregnant and 30 ewes non-pregnant. Ewes' rectal temperature ranged between 38.1 and 40.1^{o}C and averaged 39.2 ± 0.42^{o}C.

Table 1. Percentage of AI success for the rams, ewes and herds used in the experiment

	Ram 1	Ram 2	Ram 3	Ram 4	Ram 5	average rams	average ewes	Herd 1	Herd 2	Herd 3	Herd 4	Herd 5
% AI fertility	40	40	40	30	50	40	20	70	20	20	60	30

Vaginal and sperm microbiota

The most represented genera (Figure 1) in both sexes was Corynebacterium, which supposes a 10% and 11% of the total counts in ewes and rams, respectively. Ureaplasma was the second majority genera in ewes (6%) and was present, but in lower abundance, also in the sperm microbiota (3%). On the contrary, a 10% of Pseudomonas was detected in sperm but only 1% in vagina. In the work of Swartz and colleagues (Swartz et al., 2014) species from the bacterial genera Aggregatibacter, Streptobacillus, Cronobacter, Phocoenobacter, and Psychrilyobacter were found to be the predominant in ewes' vaginal tract. However, only Streptobacillus was found in our samples.

Pair wise comparisons between levels of effects

Only for PRIDs treatment (NAB vs AB), pregnancy status (P vs NP) and herds differences in microorganism abundance were significant at the phylum, family and genus levels. Table 2 shows the most significant differences at the genera level between PRIDs treatments, pregnancy status and herds. Profiles could be studied only at the genus levels, because PCR product for bacteria yielded only 400 nt which did not allow a correct identification of the species. However, putative species detected (data not shown) are commented to speculate about its possible role in AI success/failure.

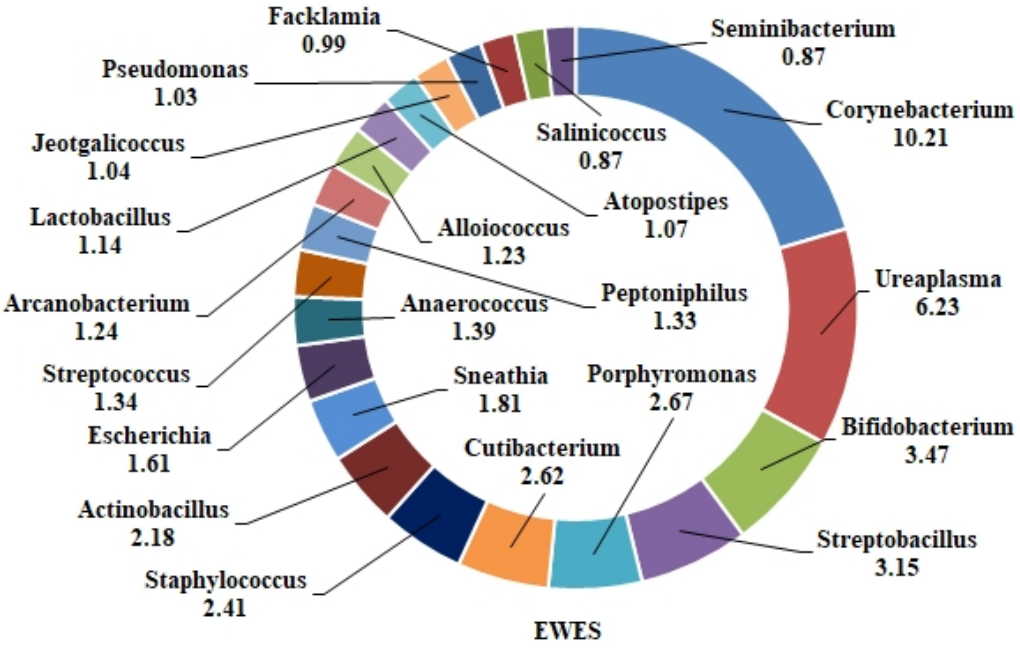
Results showed that Streptobacillus was the unique genus affected by the antibiotic used in the PRIDs, since it appears 21 times overrepresented in ewes carrying NAB-PRIDs. *Streptobacillus notomytis,* a species of this genus (data not shown), was 19 times (log_2FoldChange=4.23; p_adj=0.05) more abundant in non-pregnant than in pregnant ewes. Thus, higher pregnancy rates can be linked to the efficiency of framycetin to eliminate Streptobacillus from ewes' vagina, which could be partly responsible of AI failure. Framycetin does not seem to have any effect over the growth of the rest of microorganisms detected in vaginal samples.

