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Changes in the vaginal flora of goats following a short-term protocol of oestrus induction and synchronisation with intravaginal sponges as well as their antimicrobial sensitivity



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ABSTRACT

The aim of this study was to characterise the changes in the vaginal flora of goats subjected to the induction and synchronisation of oestrus using intravaginal sponges (IVSs). Mucous samples were collected from the vaginas of 32 Toggenburg goats using sterile swabs. Samples were obtained on the day that the IVSs inserted (D0), the day they were withdrawn (D6), and 24 h (D7), 48 h (D8), and a week (D13) after the sponges were withdrawn. The samples were transferred to the laboratory in transport medium, inoculated on 5% sheep blood agar, and incubated at 37 °C (aerobic culture only). The susceptibility of the cultured bacteria to eight of the most frequently employed antimicrobial agents was determined using the disc diffusion method in Mueller–Hinton agar. Fewer colony-forming units (CFUs) were obtained from the samples that were taken before sponge insertion (D0) compared with those obtained on the other days. After the sponges were inserted, the number of CFUs obtained from the vaginal samples increased, reaching the highest value at the time of sponge withdrawal (D6), when 62.1% (18/29) of the samples yielded $\geq 10^5$ CFU/ml. At this time, more than one type (genus) of bacterial colony was detected. The most prevalent bacterium belonged to the genus *Staphylococcus* sp., except at the time of sponge withdrawal, when the most prevalent bacterium was *Escherichia coli*. The results of this study demonstrated that changes in the prevalent bacterial isolate and the number of CFUs in the vaginal flora of goats subjected to a short-term protocol of oestrus induction and synchronisation using IVS occurred while the IVSs were present and that there was a rapid re-establishment of the normal microbiota after the IVSs were removed.

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1. Introduction

Goats are seasonal breeders and are influenced by the geographic latitude and the characteristics of their breed.

The Brazilian territories occur in a wide variety of latitudes, which may strongly or weakly influence goat reproduction. In southeastern Brazil, the commercial dairy industries that use specialised dairy breeds must regularly induce or synchronise oestrus to obtain sufficient amounts of milk throughout the year. A combination of progestagens, gonadotropins and prostaglandins is the most commonly used formulation to induce oestrus in goats (Souza et al., 2011). Intravaginal sponges (IVSs) are traditionally utilised in the oestrus synchronisation of small ruminants (Abecia

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et al., 2012). The IVSs are impregnated with progestagens, which are effective at lower doses than is natural progesterone (Wildeus, 2000). The protocols vary from short-term (6 days) to long-term (14 days); recent studies have demonstrated no differences among the protocols in their efficacy of synchronisation (Karaca et al., 2010; Vilariño et al., 2011). However, those IVSs are a predisposing factor for vaginal infections because they substantially alter the profile of uterine and vaginal bacteria (Padula and Macmillan, 2006). Vaginitis is a common disease of the reproductive tract of domestic animals that is often caused by opportunistic secondary pathogens (Kustritz, 2006), such as coliforms, mainly *Escherichia coli* (Martins et al., 2009a), which is the bacterium most frequently isolated from the vaginas of ewes (Sargison et al., 2007), goats (Ababneh and Degefa, 2006) and cows (Sheldon et al., 2008).

Therefore, many commercial intravaginal devices manufacturers recommend applying antimicrobial drugs to the sponges prior to insertion. IVSs are known to induce inflammatory responses and the accumulation of vaginal fluids, with the concomitant increase in the bacterial load. The bacterial load significantly decreases after the sponge is withdrawn and reaches normal values by the day of oestrus (Suaréz et al., 2006).

The aim of this study was to characterise the changes in the vaginal flora of goats subjected to the induction and synchronisation of oestrus using IVSs that lacked antimicrobial agents and to determine the antibiotic sensitivity of the vaginal bacteria.

2. Materials and methods

2.1. Location and experimental conditions

This study was conducted during the months of November and December of 2010, which correspond to the anoestrus season of the goats. This study was approved by the Animal Care Committee of Fluminense Federal University, and it was conducted according to the ethical principles of the Sociedade Brasileira de Ciências de Animais de Laboratório.

