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Spectrum of vaginal lactic acid bacteria in indigenous and cross bred cows of India

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Lactic acid bacteria (LAB) are beneficial microorganisms present in the bovine vagina. Documentation of LAB community in the vagina of indigenous and cross bred dairy cows of India is important to further establish its probiotic potential. The aim of this study was to isolate and identify the spectrum of LAB by phenotypic and genotypic methods. A total of 24 LAB were isolated from the vagina of 110 apparently healthy indigenous and cross bred cows. The LAB isolates belonged to the genus *Lactobacillus*, *Bacillus*, *Enterococcus* and *Weisella* and were further speciated based on their grouping with respective reference sequences available in the GenBank, on phylogenetic analysis. The species diversity of bovine vaginal LAB identified in this study was *Lactobacillus agilis* (17%), *Lactobacillus mucosae* (8%), *Lactobacillus plantarum* (21%), *Lactobacillus fermentum* (17%), *Lactobacillus pentosus* (4%), *Weisella cibaria* (8%), *Bacillus coagulans* (4%), *Bacillus cereus* (4%), *Enterococcus faecium* (13%) and *Enterococcus asini* (4%). The overall spectrum and relative abundance of the bovine vaginal LAB reported in this study provides critical information for the formulation of uterine probiotics for the prevention/treatment of bovine clinical endometritis.

Keywords: Bovine vagina, Diversity, Endometritis, *Lactobacillus*, Probiotics

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Reproductive disorders in dairy animals cause huge impact on animal health, reproductive performance, milk production and farm profitability in livestock sector. Endometritis is one of the major reproductive disorders in dairy cows, commonly known as inflammation of the uterine endometrium. This can be caused by bacterial, viral, fungal and mycoplasma infections or in any of these combinations. Clinical endometritis is an advanced state of inflammation characterised by purulent uterine discharge along with pus flakes. Traditional treatment mostly relies on administration of antibiotics in dairy cows[1]. Use of antibiotics can disturb the uterine microecology, pH of uterine fluids and most importantly reduction of beneficial microbes in the uterine microbiota. Moreover, in the past two decades, emergence of antibiotic resistance poses a significant threat to the livestock and human health. This has emerged due to random and frequent use of antibiotics in livestock and commercial poultry farms to combat the microbial infections and also as to improve growth performance. Emerging antibiotic resistance can be controlled only by reducing it's "across the counter" availability and also limiting its prescriptions to necessary cases. The alternate strategy to combat the bacterial infections and antibiotic resistance is treatment with herbal medicines[2], phytotherapeutic treatment[3], phage therapy[4] and use of probiotics[5].

Previous studies suggested that vaginal microbes have a huge impact on uterine microbial population. Application of probiotics has shown to positively impact the host physiology, immune response and digestion. In India, the use of probiotics in feed supplements to improve the gut health is its major application[6-8]. However, there is a paucity of application of probiotics in other areas such as reproductive health in animals. The isolation of LAB from the reproductive tract of indigenous cows and cross breeds maintained in the local tracts and their subsequent use in the treatment of post partum bacterial infections were reported previously[9-14].

In humans, vaginal ecosystem is dynamic and is dominated by LAB that plays a fundamental protective role against invading pathogens[15]. In cases of vaginal infection in women, studies have shown that the traditional use of yogurt and probiotics proved to be cheap and effective treatment. Previous studies have shown that *Lactobacilli* were a normal

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constituent of the vaginal microbiome of healthy cows[16]. Bovine vagina and teat canal of adult heifers, oral cavity and rectum of young healthy calves are good ecosystem for isolation of LAB[17]. Researchers could isolate and identify *Lactobacillus* and *Enterococcus* spp. exhibiting probiotic potential from equine vagina[18]. The influence of the diverse vaginal microbes present in dairy cows on the reproductive health has been previously reported[19-21]. The spectrum of LAB among the microbial population present in the vagina of indigenous breeds and cross bred cows during different stages of estrous cycle and pregnancy has not been studied so far. Hence, this work was designed to study the abundance of LAB in the bovine vagina of the Indian dairy cows based on the 16S rDNA sequencing method. Later the isolated LAB can be characterized as probiotics and used as an alternate strategy to improve post partum disease conditions in cows.

