ORIGINAL ARTICLE

Vaginal microbial communities from synchronized heifers and cows with reproductive disorders

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Abstract

Aim: To evaluate changes in the resident microbial population in the cranial vaginal mucosa induced by a progesterone-releasing intravaginal device (PRID) compared to the vaginal microbiota of cows with reproductive disorders.

Methods and Results: Vaginal discharge was evaluated by clinical examination and a Vaginitis Diagnosis Score was performed by exfoliative cytology. All samples classified as positive and some classified as negative by clinical evaluation were later diagnosed as positive for vaginitis by cytological analysis. Bacterial diversity profiles were performed by PCR-DGGE and clustered according to the reproductive health status of the specimens, revealing a correspondence between the structures of the communities in the vagina and the clinical profile. Representative bands from each group were sequenced and identified as *Ruminococcus* sp., *Dialister* sp., *Escherichia* sp./*Shigella* sp., *Virgibacillus* sp., *Campylobacter* sp., *Helcoccoccus* sp., *Staphylococcus* sp., *Bacillus* sp., *Actinopolymorpha* sp., *Exiguobacterium* sp., *Haemophilus* sp./*Histophilus* sp., *Aeribacillus* sp., *Porphyromonas* sp., *Lactobacillus* sp. and *Clostridium* sp.

Conclusion: Our results contribute to the knowledge of the vaginal microbiome in synchronized heifers showing positive or negative clinical vaginitis.

Significance and Impact of the Study: This study contributes to the understanding of a dynamic vaginal colonization by bacterial consortiums during the synchronization with a widely used PRID protocol. Also, the results reveal the presence of well-known metritis-related pathogens as well as emerging uterine opportunistic pathogens. The provided information will allow to carry out further studies to elucidate functional roles of these native micro-organisms in the bovine reproductive tract.

Introduction

Vaginal microbial composition has been widely studied and associated with reproductive tract health and fertility in both women and animals (Vitali *et al.* 2012; Penna *et al.* 2013; Lorenzen *et al.* 2015). The indigenous microbiota that colonizes the vaginal mucosa of healthy cows is an equilibrated and dynamic ecosystem which consists of a combination of aerobic, facultative anaerobic and obligate anaerobic micro-organisms including Gram-positive cocci and bacilli (*Staphylococcus* sp., *Streptococcus* sp. and *Enterococcus* sp.; and *Lactobacillus* sp.) as well as Enterobacteriaceae (Otero *et al.* 1999, 2000; Wang *et al.* 2013). However, this microbiota may be modified by both intrinsic (physiological, hormonal, immunological and nutritional) and extrinsic (antibiotic and hormonal therapies, reproductive managing and high animal density systems) factors (Nader-Macias and Otero 2009).

In addition, uterine homeostasis is frequently compromised by postpartum bacterial contamination, where the

persistence of pathogenic bacteria may cause infertility (Sheldon and Dobson 2004). The role of bacteria in the pathogenesis of uterine infections has been frequently investigated by culture-dependent studies (Sheldon et al. 2002; Williams et al. 2005; Miller et al. 2007; Santos et al. 2010). Escherichia coli and Arcanobacterium pyogenes are conventionally considered the most relevant species implicated in the pathogenesis of metritis mainly due to their ability to persist in the contaminated uterus (Sheldon et al. 2002, 2010; Williams et al. 2005; Miller et al. 2007). It has also been reported that pre- and postpartum metritis and endometritis produced by nonspecific infectious agents may lead to a Repeat Breeder (RB) (Sharma et al. 1988; Singh et al. 1996; Bhat et al. 2014). A RB is usually defined as a cow with apparently healthy genitalia that cannot conceive after three or more services but exhibits normal estrous cycles (Kumar and Singh 2009). In RB, bacterial infection can induce fertilization failure due to inflammatory exudates, causing blockade of oviducts or death of sperm by bacterial toxins and inflammatory reaction before the sperm can reach the fertilization site (Bhat et al. 2014). It was also reported that this syndrome can alter the uterine environment by causing inflammation of the endometrium, denudation of its mucosa and changes in its secretion followed by early embryonic death (Singh 1979; Bhat et al. 2014).