Pregnancy vs non-pregnancy contrast showed four genera significantly much more abundant in non-pregnant ewes. Mageebacillus, Histophilus, Actinobacilllus and Sneathia were 70, 25, 22 and 13 times more abundant in non-pregnant than in pregnant ewes, respectively. The putative species found for these genera were *Histophilus somni* (log_2FC=-5.36; p_adj=0.05), *Actinobacillus seminis* (log_2FC=4.80; p_adj=0.03), *Mageeibacillus indolicus* (log_2FC=4.72; p_adj=0.09) and *Sneathia sanguinegens* (log_2FC=4.35; p_adj=0.05) which were 41, 28, 26 and 20 times more abundant in non-pregnant than in pregnant ewes, respectively. Also all these genera, Histophilus, Sneathia, Mageeibacillus and Actinobacillus, were significantly more abundant in two herds with low AI success (H2 and H3).

Histophilus and Actinobacillus belong to the Pasteurellaceae family and to the HAP group (Haemophilus-Actinobacillus-Pasteurella) which includes a great number of human and livestock pathogens resistant to most cellular defence mechanisms (Czuprynski, 1990). *Actinobacillus seminis*, the specie detected in our work, has been related with abnormal semen, epididymitis and infertility in rams (Low et al., 1995; De la Puente et al. 2000), abortions in ewes (Foster et al., 1999) and vaginitis in cows (González-Moreno et al., 2016). Epididymitis is a contagious disease which transmission occurs venereally or by homosexual activity, and in the case of *Actinobacillus seminis*, by ewe to lamb transmission (Burgess, 1982). This results in substantial economic losses worldwide due to reproductive failure and culling of breeders. However, Actinobacillus and Histophilus have not been detected in the sperm samples from the five AI rams used in this work. Furthermore, no relationship between rams' microbiota and the AI success/failure was found. Thus, we hypothesise, that the high presence of these genera in the vaginal tract of non-pregnant ewes and in one of the herds

(herd 2) with low AI success (20%), join to the fact that all ewes had at least one lambing previous to the AI here conducted, could be due to the transmission of these microorganisms from natural mating rams exiting in the herds.

Figure 1. Relative abundance of the most representative genera found in vaginal and sperm samples



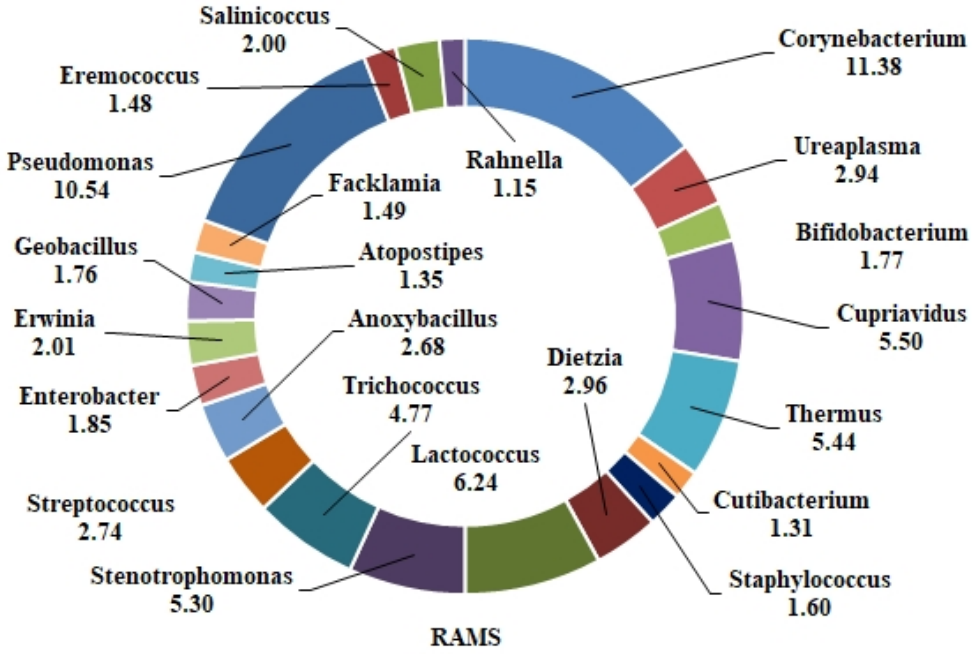


Table 2. Significant contrasts of pair wise comparisons between PRIDs treatments (NAB non antibiotic/AB antibiotic), pregnancy status (P pregnant/NP non-pregnant) and herds at the genera level. stat = log2FC/log2FCSE, padj = p_value adjusted for multiple test