Materials and Methods

Isolation and identification of LAB bacteria

Vaginal swabs collected from 110 apparently healthy cows, including heifers and cows were used as source material for isolation of LAB. Records of these animals showed that they were free from endometritis during previous calving and were not treated with antibiotics for any other infection during the last one year. These samples were collected from the dairy cows maintained at Tamil Nadu Veterinary and Animal Sciences University (TANUVAS) research farm and Private Dairy Farms in Chennai, Tamil Nadu, India. This work was carried out following the Institutional Animal Ethical committee guidelines. 190/CPCSEA

For collection of bovine vaginal microbes, initially the vulvar region was cleaned with 5% povidone-iodine and sterile water. Sterile swab was rolled against the walls of the bovine vagina after using a sterile speculum to reach the posterior part. These swabs were enriched in deMan-Ragosa-Sharpe (MRS), (Cat.No.M369, HiMedia) broth for 24 h. The enriched swabs were spread on MRS agar (Cat.No.M641, HiMedia) plates and incubated at 37°C for 1-2 days. Typical morphological characters of LAB on MRS agar were used for further subculture to obtain pure colonies of LAB after 24 h.

Phenotypic identification

Taxonomic identification of the microorganisms was done by phenotypic and biochemical assays (Colony morphology, catalase activity, nitrate reduction and indole production). Further biochemical characterization of isolates was performed using Anaero23 test kit (Cat No. 10003366 PLIVA LACHEMA Diagnostics, Czechoslovakia) following the manufacturers protocol. Bergey's Manual of Determinative Bacteriology was used as a guide for the identification of LAB[22]. Presumptive LAB colonies were identified by colony morphology, Gram-staining, catalase activity and biochemical tests.

Genotypic identification

About 2 mL of the biochemically confirmed fresh cultures were used for DNA extraction. Initially the pellet was treated with 20 mg of Lyzozyme and the subsequent steps were performed as per the manufacturer's instructions (Qiagen DNA Kit, Cat No. 51304). The isolated DNA was resuspended in 20 µL of sterile nuclease free water. *Lactobacillus* genus specific primer used were LAB F (5' CTC AAA ACT AAA CAA AGT TTC 3') and a reverse primer LAB R (5' CTT GTA CAC ACC GCC CGT TCA 3') using previously reported thermal cycling programme[23]. Similarly the 16S rDNA primers used were 11f 5'-AGAGTTTGAT(C/T)(A/C)TGGCTCAG-3' and 1492r 5'-TACCTTGTTACGACTT-3' using thermal cycling programme[24]. Genus level identification and 16S ribosomal gene amplification of presumptive LAB was done and the anticipated PCR amplicon size was 200 base pair (bp) and 1500 bp, respectively. Reaction products were electrophoresed in 1% and 2% agarose gels (for 16S rDNA and genus level PCR, respectively) and stained with ethidium bromide.

The PCR products of 24 representative strains from bovine vagina were purified using the BioBasic Gel purification Kit (Cat No. BS363, Biobasic, Canada) and the gel purified PCR products were sequenced using Big dye terminator cycle sequencing kit in an automated sequencer (ABI Prism 3100, Genetic Analyser, Applied Biosystems, USA) available at the Department of Animal Biotechnology, Madras Veterinary College, Chennai, India.

Basic Local Alignment Search Tool (BLAST) program provided in the National Center of Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/ Blast.cgi) was used to find sequence identity of the isolates with biologically similar sequences. Multiple sequence alignments and generating the phylogenetic tree were done using MEGA 6 software tool[24]. The genetically identified strains were preserved at -70°C in MRS broth with 10% (v/v) glycerol for further characterization.

Results

Isolation and identification of LAB bacteria

Twenty four LAB that were retrieved from the vagina of 110 healthy indigenous and cross bred cows are listed in Table 1. The table also shows the stages of estrum/pregnancy, age and breed of the animals from which the samples were collected.