Reproductive hormonal therapies, including progesterone-releasing intravaginal devices (PRID), have significantly improved oestrus synchronization and conception rate after the first Artificial Insemination (AI) in anovulatory cows (Folman et al. 1990; Murugavel et al. 2003). However, only a few studies have reported the effects of PRID on the vaginal microbiota (Padula and Macmillan 2006; Walsh et al. 2008; Fischer-Tenhagen et al. 2012). Using culture-dependent techniques, these authors demonstrated a high growth of pyogenic bacteria from vaginal swabs. Both Walsh et al. (2008) and Fischer-Tenhagen et al. (2012) observed purulent vaginal discharge (VD) in heifers treated with a progesterone (P4) insert for 7 days. Fischer-Tenhagen et al. (2012) reported that in all severe VD cases, pyogenic bacteria such as Arcanobacterium pyogenes, coliforms, and Streptococcus sp. were isolated. In addition, the use of these inserts during early postpartum has been held responsible for the alteration in uterine and vaginal microbiota profiles (Padula and Macmillan 2006). Nevertheless, to the best of our knowledge, no study has issued the alterations in the vaginal microbial profile due to treatment with PRID by applying culture-independent techniques. Therefore, the aim of this study was to evaluate the changes in the resident microbial population in the cranial vaginal mucosa induced by PRID treatment compared to the vaginal microbiota observed in cows with reproductive disorders.

Materials and methods

Animals and sampling

Forty vaginal samples (cranial area) were collected from crossbreed Braford heifers and cows. The animals were retrospectively selected for this study according to the following criteria: 20 samples were obtained from nulliparous heifers (20 ± 2-months-old) submitted to a PRID protocol and sampled twice, once on Day 11 and then 49 days later (Day 60). On Day 11, the animals that developed post-PRID vaginitis were selected. In addition, 10 samples from cows (4 ± 1-years-old) with metritis (MT) (up to 60 days postpartum) or RB (unable to conceive after more than 6 months postpartum) and another 10 samples were obtained from nulliparous crossbreed Braford heifers (H) (20 ± 2-months-old); which were deemed suitable for reproduction through transrectal palpation to confirm reproductive maturity. None of these animals had been previously inseminated, used for natural breeding or showed signs of vaginitis according to clinical and cytological evaluations.

Synchronization protocol

On Day 0, all heifers under the synchronization programme received a PRID vaginal insert (Pro-ciclar P4 Zoovet; containing 750 mg P4; Productos Veterinarios S.A., Santa Fe, Argentina) and an intramuscular injection with both D+cloprostenol (2 ml PGF Ciclar; Productos Veterinarios S.A.) and estradiol benzoate (1 ml Benzoato de estradiol Zoovet; Productos Veterinarios S.A.). On Day 8, the PRID device was removed and all animals were treated with D + cloprostenol (2 ml PGF Ciclar) plus estradiol cipionate (1 ml Cipionato de Estradiol Zoovet; Productos Veterinarios S.A.). Prior to sampling, the perineum and vulvar areas of each animal were washed with water and dried with paper towels. Subsequently, a steel vaginoscope was gently inserted into the vagina with a small amount of antiseptic-free lubricant gel (0·8% w/v carbopol, 1% v/v triethanolamine, 10% v/v glycerine, Sigma-Aldrich, Taufkirchen, Germany), and a long-handled cytobrush and a sterilized cotton swab were introduced into the cranial vaginal wall to obtain samples for bacteriological and cytological examinations respectively. The loaded cytobrush was collected in 1 ml of phosphate buffered saline solution (PBS), pH 7·0 and kept refrigerated (8°C) until processing. Each swab was rolled onto two microscope slides and fixed with ethanol 96°.

On Day 11 of the protocol, 10 samples were taken from heifers showing positive vaginitis (pV). On Day 60, 10 samples were collected from animals clinically assessed as negative for vaginitis (nV). Clinical vaginitis was based

on evidence of vulvar discharge by applying a Vaginal Discharge Score (VDS): 0, no mucus/+ clear, fluid mucus (little); 1, ++/+++ clear, fluid mucus (regular/profuse); 2, yellowish white fluid; 3, white dense mucus. This score was defined according to previous reports (Walsh *et al.* 2008; Fischer-Tenhagen *et al.* 2012).

The animal management procedures included in the experimental protocol (intra-vaginal treatments, hormonal injections and vaginal sampling) were conducted while cows were restrained in the cattle crush (Argentinean animal welfare legislation, Law N°14.346, SENASA-R70/2001).

Cytological studies and vaginitis diagnosis

Two slides from each sample were stained using the May-Grunwald-Giemsa technique. Briefly, in order to determine the proportion of polymorphonuclear neutrophils (PMN), 100 cells (epithelial cells and leucocytes) were counted under a microscope (×400 magnification). A vaginal cytological score (VCS) was defined for the vaginitis diagnosis based on epithelial cells richness, flaps presence, %PMN and Neutrophil Extracellular Traps (NET): I, + cellular count & %PMN < 10%; II, +/++ cellular count & PMN > 10%; III, ++/+++ cellular count & %PMN > 10% (few NET); IV, ++/+++ cellular count & %PMN > 10% (abundant large NET). For the determination of the health status of the cow vagina, VDS, VCS and NET were considered in order to define vaginitis diagnosis: Negative (VDS = 0, 1 o 2 and VCS = I); Positive (VDS = 1, 2 o 3 and VCS = II, III or IV).

