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Susceptibility of Probiotic Bovine Vaginal *Lactobacillus* to Antimicrobial Agents

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Abstract: The aim of this research was the assessment of antibiotic susceptibility profile on bovine vaginal probiotic *Lactobacillus*. Antibiotics could affect the colonization of probiotic microorganisms when the host is exposed to therapies for other infections. We studied the sensibility of four strains of bovine probiotic vaginal *Lactobacillus* to ampicillin, vancomycin, gentamicin, kanamycin, lincomycin, tetracyclin, chloramphenicol, erythromycin, sulphamethoxazole, metronidazole, nitrofurantoin, aminopenicillin/sulbactam and trimethoprim/sulfamethoxazole. The agar overlay disc diffusion and the dilution methods were performed according to the Clinical and Laboratory Standards Institute (CLSI) recommendations. The Minimal Inhibitory Concentrations (MICs) obtained showed that *L. gasseri* CRL1412, *L. gasseri* CRL1421 and *L. gasseri* CRL1460 have similar susceptibility profiles. *L. delbruekii* subsp. *delbrueckii* CRL1461 was the most susceptible strain. The four strains showed high MIC values for the inhibitors of nucleic acid synthesis (>500 μg mL^{-1} for sulfamethoxazole and metronidazole), which are widely used in bovine therapies. None of the lactobacilli was resistant to vancomycin. These results are one of the studies required for the inclusion of these strains in a probiotic product for the reconstitution of the bovine urogenital microflora with *Lactobacillus*. Thus, can prevent metritis in cows during postpartum and increases the reproductive performance in dairy herd.

Key words: *Lactobacillus*, susceptibility, antibiotic, probiotic, metritis

INTRODUCTION

Metritis is one of the diseases that affect dairy cows in postpartum (Rajala-Schultz and Grohn, 1999), resulting in the impairment of fertility performance and decreasing milk yield (Fonseca *et al*., 1983; Coleman *et al*., 1985; Lewis, 1997). Opportunistic pathogens from other areas of the animal are capable to colonize the uterus and to cause acute metritis during postpartum (Zemjanis, 1980; Kask *et al*., 1998; Bondurant, 1999). The treatments using antiseptics and antibiotics cause economic and health disadvantages such as increase in production costs, loss of milk discarded because of antibiotic residues, development of microbial resistance to antibacterial drugs, adverse effects on the uterine epithelium and its defense mechanisms, failures in the contractility of the myometrium and disturbances in the ecological balance in the normal host microflora (Oxender and Seguin, 1976; Masera *et al*., 1980; Lotthammer and Wittkowski, 1994; Witte, 2000; Sullivan *et al*., 2001; Ocal *et al*., 2004).

Several researchers have proposed alternatives to antibiotics and hormones for the treatment and prevention of infectious metritis, such as the uterine administration of *E. coli* lipopolysaccharides to induce immune cells activation in the uterus (Singh *et al*., 2000).

Lactobacilli are considered as the primary microbiological barrier against infections by genital pathogens. They exert a protective role mainly by a combination of steric exclusion, immune system stimulation and production of inhibitory substances (Lepargneur and Rousseau, 20002). In previous studies, the microbial populations of the vagina in healthy cows was described, lactobacilli being normal constituents of this microbial flora (Otero *et al*., 1999, 2000). From this microflora, four strains that shared some probiotic properties (Otero *et al*., 2006) were selected. Our research group is working on the formulation of a probiotic product for veterinary use to prevent infectious diseases in cattle, which can diminish or eliminate the need for antibiotic or hormonal treatments, thus increasing the reproductive performance, health quality and economic profitability in livestock.

The aim of the present study was to study the antibiotic susceptibility of the selected bovine vaginal *Lactobacillus* strains in order to assess their ability to remain viable in the reproductive tract of cows when administered simultaneously or not with antibiotics. In this work the levels of susceptibility to several antimicrobial agents used frequently in veterinary practice in postpartum cows are reported.