Phenotypic identification

The phenotypic characteristics showed presumptive LAB colonies as pin point, white, smooth colonies on MRS Agar. On Gram staining, *Lactobacilli* appeared as long, slender, non-spore forming, Gram-positive rods; *Enterococci* appeared as Gram-positive diplococci tetrads; *Weissella* spp., appeared as Gram-positive pleomorphic curved rods and *Bacilli* appeared as Gram-positive rods. Fermentation of carbohydrates for biochemical characterization was determined using commercially available biochemical test kits. The Gram-positive rods KF43 and 1002 were catalase and oxidase positive while all other presumptive LAB strains were negative for catalase and oxidase tests. All the isolates gave positive reactions with sugars like glucose, maltose, fructose, galactose, lactose, sucrose and negative reactions with melizitose, urease, nitrate and esculin. Isolates KF14, R8, S14 and S24 showed negative reaction to xylose. Isolates 181, 854, 411 and 141 showed negative reactions to maltose.

Genotypic identification

For genotypic identification, biochemically characterized LAB from bovine vagina was subjected to *Lactobacillus* genus -specific and 16S rDNA PCRs. The presumptive LAB showed 200 bp PCR amplicon with genus specific primers (Fig. 1). Further confirmation of the LAB using species specific 16S

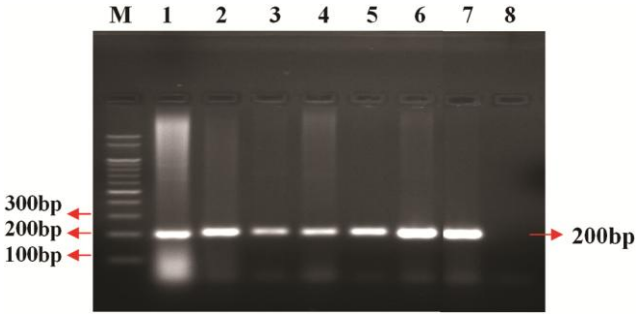

Fig. 1 — *Lactobacillus* Genus specific PCR showing 200 bp products on agarose gel; M – 100 bp Ladder, LAB strains specific PCR amplified products from UCBSS1- Lane 1, MMR181- Lane 2, MMR 854 – Lane 3, UCBSS2- Lane 4, URFS2- Lane 5, LRSDS1- Lane 6, LRS955 - Lane 7 and No Template Control - Lane 8

Table 1 — List of the LAB isolates from vagina of healthy cows housed at different farms

*X- cross breed; ND- Non Descript; URF-University Research Farm, Madhavaram; PGRIAS – Post Graduate Research Institute in Animal Sciences Kattupakkam; MMR- Melmaruvathur Adhiparasakthi Trust Farm; MVC- Madras Veterinary College.

S No.	Breed	Sample ID	Place of Collection*	Age (years)	No. of calving	Stage of estrous cycle/ pregnancy	LAB isolated with ID No.
1	Kangeyam	KF43	URF	8	6	Estrum	*B. coagulans* URF KF43
2		KF14		7	2	Diestrum	*L. agilis* URFKF14
3	Rathi	R3	PGRIAS	9	2	Estrum	*L. plantarum* UCBSS1
4		R2		9	4	Diestrum	*L. plantarum* UCBSS2
5		R8	URF	9	3	Estrum	*L. agilis* URF R8
6		R7	PGRIAS	9	2	Early pregnancy	*L. plantarum* UCBSS5
7	Friesian X	181	MMR	4.5	3	Diestrum	*L. fermentum* MMR 181
8		854		5.5	5	Diestrum	*L. fermentum* MMR 854
9		411		4	3	Proestrus	*L. fermentum* MMR 411
10		141		3.5	3	Diestrum	*L. fermentum* MMR 141
11	Jersey	1002	PGRIAS	6.5	5	Full term pregnant	*Bacillus cereus* LRS 1002
12		955		6	4	Proestrum	*Weisella cibaria* LRS955
13		987		4.5	3	Estrum	*Weisella cibaria* LRS987
14		DS1		3.5	2	Diestrum	*Enterococcus faecium* LRS DS1
15		P9	URF	5	4	Full term pregnant	*Enterococcus faecium* UCB P9
16	Jersey X	843	MMR	4.5	3	Full term pregnant	*Enterococcus faecium* MMR S9
17		954	PGRIAS	4	2	Diestrum	*Enterococcus asini* LRS R8
18	Sahiwal	LRS6		5	3	Estrum	*L. pentosus* UCBSS6
19		S14	URF	9	4	Diestrum	*L. agilis* URFS14
20		S24		8	2	Diestrum	*L. agilis* URF S24
21		S23		8	2	Diestrum	*L. mucosae* URF S 23
22	Gir	G6	PGRIAS	9	5	Late Estrum	*L. plantarum* UCBSS3
23		G14		9	2	Diestrum	*L. plantarum* UCBSS4
24	ND	152101	MVC	3	8	Estrum	*L. mucosae* MVCS8