Microbial communities

Culture-dependent methods

The material from the cytobrushes was homogenized and serial 10-fold dilutions were performed and plated on MacConkey agar for Enterobacteriaceae and Columbia agar supplemented with 5% sheep blood for total micro-aerophilic and haemolytic microbial counts. Plates were incubated for 48 h at 37°C in aerobic conditions, except for the blood agar plates, which were incubated in micro-aerophilic conditions (5% CO_2-enriched chamber). Representative colonies from each medium were evaluated by morphology, haemolysis and Gram staining.

Culture-independent techniques

DNA extraction from vaginal samples

The material retrieved from the cytobrush was vigorously agitated for 2 min to dislodge cells and recovered by centrifugation (3000 *g*) for 10 min at 4°C, washed in sterilized PBS and centrifuged for 5 min. Finally, the pellets were suspended in 200 μl PBS. Bacterial DNA was extracted using a modified DNA purification from buccal swabs protocol (QIAamp® DNA Mini Kit; Qiagen, Hilden, Germany) as follows: 20 μl proteinase K (Qiagen Protease; Qiagen) and 200 μl of lysis buffer were added to 200 μl of sample and incubated at 56°C for 1 h. Samples with a high mucous content were treated with 30 μl proteinase K for 2 h at 56°C. DNA was purified and eluted according to the manufacturer's instructions, and stored in elution buffer at −20°C. Quantification and integrity were checked before amplification reactions.

PCR amplification, denaturing gradient gel electrophoresis (DGGE) and cluster analysis

A nested PCR was applied using two primer-pairs set for the 16S rRNA gene amplification from the 1 : 100-diluted DNA extracted from each vaginal sample. The first round of PCR was performed with a universal primer-pair P0-P4 (Klijn *et al.* 1991). One microlitre of the PCR products were separately utilized as templates for the second PCR round, using the DGGE-primers: V3f (GC), (5′-CC GGG GGG CGC GCC CCG GGC GGG GCG GGG A CGG GGG GCC TAC GGG AGG CAG CAG) and Uni-0515r (5′-ATC GTA TTA CCG CGG CTG CTG CTG GCA-3′) (Fontana *et al.* 2005). All PCR amplifications were performed with the PCR Master Mix (Promega, Fitchburg, WI, USA) in GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA) programmed as follows: initial denaturation of DNA for 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 58°C and 45 s at 72°C, for the first PCR, while the amplification program for the second one was: 5 min at 94°C, 35 cycles of: 30 s at 95°C, 30 s at 56°C and 1 min at 72°C; finally, both reactions were concluded with an extension of incomplete products for 7 min at 72°C. PCR products were quantified by electrophoresis on a 1% (w/v) agarose gel containing SYBER Safe (Invitrogen, Carlsbad, CA, USA). DGGE was performed using an INGENY phorU-2 (Ingeny International BV, Goes, Netherlands) DGGE system. PCR products were run on 8% (w/v) acrylamide gel with a 35–60% linear gradient of urea-formamide and electrophoresed at 80 V, 60°C for 18 h in 1× TAE buffer. Denaturing gradient gels were stained with 1× SYBR Green I (Roche, Basel, Switzerland) for 15 min and photographed under UV illumination. The DGGE banding profiles were analysed with FINGERPRINTING II software (Bio-Rad Laboratories, Hercules, CA, USA), using the Pearson correlation coefficient and the unweighted-pair group method with averages (UPGMA) for dendrograms generation.

Sequencing of DGGE bands

Different DGGE bands were excised from the gel, placed in 50 μl of nuclease-free water and stored at 4°C overnight to allow DNA elution. Five microlitres of eluted DNA were re-amplified using the DGGE primers without GC-clamp in the above PCR conditions and finally checked by agarose gel electrophoresis. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced (BMR Genomics, Padoa, Italy). The sequences were compared with those available in the GenBank using BLAST searches (http://www.ncbi.nlm.nih.gov/) and those in the Ribosomal Database Project (RDP) using the Sequence Map tool (http://rdp.cme.msu.edu/). The identification at genus level was based on 97% or greater sequence homology.