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MATERIALS AND METHODS

Bacterial strains and culture conditions: Four selected strains: *Lactobacillus gasseri* CRL1412, CRL1421 and CRL1460 and *Lactobacillus delbruekii* subsp. *delbrueckii* CRL1461, isolated from bovine vagina were examined in this study. The three *L. gasseri* strains have been identified to the species level by the ARDRA-PCR system and *L. delbruekii* subsp. d*elbrueckii* by the carbohydrate fermentation profile (API CH 50, BioMérieux) (Otero *et al*., 2006). The four strains of lactobacilli used showed some probiotic properties (Otero *et al*., 2006; Otero and Nader-Macias, 2006). Lactobacilli were cultured in MRS broth (De Man *et al*., 1960) at 37°C for 12-14 h. The cultures were stored in milk-yeast extract (13% (w/v) nonfat milk, 1% (w/v) yeast extract) at -20°C. Two reference strains were used to validate the culture medium in compliance with the CLSI (Clinical Laboratory Standards Institute) recommendations: *Staphylococcus aureus* ATCC25923 and *Escherichia coli* ATCC25922 (CLSI, M2-A7). They were stored at -20°C in BHI broth (Brain-Heart Infusion) with 25% glycerol and cultured in BHI (Brain-Heart infusion) broth at 37 °C for 8 h.

Antimicrobial susceptibility testing: As there are not standard methods for testing the antimicrobial susceptibility described for the genus *Lactobacillus,* several methods were assayed. A semi-quantitative and a quantitative method suggested by the CLSI (CLSI, M2-A7 document) modified for their application in lactobacilli were used.

The semi-quantitative disc diffusion method was modified by using MRS agar. Agar plates containing MRS agar were seeded on their surface with a swab of the bacterial suspension with an Optical Density equivalent to 0.5 turbidity in the Mc Farland scale. The suspensions were obtained from each reference microorganism and from the lactobacilli. The plates were kept at room temperature for 15 min. Then the antibiotic-containing discs were dispensed with sterile forceps. The antibiotic discs used were: ampicillin (10 μg mL^{-1}), aminopenicillin/sulbactam (20 μg mL^{-1}), vancomycin (30 μg mL^{-1}), ceftazidime (30 μg mL^{-1}), gentamicin (10 μg mL^{-1}), erythromycin (15 μg mL^{-1}), chloramphenicol (30 μg mL^{-1}), trimethoprim/sulphamethoxazole (25 μg mL^{-1}), piperacillin (100 μg mL^{+}) and nitrofurantoin (300 μg mL^{-1}) (Britania Laboratories S.A, Argentina). The plates were then incubated at 37°C for 24 h. The diameters of the inhibition halos were measured and compared with the standard values suggested for the reference strains (CLSI, 2000).

The MIC values were determined by the quantitative agar dilution method and by the broth micro-dilution method, following the CLSI recommendations (CLSI, M2-A7 document). Stock solutions of the antibiotics were prepared by using the solvents suggested by CLSI (M100-S10, for use with M7-A5) and MRS broth was used as diluent. The antibiotics assayed in this test were ampicillin, vancomycin, gentamicin, kanamycin, lincomycin, tetracyclin, erythromycin, sulphamethoxazole, metronidazole and nitrofurantoin (Sigma-Aldrich Co., USA).

For the agar dilution method, MRS agar plates supplemented with the antibiotic were prepared. Each antibiotic was assayed within a concentration range of 0.0005-200 μg mL^{-1}. Ten microlitres of bacterial suspension (Optical density equivalent to 0.5 turbidity in Mc Farland scale) were cultured on the plates. This suspension was prepared from a 12 h subculture in MRS. The MIC was defined as the lowest antibiotic concentration showing a visible inhibition of the growth after incubation for 18-24 h at 37°C.

The MIC in broth was also determined by the application of the micro-dilution method following CLSI recommendations. The range of the antibiotic concentrations assayed was 0.244-500 μg mL^{-1} by dilution in MRS broth. Fifty microlitres of each dilution and an equal volume of a bacterial suspension of 1×10^{6} CFU mL^{-1} in MRS broth were added to each well under sterile conditions. Consequently, the final antibiotic concentration was reduced to the half. The micro-plates were incubated at 37°C for 20 h. Negative (MRS broth used for the dilutions) and positive (bacterial suspension) growth controls were included. The MIC was defined as the lowest antibiotic concentration added to the wells with no visible growth.

Breakpoints (MIC interpretative standards) for Gram positive clinical isolates (particularly those genera that are bovine pathogens) defined by the CLSI (2000) were used as guidelines for the interpretation of the results.