The goats were kept in an intensive system within suspended pens. The goats were fed corn silage, *Pennisetum purpureum* and *Saccharum officinarum* were available for forage. Additionally, a balanced concentrate supplement was provided according to their milk production (NRC, 2007). Mineralised salt (Salminas Goats®, Nutriplan, Juiz de Fora, MG, Brazil) and drinking water were available *ad libitum*.

2.2. Animals

The samples were obtained from 32 Toggenburg goats that were apparently free of reproductive diseases. Oestrus was induced and synchronised using IVSs impregnated with 60 mg of medroxyprogesterone acetate (Progespon®, Tecnopec, São Paulo, SP, Brazil) lacking antibiotics, which were left in the dams for 6 days. Twenty-four hours before the removal of the sponges, the goats were given an intramuscular injection of equine chorionic gonadotropin (eCG

- 200 UI - Novormon 5000®, Tecnopec, SP, Brazil) and 30 µg of d-cloprostenol (Prolise®, ARSA S.R.L., Buenos Aires, Argentina).

2.3. Collection of the vaginal mucous samples

The mucous samples were collected from the posterior vaginal region using a sterile cotton swab. Sampling was conducted on the day of the intravaginal sponge insertion (D0), at sponge withdrawal (D6), 24 h (D7), 48 (D8) hours and a week (D13) after sponge withdrawal. The samples were maintained at 4 °C until the microbiological tests were performed.

2.4. Bacterial culture

The samples were transferred to the laboratory in transport medium (Stuart's medium, Copan, Italy), inoculated on 5% sheep blood agar (Merck, São Paulo, SP, Brasil), and incubated at 37 °C (aerobic culture only). If bacterial growth was apparent after 24 or 48 h of incubation, smears were made, Gram-stained, and examined by microscopy. The samples containing bacteria with morphologies consistent with Gram-negative rods were transferred to EMB Teague agar (Merck, São Paulo, SP, Brasil), whereas those with morphologies suggestive of *Staphylococcus* sp. were transferred to mannitol-salt agar (Merck, São Paulo, SP, Brasil).

2.4.1. Bacterial identification

The bacteria were identified based on their colony characteristics, Gram-staining, pigment production and biochemical reactions, using Triple Sugar Iron (TSI) agar, the citrate, urease, indole, Methyl Red (MR), Voges Proskauer (VP), nitrate and motility tests, the catalase activity test, the tube coagulase test, and assays of their aerobic fermentation of several carbohydrates. The bacteria were classified as described in previous studies (Martins et al., 2009a), according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

2.5. Antibiotic sensitivity test

The susceptibility to a panel of eight of the most frequently used antimicrobial agents was determined using the disc diffusion method with Mueller-Hinton agar (Merck, São Paulo, SP, Brasil), in accordance with the protocols of the Clinical and Laboratory Standards Institute (2008). Briefly, three to five well-isolated colonies of the same morphological type were selected from the agar plate culture and transferred to a tube containing Brain Heart Infusion broth (Merck, São Paulo, SP, Brasil). Standardised inoculum cultures with a turbidity equivalent to a 0.5 McFarland standard were used (corresponding to a concentration of approximately $(1-2) \times 10^6$ CFU/mL). All of the isolates were tested using disks with the following drug concentrations and minimum inhibitory zones: gentamicin – GEN (10 µg, 15 mm), cefalotin – CFL (30 µg, 18 mm), tetracycline – TET (30 µg, 15 mm), ciprofloxacin – CIP (5 µg, 21 mm), florfenicol – FLF (30 µg, 18 mm), and trimethoprim-sulfamethoxazole – SUT (25 µg, 17 mm). In

Table 1

Colony forming units obtained from the vaginal samples of Toggenburg goats taken at different times relative to a short-term protocol of oestrus induction and synchronisation using an IVS.

	Lowest bacterial concentration		Total	Highest bacterial concentration
	CFU $\leq 2.0 \times 10^4$	CFU $> 2.0 \times 10^4$		CFU $\geq 10^5$
D0 ^a	54.5% (18/33)	39.4% (13/33)	93.9% (31/33) ^g	6.1% (2/33) ^f
D6 ^b	10.3% (3/29)	27.6% (8/29)	37.9% (11/29)	62.1% (18/29) ^f
D7 ^c	9.3% (3/32)	50.0% (16/32)	59.4% (19/32)	40.6% (13/32)
D8 ^d	24.0% (6/25)	40.0% (10/25)	64.0% (16/25)	36.0% (9/25)
D13 ^e	37.5% (9/24)	29.1% (7/24)	66.7% (16/24) ^g	33.3% (8/24)

^a Sponge insertion.