Statistical analysis

Microbiological data are presented as median values and interquartile (IQ) ranges; also, a correlation analysis was performed between total microaerophilic and Enterobacteriaceae populations, while Pearson coefficient was calculated for each samples group with a significance of $P < 0.05$. A contingency table was used to evaluate the relationship between health status and phylum/genus prevalence (the contribution to Chi-square was calculated) and a multivariate of correspondence analysis was performed to show health status-associated taxa. Data processing was carried out with MINITAB (ver. 14) and INFOSTAD (2015p ver.) software.

Results

Culture-dependent bacteriological analysis

Counts of total microaerophilic micro-organisms recovered from vagina (Fig. 1a) showed similar mean values in synchronized heifers with and without clinical vaginitis (pV and nV respectively) (mean = 3·98 $\pm$ 0·89 and 3·89 $\pm$ 1·02 log CFU sample^{-1} respectively). However, median values were 3·11 (nV) and 4·5 (pV) while the whiskers ranged between 1–7·84 and 1–9 respectively. A different behaviour was observed for the group of Enterobacteriaceae (Fig. 1b), the mean and median values (log CFU sample^{-1}) were similar between nV and pV and the whiskers ranged between 1–4·32 and 0–5·55 respectively. The relationship between both microbial populations was also evaluated (Fig. 1c). Thus, a high Pearson correlation factor was obtained for the nV group (0·711, $P < 0.05$); in contrast to the pV group, the correlation factor was not significant (0·52, $P > 0.05$). Higher percentages of both α- and β-haemolytic micro-
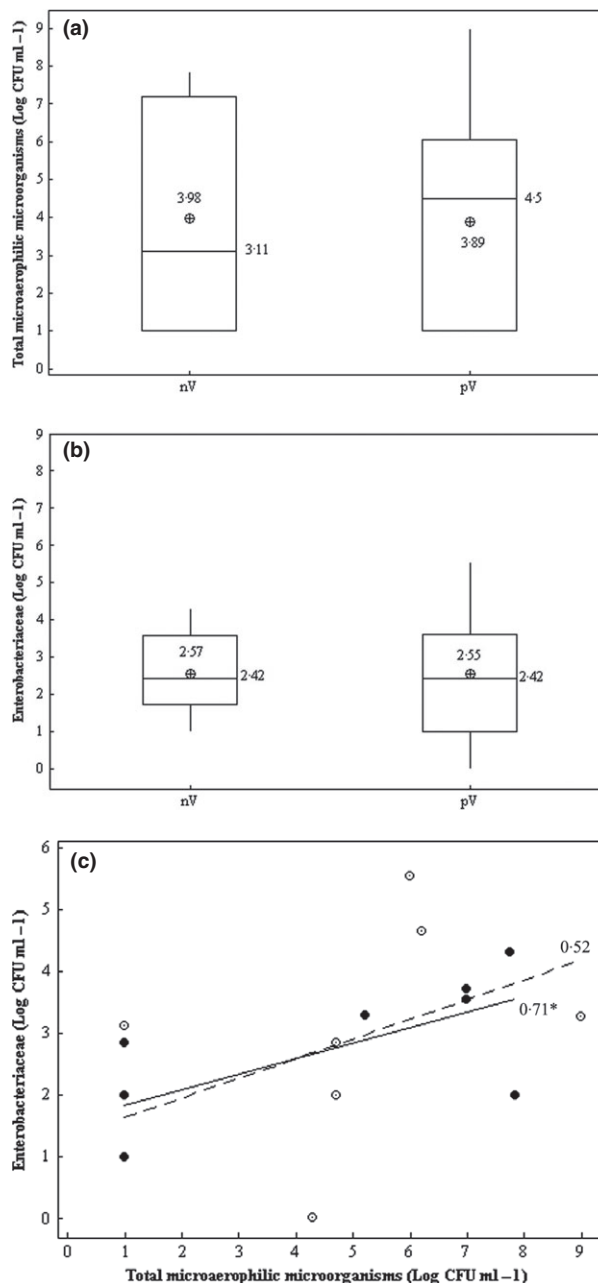

Figure 1 Total microaerophilic (a) and Enterobacteriaceae (b) populations from cranial vagina of heifers with (pV) and without clinical vaginitis (nV); digits in and outside the boxes represent the mean and median values respectively. (c) Relationship between both microbial populations in the groups of animals nV [—] and pV [---]; Pearson correlation coefficient and the statistical significance (*$P < 0.05$) are indicated.

organisms were found in the pV group (60%) than in the nV group (20%).