rDNA PCR revealed an amplicon of 1500 bp (Fig. 2). Twenty four 16S rDNA PCR amplicons were sequenced, the sequenced regions were compared with similar sequences in the GenBank (NCBI) database and sequence similarity above 98% were considered for *in silico* analysis. These sequences were used for the construction of phylogenetic tree using MEGA 6 analysis tool. Twenty four LAB isolates belonging to the genus *Lactobacillus*, *Bacillus*, *Enterococcus* and *Weisella* were speciated based on their grouping with respective reference sequences available in the GenBank, on phylogenetic analysis (Fig. 3).

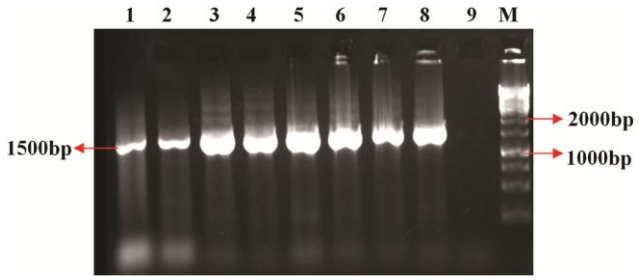

Fig. 2 — Lab 16S rDNA gene PCR showing 1500 bp product on 1% agarose gel; M–1 kb Ladder, PCR products amplified from LAB strains UCBSS1- Lane 1, MMR181-Lane 2, MMR 854- Lane 3, UCBSS2- Lane 4, URFS2- Lane 5, LRSDS1- Lane 6, LRS955 - Lane 7 UCB SS3 – Lane 8 and Negative Control - Lane 9