^b Sponge withdrawal.

^c 24 h after sponge withdrawal.

^d 48 h after sponge withdrawal.

^e One week after sponge withdrawal.

^f $P < 0.001$.

^g $P < 0.05$.

addition, the presence of Gram-negative rods was tested with the following drug concentrations and minimum inhibitory zones: ampicillin – AMP (10 µg, 17 mm), amoxicillin and clavulanic acid – AMC (30 µg, 18 mm); penicillin G – PEN (10 U, 29 mm) and cefoxitin – CFO (30 µg, 22 mm). *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as the quality control organisms.

2.6. Statistical analysis

The data from the bacterial concentration (low and high concentration) assays and the tests for grouping the bacteria (Gram-positive and Gram-negative) using the samples taken at different times relative to the short-term protocol were compared using the chi-square test (X^2 - Pearson).

3. Results

Fewer colony-forming units (CFUs) were obtained before sponge insertion (D0) compared with those obtained on other days. The number of CFUs obtained was highest at the time the sponges were withdrawn (D6), when 62.06% (18/29) of the samples possessed $\geq 10^5$ CFU/ml ($P < 0.001$; Table 1). Additionally, the concentration of bacteria obtained from the D6 samples was

significantly different ($P < 0.05$) from that of the D13 samples (one week after the sponges were withdrawn). At D0, more than one type of bacterial colony was detected. The most prevalent bacterium belonged to the genus *Staphylococcus* sp., except at the time the sponges were withdrawn (D6), when the most prevalent bacterium was *E. coli* ($P < 0.05$; Table 2). Therefore, a significant ($P < 0.05$) reduction in the content of Gram-negative bacteria occurred between D6 and D13 (one week after the sponges were withdrawn).

The results for the percentage of bacteria with resistance to each of the antibiotics assessed using the sensitivity tests are shown in Table 3.

4. Discussion

The changes in the vaginal flora may be attributed to the physical effects of the intravaginal sponge and/or to the constant absorption and retention of vaginal secretions by the intravaginal sponge, which establishes an environment that favours bacterial growth (Suárez et al., 2006).

The results of this study revealed that changes in the prevalent bacterial isolate and the load of CFUs in the vaginal flora of goats subjected to a short-term protocol of oestrus induction and synchronisation with IVSs

Table 2

Bacterial colony type isolated from goats' vaginas and their prevalence at different times relative to a short-term protocol of oestrus induction and synchronisation using an IVS.

	D0 ^a	D6 ^b	D7 ^c	D8 ^d	D13 ^e
Gram-positive bacteria					
<i>Staphylococcus</i> sp.	63.6% (21/33) ^f	17.2% (5/29) ^f	53.1% (17/32)	56.0% (14/25)	76.0% (19/24)
Gram-negative bacteria					
<i>Klebsiella pneumoniae</i>	12.1% (4/33)	27.5% (8/29)	12.5% (4/32)	24.0% (6/25)	12.5% (3/24)
<i>Escherichia coli</i>	12.1% (4/33)	44.8% (13/29)	34.3% (11/32)	16.0% (4/25)	8.3% (2/24)
<i>Proteus vulgaris</i>	12.1% (4/33)	3.4% (1/29)	0.0% (0/32)	0.0% (0/25)	0.0% (0/24)
<i>Pseudomonas aeruginosa</i>	0.0% (0/33)	6.9% (2/29)	0.0% (0/32)	0.0% (0/25)	0.0% (0/24)
Total of Gram-negative bacteria	33.4% (12/33)	82.7% (24/29) ^g	46.8% (15/32)	40.0% (10/25)	20.8% (5/24) ^g

^a Sponge insertion.

^b Sponge withdrawal.

^c 24 h after sponge withdrawal.

^d 48 h after sponge withdrawal.

^e One week after sponge withdrawal.

^f $P < 0.05$.