Effect	Genera	log2FC	log2FCse	stat	pvalue	padj
NAB vs AB	Leptotrichiaceae;**Streptobacillus**	4.424	0.854	5.183	2.10E-07	0.0002
P vs NP	Leptotrichiaceae;**Sneathia**	-3.757	1.19	-3.158	0.0015	0.0355
P vs NP	Pasteurellaceae;**Actinobacillus**	-4.492	1.114	-4.033	5.50E-05	0.005
P vs NP	Pasteurellaceae;**Histophilus**	-4.663	1.436	-3.246	0.0011	0.0298
P vs NP	Ruminococcaceae;**Mageeibacillus**	-6.121	1.34	-4.568	4.90E-06	0.0008
H2 vs H1	Enterobacteriaceae;Escherichia	5.34	1.158	4.613	3.90E-06	0.0003
H2 vs H1	Fusobacteriaceae;Fusobacterium	6.375	1.516	4.207	2.60E-05	0.0011
H2 vs H1	Pasteurellaceae;**Actinobacill**us	6.48	1.419	4.567	4.90E-06	0.0003
H2 vs H1	Ruminococcaceae;**Mageeibacillus**	6.487	1.596	4.064	4.80E-05	0.0016
H2 vs H1	Mycoplasmataceae;Mycoplasma	7.233	1.734	4.172	3.00E-05	0.0011
H2 vs H1	Leptotrichiaceae;**Sneathia**	7.326	1.357	5.4	6.60E-08	1.50E-05
H2 vs H1	Pasteurellaceae;**Histophilus**	8.162	1.572	5.191	2.00E-07	2.40E-05
H2 vs H 4	Ruminococcaceae;**Mageeibacillus**	8.337	1.605	5.195	2.00E-07	0.0001
H2 vs H 4	Pasteurellaceae;**Histophilus**	9.501	1.578	6.02	1.70E-09	1.80E-06
H3 vs H2	Actinomycetaceae;Actinomyces	-4.919	1.156	-4.256	2.00E-05	0.0015
H3 vs H2	Leptotrichiaceae;**Streptobacillus**	-5.729	1.231	-4.654	3.20E-06	0.0003
H3 vs H2	Leptotrichiaceae;Leptotrichia	-6.061	1.455	-4.167	3.00E-05	0.0015
H3 vs H2	Fusobacteriaceae;Fusobacterium	-6.422	1.517	-4.232	2.30E-05	0.0013
H3 vs H2	Pasteurellaceae;**Histophilus**	-7.644	1.569	-4.872	1.10E-06	0.0001
H3 vs H2	Leptotrichiaceae;**Sneathia**	-8.273	1.363	-6.068	1.30E-09	3.80E-07
H5 vs H2	Actinomycetaceae;Actinomyces	-4.166	1.15	-3.622	0.0003	0.0063
H5 vs H2	Mycoplasmataceae;Mycoplasma	-5.68	1.723	-3.296	0.0009	0.0193
H5 vs H2	Enterobacteriaceae;Escherichia	-6.109	1.156	-5.283	1.20E-07	4.60E-06
H5 vs H2	Ruminococcaceae;**Mageeibacillus**	-6.941	1.59	-4.366	1.20E-05	0.0004
H5 vs H2	Leptotrichiaceae;**Streptobacillus**	-7.201	1.23	-5.853	4.80E-09	2.10E-07
H5 vs H2	Leptotrichiaceae;Leptotrichia	-8.55	1.46	-5.857	4.70E-09	2.10E-07
H5 vs H2	Pasteurellaceae;**Actinobacillus**	-9.156	1.421	-6.445	1.10E-10	8.30E-09
H5 vs H2	Leptotrichiaceae;**Sneathia**	-9.61	1.362	-7.057	1.70E-12	1.80E-10
H5 vs H2	Pasteurellaceae;**Histophilus**	-11.262	1.588	-7.093	1.30E-12	1.80E-10
H5 vs H3	Pasteurellaceae;**Actinobacillus**	-7.885	1.421	-5.551	2.80E-08	2.90E-05

Mageeibacillus is a genus belonging to the order Clostridiales and the family Ruminococcaceae, and has been recovered from the genital tract of women with bacterial vaginitis (Austin et al., 2015) .Sneathia are part of the normal microbiota of the genitourinary tracts of men and women, but they are also associated with a variety of clinical conditions including bacterial vaginosis, spontaneous abortion and other invasive infections in woman (Zongxin et al., 2010). Up to now, any relationship between these genera and reproductive diseases in sheep have been described.