Viability testing: In the suspensions without visible growth, the viability of the microorganisms was determined by the plate dilution method by using MRS agar. The lowest concentration with no viable cells was defined as the Minimal Bactericidal Concentration (MBC). The mortality was calculated by the formula:% mortality = 100-[viable cells at 18 h (CFU mL^{-1})/initial inoculum (CFU mL^{-1})] × 100.

RESULTS

The results obtained in the quality control assays suggest that the disc diffusion test is not suitable when

a medium different from the recommended Mueller-Hinton is used. The diameter of the halos obtained by the disc diffusion test for the reference strains were within the quality control limits suggested by the CLSI (M2-A7) only for gentamicin and trimethoprim/sulphamethoxazole, as indicated in Table 1. The other antibiotics produced wider halos than the limit values recommended, suggesting a higher antibiotic diffusion. Table 2 shows the halos obtained for the bovine lactobacilli, no significant differences being observed in gentamicin sensibility among the four strains assayed. No inhibition halo was obtained for trimethoprim/sulphamethoxazole on lactobacilli cultures (Table 2).

The MIC values obtained with the reference strains by the agar dilution method and the broth micro-dilution method were within the acceptable quality control limits recommended for the antibiotics studied in this assay. The MIC values obtained for the bovine vaginal *Lactobacillus* strains are shown in Table 3 and 4. Although there are differences between the values obtained with the two techniques applied, the behaviour of the strains showed similar tendencies when compared with the breakpoints (MIC interpretative standards) for Gram positive clinical isolates (Table 5).

Table 1: Diameters of inhibition zone on two standard microorganisms cultures recommended for the quality control

	Inhibition zone (mm)			
	S. aureus	ATCC 25923	*E. coli*	ATCC 25922
Ampicillin	48±2[a]	27-35[b]	36±9.5[a]	16-22[b]
Aminopenicillin+ sulbactam	52±4[a]	29-37[b]	42±4[a]	20-24[b]
Piperacilin	48±12[a]	-	42±4[a]	24-30[b]
Ceftazidime	28±9[a]	16-20[b]	44±3[a]	25-32[b]
Vancomycin	36±1[a]	17-21[b]	14±4[a]	-
Gentamicin	27±1[a]	19-27[b]	22±2.5[a]	19-26[b]
Chloramphenicol	40±1[a]	19-26[b]	42±2.5[a]	21-27[b]
Erythromycin	40±3.5[a]	22-30[b]	18±1.5[a]	-
Trimethoprim+ sulfametoxazole	32±1[a]	24-32[b]	30±1[a]	24-32[b]
Nitrofurantoin	38±2.5[a]	18-22[b]	40±1[a]	20-25[b]

Table 2: Diameters of the inhibition zone in vaginal bovine lactobacilli cultures in MRS agar

	Diameters of inhibition zone (mm)			
Antibiotic	*L. gasseri* CRL1421	*L. gasseri* CRL1412	*L. delbrueckii* subsp. *delbrueckii* CRL1461	*L. gasseri* CRL1460
Ampicillin	36±1	34±1	40±1	38±2
Aminopenicillin/ sulbactam	40±1	40±2	42±4	30±4
Piperacillin	40±7	42±5	42±1	44±5
Ceftazidime	24±2	16±7	28±2	26±3
Vancomycin	16±3	36±1	24±1	34±1
Gentamicin	14±3	16±1	18±2	16±1
Chloramphenicol	34±1	38±1	38±1	Nd
Erythromycin	44±1	44±3	46±2	Nd
Trimethoprim/ sulfamethoxazole	0±0	0±0	0±0	0±0
Nitrofurantoin	16±1	20±1	32±1	26±1

[a]Diameters of the inhibition halos obtained in MRS agar; [b]Acceptable zone diameter (mm) quality control limits in Mueller-Hinton medium (CLSI, 2000)

Table 3: Antimicrobial susceptibility of bovine vaginal *Lactobacillus* expressed as MIC values obtained by the agar dilution method (MRS)