Table 3

Percentages of various antibiotic-resistant bacterial isolates from goats' vaginas.

	PEN ^a	CFO ^b	AMC ^c	AMP ^d	CFL ^e	GEN ^f	SUT ^g	FLF ^h	TET ⁱ	CIP ^j
<i>Staphylococcus</i> sp.	64.7%	41.2%	23.5%	25.9%	49.4%	47.0%	23.5%	16.5%	34.1%	35.3%
<i>K. pneumoniae</i>	66.7%	37.0%	37.0%	48.1%	55.5%	48.1%	22.2%	26.0%	51.9%	18.5%
<i>E. coli</i>	92.3%	23.1%	23.1%	53.9%	53.9%	38.5%	38.5%	7.7%	53.9%	0.0%
<i>P. vulgaris</i>	100.0%	20.0%	20.0%	20.0%	60.0%	20.0%	20.0%	20.0%	60.0%	40.0%
<i>P. aeruginosa</i>	50.0%	0.0%	0.0%	50.0%	0.0%	0.0%	0.0%	50.0%	100.0%	0.0%

^a Penicillin G.^b Cefoxitin.^c Amoxicillin and clavulanic acid.^d Ampicillin.^e Cefalotin.^f Gentamicin.^g Trimethoprim-sulfamethoxazole.^h Florfenicol.ⁱ Tetracycline.^j Ciprofloxacin.

occurred while the IVSs were present. The results demonstrated that the CFU counts were significantly lower before sponge insertion compared to the counts present when the sponges were withdrawn, when a high percentage of the samples possessed $\geq 10^5$ CFU/ml. After the sponges were removed, the CFU content decreased significantly, and after one week, most of the samples (66.7% - 16/24) exhibited low CFU counts. Progesterone has an immunosuppressive effect that, in addition to the presence of the IVS, may induce a local inflammatory response in the vagina and plays a role in this pattern of variation in the CFU counts (Lewis, 2003). After the sponges were removed, the concentration of progesterone decreased, and the local inflammatory stimulus was absent. Initially, *Staphylococcus* sp. was the most prevalence bacterial type. Martins et al. (2009b) confirmed the presence of staphylococcal isolates in the vaginal microflora of clinically healthy ewes. When the sponges were withdrawn, we noticed that the CFU counts were significantly increased and that bacteria of the Enterobacteriaceae family were prevalent. This finding is in agreement with those of Martins et al. (2009a) and Manes et al. (2010) and has been attributed to the local inflammatory effect of the device promoting the growth of opportunistic bacteria. Another hypothesis for the prevalence of opportunistic bacteria is that the string of the progestagen-containing device, which remains exposed, is a predisposing factor that carries external microorganisms into the reproductive tract. The normal microbiota was re-established within a week after the sponges were removed, with the return of the staphylococci as the predominant microorganism ($P < 0.05$).

Because *Staphylococcus* sp. are members of the normal vaginal microbiota of small ruminants, it is necessary to prevent the growth of opportunistic microorganisms such as *E. coli*.

The results of the antibiotic sensitivity tests are remarkable in that most of the bacteria were resistant to PEN and TET, which are the antibiotics most commonly used by breeders (*Staphylococcus* sp. PEN 64.7%, TET 34.1%; *Klebsiella pneumoniae* PEN 66.7%, TET 51.9%; *E. coli* PEN 92.3%, TET 53.9%; *Proteus vulgaris* PEN 100%, TET 60%; and *Pseudomonas aeruginosa* PEN 50%, TET 100%). Therefore, these are not the appropriate antibiotics to include in the

intravaginal devices. *E. coli* was the most resistant bacterium of the isolates, followed by *K. pneumoniae* and *Staphylococcus* sp.

The resistance of *P. vulgaris* and *P. aeruginosa* may have been over- or under-estimated due to their low numbers of CFUs.

5. Conclusions

Intravaginal sponges employed in oestrus induction and synchronisation protocols cause changes in the vaginal microbiota of goats for a short period. After removing the sponges, there was a rapid re-establishment of the normal microbiota. These results indicate that this protocol would not affect the microbiotic status at the time of natural mating or artificial insemination; the breeders may thus choose to associate an antibiotic with the progestagen-containing devices.