The qualitative comparison between microbial populations from H and MT are shown in Table 1. The number

Table 1 Microbial populations in healthy heifers (H) and cows with reproductive disorders (MT) on MacConkey agar and Blood agar. Presence or absence of α/β haemolysis is indicated

Sample	MacConkey	Blood agar	α/β haemolysis
4 H*	+‡	+	−
9 H	−§	−	−
12 H	+	+	−
8 H	+	+	+¶
11 H	+	+	−**
5 H	−	−	−
6 H	+	+	−
1 H	+	+	−
7 H	+	+	−
10 H	+	+	+
7 MT†	+	+	−
8 MT	+	+	−
9 MT	+	+	−
10 MT	+	+	−
6 MT	+	+	−
4 MT	−	−	−
2 MT	−	−	−
3 MT	−	+	−
1 MT	−	−	−
5 MT	+	+	−

*H, nulliparous clinically healthy heifers.
†MT, cows diagnosed with metritis or RB.
‡+, presence of representative colonies.
§−, absence of representative colonies.
¶+, presence of α/β haemolytic colonies.
**−, absence of α/β haemolytic colonies.

of samples with positive growth in MacConkey agar was higher in the H group than in the MT group. However, few samples from both groups failed to grow in blood agar. Also, β-haemolytic micro-organisms were only detected in two samples from the H group.

Vaginal bacterial composition and cytological profile in synchronized heifers with and without clinical vaginitis

The bacterial diversity profile obtained by PCR-DGGE revealed differences in the structure of the communities present in nV and pV samples (Fig. 2). Cluster analysis showed that the profiles of the animals from the nV group were associated in a separate cluster, which also included the pV10 sample. However, five out of these 11 samples were diagnosed as positive for vaginitis when cytological and discharge scores were considered together. Moreover, 90% of the DGGE profiles from the pV group were pooled in 2 clusters different from the one above. All samples included in these groups were positive for vaginitis diagnosis and NET were present with the exception of the pV7 sample (Fig. 2).

Vaginal bacterial composition and cytological profile in clinically healthy heifers and cows with reproductive disorder

DGGE profiles of the microbiota showed two distinctive clusters corresponding to the health status of the animals. Therefore, samples from cows with MT were associated in a single cluster different from the one that included H samples. Also, high variability was observed between the microbiota profiles from samples of the H group, where only four of them presented a correlation ≥60%, while in the MT group this similarity level was observed in eight out of the 10 samples (Fig. 3). In the MT group, only two samples were diagnosed as negative for vaginitis, and in six samples NET were detected. Also, three out of 10 samples from the H group were positively diagnosed with vaginitis (Fig. 3).

Identification of bacterial populations

The correspondence analysis (Fig. 4) revealed a correspondence between health status and prevalence of bacterial phyla. Thus, taxonomic assignment showed that Bacteroidetes, Proteobacteria and Firmicutes were the dominant phyla. Moreover, sequences from Actinobacteria were also present, but less frequently and only in some samples from the nV group. In contrast, Firmicutes and Bacteroidetes were present in samples from the MT, H and pV groups. Moreover, the phylum Proteobacteria was found to be almost exclusively associated with the MT group (Fig. 4).

A similar correspondence analysis was applied to evaluate the health status-associated bacterial genera (Fig. 5). Band sequences with more than 97% homology with *Ruminococcus* sp. and *Dialister* sp. were only associated with the H group. Also, *Escherichia* sp./*Shigella* sp., *Virgibacillus* sp. and *Campylobacter* sp. were exclusively related to the MT group, whereas *Lactobacillus* sp. was associated with samples from the pV group. The band sequences obtained from both pV and nV samples showed homology with *Helcoccoccus* sp., *Staphylococcus* sp., *Bacillus* sp., *Actinopolymorpha* sp. and *Exiguobacterium* sp. In addition, *Haemophilus* sp./*Histophilus* sp. was related to the MT and pV groups. *Aeribacillus* sp. was associated with the MT, pV and H groups while *Porphyromonas* sp. was related to the four groups.