	MIC (μg mL^{-1})			
Antibiotic	*L. gasseri* CRL1421	*L. gasseri* CRL1412	*L. delbrueckii* subsp. *delbrueckii* CRL1461	*L. gasseri* CRL1460
Ampicillin	25	25	0.25	25
Vancomycin	2	1	2	2
Gentamicin	200	200	25	200
Kanamycin	>200	>200	>200	>200
Lincomycin	10	10	2	10
Tetracycline	100	100	2	100
Chloramphenicol	5	5	5	5
Erythromycin	5	5	0.5	5
Sulfamethoxazole	>200	200	>200	>200
Metronidazole	>200	>200	>200	>200
Nitrofurantoin	>200	200	>200	>200

Table 4: Antimicrobial susceptibility of bovine *Lactobacillus* expressed as MIC values obtained by the broth dilution method (MRS)

	MIC (μg mL^{-1})			
Antibiotic	*L. gasseri* CRL1421	*L. gasseri* CRL1412	*L. delbrueckii* subsp. *delbrueckii* CRL1461	*L. gasseri* CRL1460
Ampicillin	15.62	15.62	15.62	15.62
Vancomycin	15.62	-	15.62	15.62
Gentamicin	62.50	62.50	31.25	62.50
Kanamycin	250	250	125	125
Lincomycin	31.25	-	15.62	15.62
Tetracycline	125	125	31.25	125
Erythromycin	3.9	3.9	3.9	3.9
Sulfamethoxazole	>500	>500	>500	>500
Metronidazole	>500	>500	>500	>500
Nitrofurantoin	250	250	250	250

(-) Not determined

Table 5: Breakpoints applied for the classification of the antibiotic susceptibility of bovine vaginal lactobacilli

	Breakpoint for resistance (μg mL^{-1})		
	Sensitive	Intermediate	Resistant
Ampicillin[1]	≤0.25	0.5-4	≥8
Vancomycin[3]	≤4	8-16	≥32
Gentamicin[2]	≤4	8	≥16
Kanamycin[2]	≤16	32	≥64
Lincomycin[4]	≤0.25	0.5	≥1
Tetracycline[1]	≤2	4	≥8
Erythromycin[1]	≤0.25	0.5	≥1
Sulfamethoxazole[2]	≤38	-	≥76
Metronidazole	-	-	-
Nitrofurantoin[3]	≤32	64	≥128

[1]Breakpoints defined by the CLSI (2000) for *Streptococcus* sp.; [2]Breakpoints defined by the CLSI (2000) for *Staphylococcus* sp.; [3]Breakpoints defined by the CLSI (2000) for *Enterococcus* sp.; [4]The values corresponding to clindamycin breakpoints were defined by the CLSI (2000) for *Streptococcus* sp.; -No breakpoint values were available

Table 6: Bacterial viability after 18 h of incubation under MIC of each antibiotic

	Viability[2] (Log CFU mL^{-1}) and mortality percentage (%)[1]			
Antibiotic	*Lactobacillus gasseri* CRL1421	*Lactobacillus gasseri* CRL1412	*Lactobacillus delbrueckii* subsp. *delbrueckii* CRL1461	*Lactobacillus gasseri* CRL1460
Ampicillin	6.67±0.05[2]	5.86±0.05[2]	4.79±0.07(87.4)[1]	3.45±0.21(99.4)[1]
Vancomycin	6.69±0.12[2]	-	3.54±0.34(99.2)[1]	6.19±0.83[2]
Gentamicin	6.85±0.0[2]	0(100)[1]	0(100)[1]	0(100)[1]
Kanamycin	3.45±0.21(99.4)[1]	0(100)[1]	0(100)[1]	5.58±0.04(23.6)[1]
Lincomycin	4.97±0.06(81)[1]	-	0(100)[1]	6.86±0.03[2]
Tetracycline	4.15±0.21(97)[1]	3.45±0.21(99.4)[1]	5.37±0.02(52)[1]	3.87±0.38(98.2)[1]
Erythromycin	3.88±0.15(98.4)[1]	0(100)[1]	0(100)[1]	4.96±0.05(81.4)[1]
Nitrofurantoin	6.53±0.1[2]	5.14±0.10(72)[1]	0(100)[1]	4.89±0.15(83.8)[1]

Initial inoculum 2.5×10^5 CFU mL^{-1}; [1]% of mortality = 100-[viable cells at 18 h (CFU mL^{-1})/initial inoculum (CFU/mL)]×100; [2]The viability increased with respect to initial inoculum (Log initial inoculum = 5.4); (-) Not determined

L. delbrueckii subsp. *delbrueckii* CRL1461 exhibited an intermediate sensibility to erythromycin and was sensitive to ampicillin, vancomycin and tetracyclin when tested in the agar medium, while it showed an intermediate sensibility to vancomycin and was resistant to other antibiotics when assayed in broth (Table 3 and 4).