If the choice is made to incorporate antibiotics, an antibiotic sensitivity test should be performed to select the appropriate compound(s).

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References

- Ababneh, M.M., Degefa, T., 2006. Bacteriological findings and hormonal profiles in the postpartum Balady goats. *Reprod. Domest. Anim.* 41, 12–16.
- Abecia, J.A., Forcada, F., González-Bulnes, A., 2012. Hormonal control of reproduction in small ruminants. *Anim. Reprod. Sci.* 130, 173–179.
- Clinical and Laboratory Standards Institute, 2008. Performance Standards for Antimicrobial Susceptibility Testing. CLSI Publication M100-S18, Wayne, PA, USA. p.188.
- Holt, J.C., Krieg, N.K., Sneath, P.H.A., Stanely, J.F., Williams, S.T., 1994. *Bergey's Manual of Determinative Bacteriology*, ninth ed. Williams and Wilkins, Baltimore.
- Karaca, F., Doğruer, G., Sarıbay, M.K., Ateş, C.T., 2010. Oestrus synchronisation with short-term and long-term progestagen treatments in goats:

- the use of GnRH prior to short-term progestagen treatment. *Italian J. Anim. Sci.* 9, 22–23.
- Kustritz, M.V., 2006. Collection of tissue and culture samples from the canine reproductive tract. *Theriogenology* 66, 567–574.
- Lewis, G.S., 2003. Steroidal regulation of uterine resistance to bacterial infection in livestock. *Reprod. Biol. Endocrinol.* 1, 117.
- Manes, J., Fiorentino, M.A., Kaiser, G., Hozbor, F., Alberio, R., Sanchez, E., Paolicchi, F., 2010. Changes in the aerobic vaginal flora after treatment with different intravaginal devices in ewes. *Small Rum. Res.* 94, 201–204.
- Martins, G., Figueira, L., Penna, B., Brandão, F., Varges, R., Vasconcelos, C., Lilenbaum, W., 2009a. Prevalence and antimicrobial susceptibility of vaginal bacteria from ewes treated with progestin-impregnated intravaginal sponges. *Small Rum. Res.* 81, 182–184.
- Martins, G., Brandão, F., Figueira, L., Penna, B., Varges, R., Vasconcelos, C., Lilenbaum, W., 2009b. Prevalence and antimicrobial susceptibility of *Staphylococci* isolated from the vagina of healthy ewes. *R. Bras. Ci. Vet.* 1, 37–40.
- National Research Council, NRC, 2007. Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New Worlds Camelids. National Academic Press, Washington, p. 384.
- Padula, A.M., Macmillan, K.L., 2006. Effect of treatment with two intravaginal inserts on the uterine and vaginal microflora of early postpartum beef cows. *Aust. Vet. J.* 84, 204–208.
- Sargison, N.D., Howie, F., Mearns, R., Penny, C.D., Foster, G., 2007. Shiga toxin-producing *Escherichia coli* as a perennial cause of abortion in a closed flock of Suffolk ewes. *Vet. Rec.* 160, 875–876.
- Sheldon, I.M., Williams, E.J., Miller, A.N., Nash, D.M., Herath, S., 2008. Uterine diseases in cattle after parturition. *Vet. J.* 176, 115–121.
- Souza, J.M.G., Torres, C.A.A., Maia, A.L.R.S., Brandão, F.Z., Bruschi, J.H., Viana, J.H.M., Fonseca, J.F., 2011. Autoclaved, previously used intravaginal progesterone devices induces estrus and ovulation in anestrus Toggenburg goats. *Anim. Reprod. Sci.* 129, 50–55.
- Suaréz, G., Zunino, P., Carol, H., Hungerfeld, R., 2006. Changes in the aerobic vaginal bacterial mucous load and assessment of the susceptibility to antibiotics after treatment with intravaginal sponges in anestrus ewes. *Small Rum. Res.* 63, 39–43.
- Vilariño, M., Rubianes, E., Menchaca, A., 2011. Re-use of intravaginal progesterone devices associated with the Short-term Protocol for timed artificial insemination in goats. *Theriogenology* 75, 1195–2000.
- Wildeus, S., 2000. Current concepts in synchronization of estrus: sheep and goats. *J. Anim. Sci.* 77, 1–14.