Future experimental work may be conducted to determine the importance of the transmission of microorganisms from natural mating rams to ewes and if this produces transitory or permanent changes in the vaginal microbiota of ewes. The responsibility of the

host genome in microbiome differences, and the possibility to implement a genetic selection of animals that promotes a reproductive beneficial microbiota, may be explored. Furthermore, probiotics and prebiotics may be tested to replace antibiotics. The incorporation of probiotics such as certain bacteria of the Bifidobacterium and Lactobacillus genera would enable these bacterial groups to outcompete other species, and inhibiting the growth of potentially pathogenic bacteria associated with infertility.

Acknowledgements

This work has been supported by RTA2013-00041 INIA project. We thank OVIGEN to make inseminations and ultrasound and LifeSequencing to make metagenome sequencing.

References

Anders, S. & W. Huber 2010. Differential expression analysis for sequence count data. Genome Biology 11(10): R106.

Austin, M.N., L.K. Rabe, S. Srinivasan, D.N. Fredricks, H.C. Wiesenfeld & S.L. Hillier 2015. Mageeibacillus indolicus gen. nov., sp. nov.: a novel bacterium isolated from the female genital tract. Anaerobe 32: 37-42.

Burgess, G.W. 1982. Ovine contagious epididymitis: a review. Vet Microbiol 7(6): 551-75.

Czuprynski, C.J. & A.K. Sample 1990. Interactions of Haemophilus-Actinobacillus-Pasteurella bacteria with phagocytic cells. Can J Vet Res 54 Suppl: S36-40.

De la Puente-Redondo, V.A., N. Garcia del Blanco, C. Pérez-Martinez, M.C. González-Rodriguez, E.F. Rodriguez-Ferri & C.B. Gutiérrez-Martin 2000. Isolation of Actinobacillus seminis from the Genital Tract of Rams in Spain. Journal of Comparative Pathology 122(2–3): 217-222.

Edgar, R.C., B.J. Haas, J.C. Clemente, C. Quince & R. Knight 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27 (16): 2194-2200.

Foster, G., M.D. Collins, P.A. Lawson, D. Buxton, F.J. Murray & A. Sime 1999. Actinobacillus seminis as a cause of abortion in UK sheep flock. Veterinary Record 144: 479-480.

Granleese, T., A. Samuel, S.A. Clark, A.A. Swan & J.H.J. Van der Werf 2015. Increased genetic gains in sheep, beef and dairy breeding programs from using female reproductive technologies combined with optimal contribution selection and genomic breeding Genet Sel Evol 47(1): 70

Hyman, R.W., M. Fukushima, L. Diamond, J. Kumm, L.C. Giudice & R.W. Davis 2005. Microbes on the human vaginal epithelium. Proc Natl Acad Sci USA 102(22): 7952–7957.

Klindworth, A., E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn & FO. Glöckner 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41(1): e1.

Low, J.C., D. Somerville, M.J. Mylne & W.A. McKelvey 1995. Prevalence of Actinobacillus seminis in the semen of rams in the United Kingdom Vet Rec. 136(11): 268-269.

Swartz, J.D., M. Lachman, K. Westveer, T. O'Neill, T. Geary, R.W. Kott, J.G. Berardinelli, P.G. Hatfield, J.M. Thomson, A. Roberts & C.J. Yeoman 2014. Characterization of the vaginal microbiota of ewes and cows reveals a unique microbiota with low levels of lactobacilli and near-neutral pH. Frontiers in Veterinary Science 1: 19.

Zongxin, L., K. Jianming, F. Liu, H. Zhu, X. Chen, Y. Wang, L. Li, K. E. Nelson, Y. Xia & C. Xiang 2010. Molecular analysis of the diversity of vaginal microbiota associated with bacterial vaginosis. BMC Genomics 11: 488.