The three *L. gasseri* strains, CRL1412, CRL1421 and CRL1460, showed identical sensibility profiles to the antibiotics assayed. They were classified as resistant to ampicillin, gentamicin, kanamycin, lincomycin, tetracyclin, erythromycin, sulphamethoxazole, metronidazole and nitrofurantoin by both the agar and the broth methods. They were sensitive or intermediate to vancomycin when assayed in agar or broth, respectively (Table 3, 4 and 5).

Susceptibility to inhibitors of the cell wall synthesis: With regards to the susceptibility to inhibitors of the cell wall synthesis, *L. gasseri* CRL1421 and *L. gasseri* CRL1412 showed a lower sensibility than other microorganisms, because they increased their viability after 18 h of incubation in the presence of an antibiotic concentration equivalent to MIC (Table 6). *L. delbrueckii* subsp. *delbrueckii* CRL1461 showed the highest sensibility, with bacterial mortality percentages of 87.4% and 99.2% for ampicillin and vancomycin, respectively (Table 6).

Susceptibility to inhibitors of protein synthesis: *L. delbrueckii* subsp. *delbrueckii* CRL1461 was the most sensitive strain to this group of antibiotics, except for tretracyclin, which elicited a bacterial mortality percentage of 52% after 18 h of incubation. *L. gasseri* strains exhibited a variable behaviour: *L. gasseri* CRL1421 retained the viability with aminoglycosides, which had a lethal effect on the other strains. Lincomycin induced 100% mortality in *L. delbrueckii* subsp. *delbrueckii* CRL1461. However, this strain showed the lowest MIC to tretracycline and this effect is bacteriostatic.

Susceptibility to inhibitors of nucleic acid synthesis: No halos were obtained with trimethoprim/sulphamethoxazole (25 μg mL^{-1}) discs on lactobacilli cultures (Table 2). The four bovine lactobacilli strains were resistant to sulphamethoxazole when the agar and broth dilution methods were applied. The MIC values of metronidazole were higher than 500 μg mL^{-1} for the four strains (Table 3 and 4).

DISCUSSION

The lack of a standardized methodology to determine the antibiotics susceptibility in the genus *Lactobacillus* is mainly due to the fact that this genus is recognized as safe (Swerson *et al*., 1990; Reid *et al*., 2003). In the last years, many authors have studied the antibiotic susceptibility of lactic acid bacteria (LAB) by different methods including disc diffusion (Sozzi and Smiley 1980; Orberg and Sandine, 1985; Perreten *et al*., 1998), E-test (Katla *et al*., 2001), broth dilution (Perreten *et al*., 1997) and agar dilution (Butaye *et al*., 2000) tests. We applied the disc diffusion method because is the most widely used and is relatively easy to perform. The Mueller-Hinton medium is the one recommended for the disc diffusion test by CLSI, but as lactobacilli cannot grow in this medium, it was replaced by MRS medium in this study. Other researchers have used MRS agar for this test (Katla *et al*., 2001; Vescovo *et al*., 1982; Danielsen and Wind, 2003). The results obtained by the disc diffusion test are affected by the medium conditions such as pH, humidity, presence of divalent ions and other components, which can interfere with the diffusion of the antibiotics. For this reason, two reference strains (recommended by CLSI guidelines) were included in the assays as quality control of the medium. Our results agree with those published by Huys *et al*. (2002), because the effects of the growth medium on the zones obtained by the disc diffusion test depend on the antibiotic compound

tested. In present experiments, only gentamicin and the combination of trimethoprim/sulphamethoxazole produced inhibition zones within the acceptable limits for *Staphylococcus aureus* ATCC25923 and *Escherichia coli* ATCC25922 control cultures. This test was not specific enough to evidence the higher susceptibility to gentamicin of *L. delbrueckii* subsp. *delbrueckii* CRL1461 strain, which is shown by the dilution tests in agar and broth.