Discussion

Several studies have extensively described the uterine microbiota of healthy and metric cows (Machado *et al.* 2012; Santos *et al.* 2011). However, very few studies on the composition of the vaginal microbial consortium in
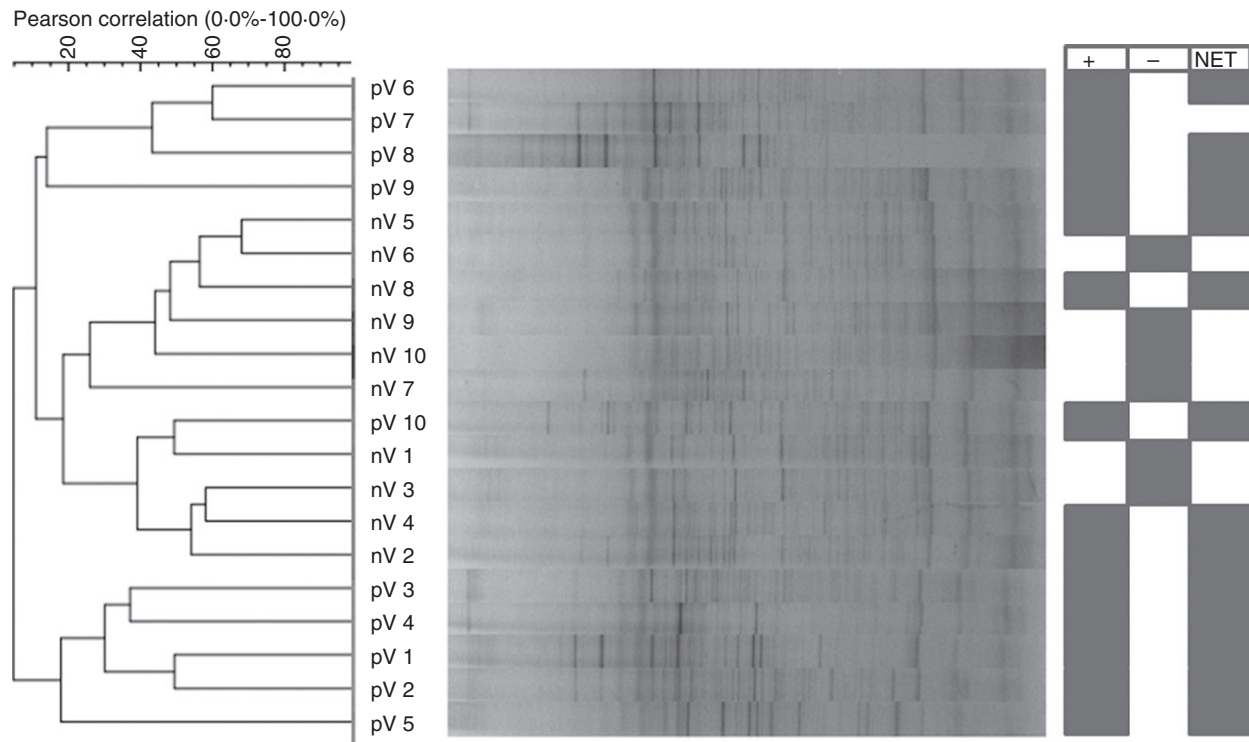
Figure 2 Denaturing gradient gel electrophoresis fingerprinting and clustering analysis of banding profile of the amplified bacterial 16S rRNA gene fragments from total genomic DNA extracted from vaginal microbiota of heifers from the pV and nV groups. The dendrogram shows similarities among the bacterial profiles in heifers with different health status, which were calculated from the intensity and position of each band in the gel. The patterns were compared with the Pearson correlation coefficient, and the matrix was clustered with the UPGMA method. Scale bar indicates similarity percentage. The matrix on the right (filled-in boxes) indicates vaginitis diagnosis (VD): + = positive; − = negative. The presence of NET is also shown.

cows with different inflammation status have been reported (Wang *et al.* 2013, 2016). To the best of our knowledge, no available study has used culture-independent techniques to analyse the composition of bacterial vaginal communities in oestrus synchronized cows. Therefore, this work evaluated the alterations in the resident vaginal microbial community induced by PRID treatment in heifers compared to the vaginal microbiota observed in cows with reproductive disorders using both PCR-DGGE and culture-dependent techniques. Overall, the use of PCR-DGGE showed a wide microbial diversity. Most of the sequences belonged to the phyla Proteobacteria, Firmicutes, and Bacteroidetes while Actinobacteria was less frequently observed. Similar results were reported for bovine uterine fluid in metritic, endometritic and healthy cows (Santos and Bicalho 2012).

In this work, bacterial community profiles were more homogeneous in the MT group than in the H group. Wang *et al.* (2016) reported similar findings when evaluated the vaginal microbiota in both healthy and endometritic postpartum dairy cows.

Escherichia coli is known as a crucial agent in the establishment of postpartum metritis (Santos *et al.* 2010; Sheldon *et al.* 2010). In our study, sequences related to *Escherichia* sp. were only observed by PCR-DGG in MT samples. The presence of *E. coli* among the vaginal Enterobacteriaceae population showed a significant increase in infected postpartum cows (Wang *et al.* 2013). However, we found lactose-positive bacteria in MacConkey agar in almost all samples from the H group. These could mean that the PCR-DGGE technique underestimated the presence of *E. coli* in this cow group. These discrepancies were also observed by Fontana *et al.* (2010) when comparing the results obtained by culture-dependent and culture-independent analyses (PCR-DGGE) of bacterial communities in other niches. Taking into consideration that *E. coli* belongs to the normal vaginal microbiota of healthy heifers (Otero *et al.* 2000) and that specific virulence factors associated with bovine metritis and endometritis have been found in *E. coli* from uterus (Bicalho *et al.* 2010; Sheldon *et al.* 2010), it would be interesting to determine if the vaginal *E. coli* strains from
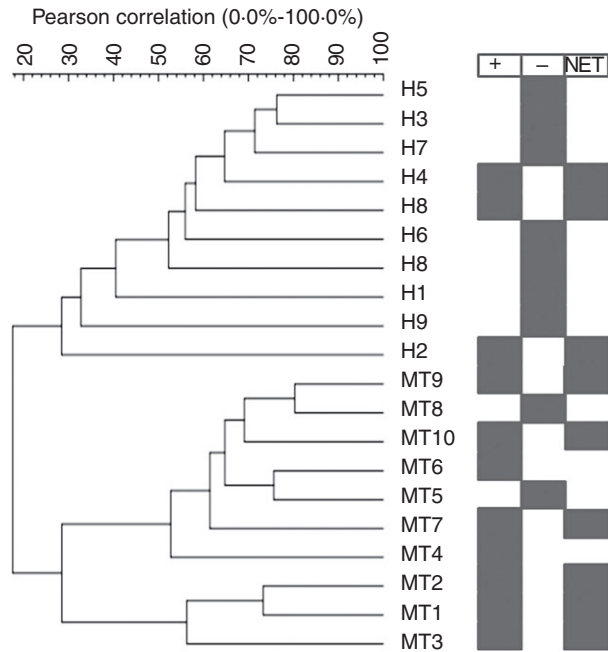
Figure 3 Dendogram shows the clustering analysis of banding profile obtained by denaturing gradient gel electrophoresis from total genomic DNA extracted from the vaginal microbiota of nulliparous clinically healthy heifers (H) and cows diagnosed with metritis or RB (MT). Scale bar indicates similarity percentage. The matrix on the right (filled-in boxes) indicates vaginitis diagnosis (VD): + = positive; − = negative. The presence of NET is also shown.
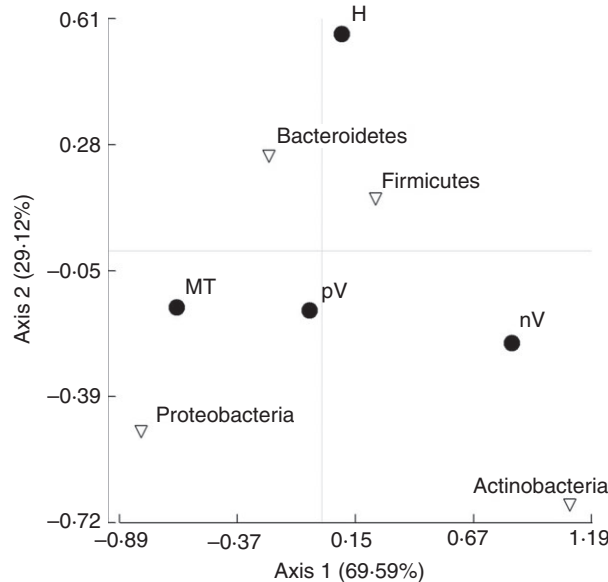
Figure 4 Correspondence analysis biplot of clinical profiles (●) and bacterial phyla (▽). Contribution to Chi-square is indicated in brackets. pV: synchronized heifers diagnosed with clinical vaginitis; nV: synchronized heifers diagnosed with no clinical vaginitis; H: nulliparous clinically healthy heifers; MT: cows diagnosed with metritis or RB.

both the H and MT groups differ in their virulence factors.

Previous studies have associated low *Lactobacillus* sp. counts in the vagina of endometritic cows and high counts in healthy ones (Wang *et al.* 2016). Although in our work this bacterial population was not associated with the H group, it was not related to the MT group either.

Helcoccoccus sp., *Staphylococcus* sp., *Bacillus* sp., *Exiguobacterium* sp. and *Actinopolimorpha* sp. were found in the DGGE profiles in both pV and nV groups, *Helcoccoccus* sp. and *Staphylococcus* sp. being more closely associated with nV samples. This last genus was previously described in the native vaginal microbiota of heifers and synchronized cows (Otero *et al.* 2000) and therefore, in agreement with previous reports (Paisley *et al.* 1986), it could be a potential uterine pathogen. Moreover, *Helcoccoccus* sp. was described in both healthy and metritic postpartum cows (Machado *et al.* 2012). Also, it was proposed as a novel cause of bovine abortion and puerperal metritis (Locatelli *et al.* 2013; AHVLA Disease Surveillance Report-April 2014). It was also suggested that the concomitant presence of *Helcoccoccus* sp. with frequent primary uterine pathogens such as *E. coli* and *Trueperella pyogenes* could be due to synergism (Locatelli *et al.* 2013). However, in the present work, *Helcoccoccus* sp. was isolated from heifers from the pV and nV groups but not from the MT group. This may indicate that *Helcoccoccus* sp. could belong to the indigenous vaginal microbiota, and thus participate as an opportunistic pathogen.