The MIC values obtained with the reference microorganisms were within the acceptable limits for most of the antibiotics assayed by the agar and broth dilution methods (data not shown). Antibiotics that showed MICs outside the limit values were excluded for the assays with the *Lactobacillus* strains.

The susceptibility to vancomycin was proposed as a requisite for the selection of probiotic microorganisms (De Vuyst *et al*., 2003). Our results show that the MIC of vancomycin in agar for the bovine *Lactobacillus* were between 1 and 2 μg mL^{-1}. Similar values were published by Katla *et al.* (2001), who, using E-test strips in MRS agar, obtained values of 1.5 μg mL^{-1} for 90% of the commercial *Lactobacillus* strains tested. When Danielsen and Wind (2003) assayed the vancomycin susceptibility in MRS agar by a similar method, they found values for MICs of 1-4 μg mL^{-1} for the *L. acidophilus* group. Bovine *Lactobacillus* strains were classified as vancomycin sensitive when their MICs were compared with the breakpoints for *Enterococcus ssp* (recommended by the CLSI). This is an advantage for their inclusion in a probiotic product for application in animals intended to human consumption (Reid *et al*., 2003).

Due to the multiplicity of methods used, there is still a lack of agreement in the resistance-susceptibility breakpoints of lactobacilli to most antibiotics (Danielsen and Wind 2003; Charteris *et al*., 1998). Therefore, the MIC values obtained were compared with the breakpoints reported for others Gram positive bacteria (Table 3).

The MICs for ampicillin were 15-25 μg mL^{-1} for the more sensitive strains. These values were higher than those reported for *Lactobacillus* from dairy commercial cultures (Katla et al., 2001) and from human vagina (Choi *et al*., 2003).

The variability in the values obtained for gentamicin agrees with the results reported by Katla *et al.* ((2001). Moreover the low susceptibility for kanamycin was also observed by other authors (Danielsen and Wind 2003; Choi *et al*., 2003).

The susceptibility to tetracyclin in bovine vaginal *Lactobacillus* was lower than the values reported by other authors, being thus an advantage because chlortetracyclin is an antibiotic used to increase weight gain, efficiency increase, carcass grade and conception rates (Reid *et al*., 2006). *L. delbrueckii* subsp. *delbrueckii* CRL1461 showed a similar susceptibility to human vaginal *Lactobacillus* (Choi *et al*., 2003). A similar behaviour was observed with erythromycin, although *L. delbrueckii* subsp. *delbrueckii* CRL1461 was more resistant than other *Lactobacillus* (Katla *et al*., 2001; Danielsen and Wind, 2003; Choi *et al*., 2003).

None of the *Lactobacillus* strains studied was susceptible to the inhibitors of the nucleic acid synthesis (sulphamethoxazole and metronidazole), a behaviour similar to that reported by other researchers (katla *et al*., 2001; Danielsen and Wind, 2003; Charteris *et al*., 1998; Delgado *et al*., 2005). Human vaginal lactobacilli were resistant to metronidazole in a study performed by Horowitz *et al.* (1994). The large number of current reports support the hypothesis that the genus *Lactobacillus* has an intrinsic resistance against the inhibitors of the nucleic acid synthesis.

The MIC is an easy to perform parameter that is useful to correlate the *in vitro* values with the *in vivo* plasmatic concentrations. It is well known that the effect of the antibiotic agent on each microorganism depends on specific factors related to both, as well as on the host. In the present paper, this criteria was applied to predict the behaviour of lactobacilli against antibiotic therapies, comparing lactobacilli sensibilities with the standardized MIC values for pathogenic microorganisms. For this reason, the assays were performed following the conditions suggested by the CLSI guidelines.

The vancomycin susceptibility of the four strains studied suggests that these microorganisms could be used in food-producing animals.

CONCLUSIONS

This study is the first to describe the antibiotic susceptibility of *Lactobacillus* strains isolated from the vagina of cattle. The results obtained allow us to predict the behaviour of the bovine vaginal lactobacilli through *in vivo* situations once they are included as probiotics in the vaginal of animals undergoing antibiotic therapies. These results allow us to propose the inclusion of these strains in a probiotic product for the prevention of bovine metritis.

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