According to a report on microbial diversity in postpartum cows, *Porphyromonas* sp. and other related anaerobes colonize the bovine reproductive tract (Machado *et al.* 2012). In agreement with the above investigation, *Porphyromonas* sp. was present in all the groups analysed in the present work. Other studies have reported that this genus has been frequently isolated from bovine reproductive infections and retained fetal membranes (Drillich *et al.* 2001; Blum *et al.* 2012). *Falsiporphyromonas endometrii*, isolated from the post-partum bovine uterus and highly related to the *Porphyromonas* genus, has been recently proposed as a metritis-related pathogen (Wagener *et al.* 2014). Moreover, *Porphyromonas levii*, known as an emerging pathogen of human and veterinary relevance, has been related to cases of bovine necrotic vulvovaginitis (Elad *et al.* 2004).

Sequences related to *Histophilus* sp. were obtained from both MT and pV samples. Phylogenetic analysis (data not shown) of the related OTUs revealed its affiliation with *Histophilus somni*, in agreement with van der Burgt *et al.* (2007), who described this micro-organism in purulent vaginal discharge of cows with infertility and endometritis.
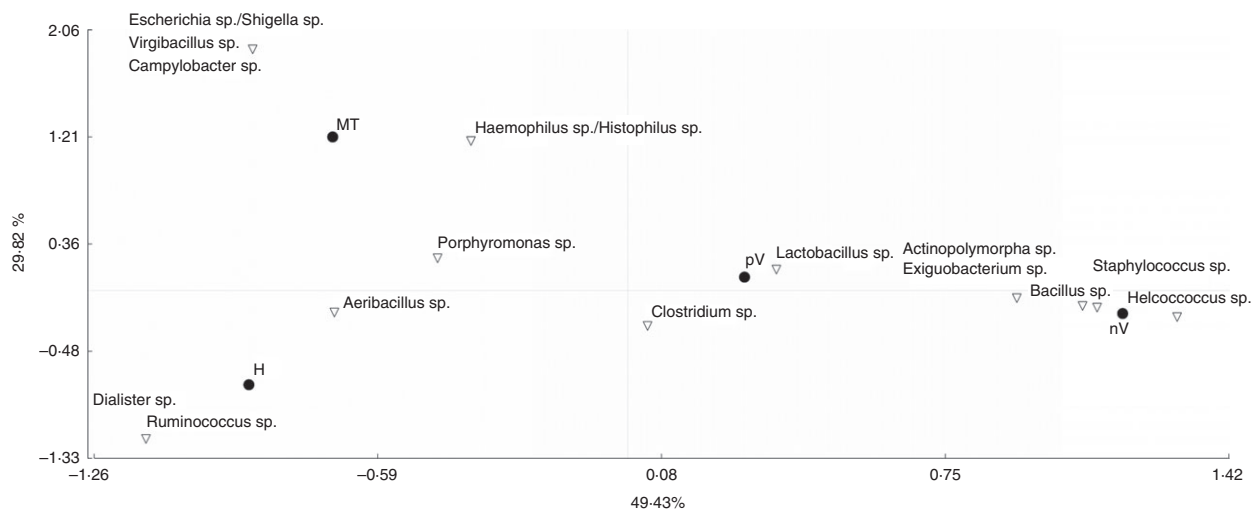
Figure 5 Correspondence analysis biplot of clinical profiles (●) and vaginal microbiota composition (▽). Contribution to Chi-square is indicated in brackets. pV: synchronized heifers diagnosed with clinical vaginitis; nV: synchronized heifers diagnosed with no clinical vaginitis; H: nulliparous clinically healthy heifers; MT: cows diagnosed with metritis or RB.

The results presented contribute to the knowledge of the vaginal microbiome in synchronized heifers with (pV) or without (nV) clinical vaginitis. This study reports the existence in the vaginal microbial consortium of bacteria that can act as uterine pathogens. Our data provide the first report about the presence of the genera *Dialister* sp. and *Ruminococcus* sp. as constituents of the vaginal microbiota of healthy cows.

Acknowledgements

Conflict of Interest

None declared.

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