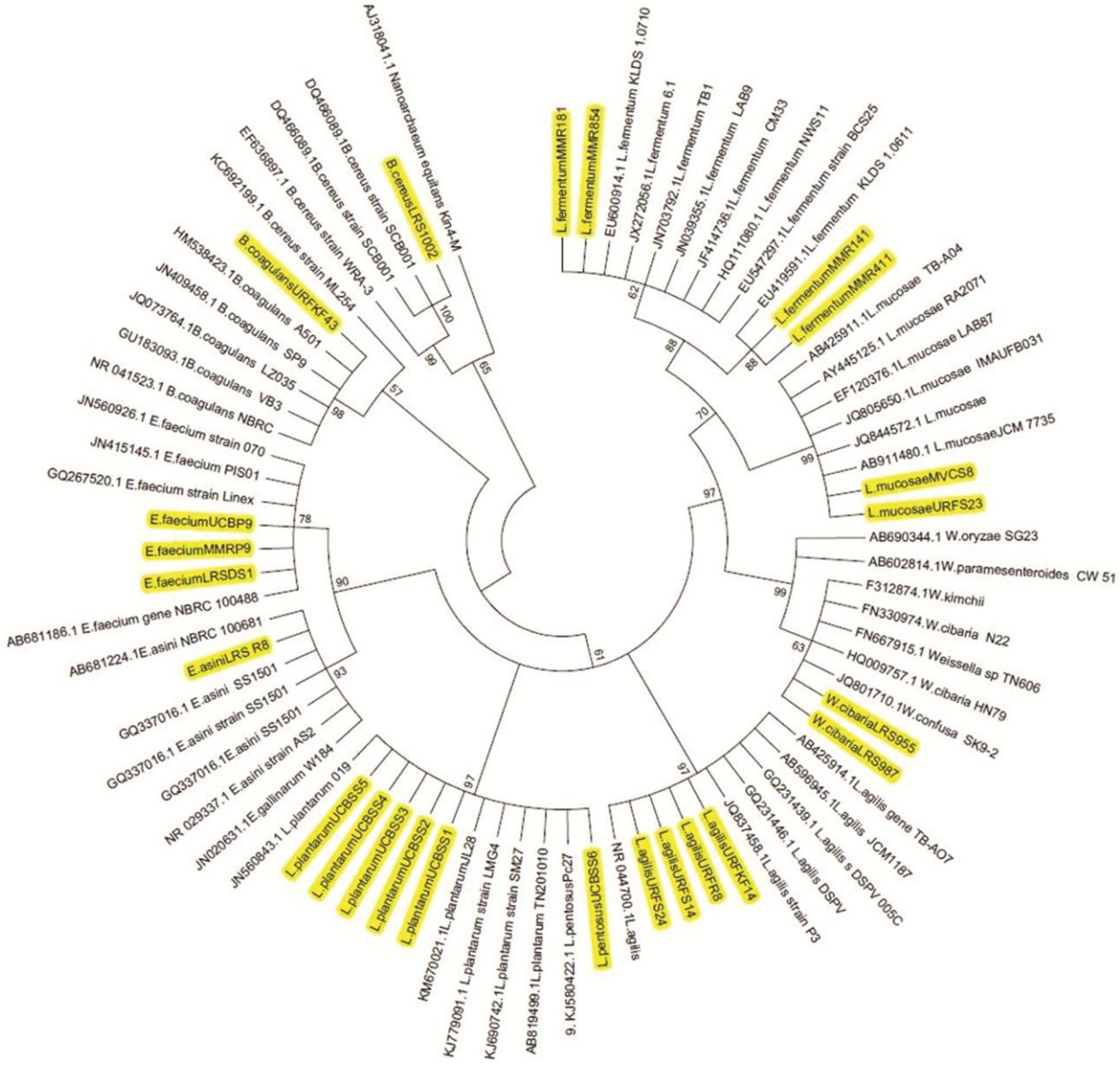

Fig. 3 — Maximum likelihood phylogenetic tree showing the positions of the 24 bovine vaginal LAB isolates related to the 16S rDNA gene sequences in the GenBank. Boot strap values are shown in 1000 replicates. Isolate names in color indicate LAB isolated in this study

Discussion

In India, use of LAB on non-gut-related applications is very limited. Considering the pivotal role of vaginal microbiome in human vaginal health, extensive research has been done on the application of LAB for bacterial vaginosis[25,26]. Since application of LAB as an alternate to improve bovine reproductive health has been reported to be effective in dairy cows[27,28], it is important to isolate LAB from their respective target tissue. It is highly debatable whether the LAB characterized as probiotic organisms need to be isolated from the target tissues of specific animal population on which it is intended to be used. Although some reports have isolated and characterized the LAB organisms from their respective target tissues[29-31,17], the available commercial probiotic products in the Indian market contain probiotic organisms obtained from other countries. A well-known example is 'Yakult' which is a fermented probiotic dairy drink made from *L. caesei* strain Shirota of Japan. Even commercial products for the human vaginal application marketed by Tablets India Pharmaceuticals includes patented strains of *L. rhamnosus GR-1* and *L. reuteri RC-14* isolated from urogenital tract of women of non-Indian origin[32]. However, the advantage of using local isolates from Indian breeds of animals is that it would probably facilitate their colonization more easily. Isolation from Indian breeds would also give us information on the spectrum of LAB present as commensals in our animals. A total of 24 LAB were isolated from bovine vagina of which *L. plantarum*, *L. fermentum* and *L. agilis* accounted for more than 55% of the entire isolates. Based on previous studies, it is evident that the majority of LAB isolated from the bovine vaginal microbiota were *L. fermentum*, *L. plantarum*[14,17,33-34] *L. rhamnosus*, *L. johnsonii* and *L. mucosae*[12,35]. The samples analysed in this study indicates that the major LAB colonizing our animals are not the same as reported elsewhere. Although *L. fermentum* was commonly found in Indian cows, *L. johnsonii* and *L. rhamnosus*, could not be isolated. Breed, stage of oestrous cycle/pregnancy and number of calvings had no apparent correlation with the LAB strains isolated.

Preliminary studies showed that individual anatomical and physiological variation had more influence on the general vaginal microbiota in cows and heifers than age or pregnancy status[35]. Although some reports suggest that the presence of genus *Lactobacillus* was relatively low during the estrous period in Holstein-Friesian dairy heifers[36]. Otero and colleagues related the level of *Lactobacillus* in the bovine vaginal microbiome to the progesterone concentration[15]. Although reports show limited population of *Lactobacillus* spp. in the bovine reproductive tract[37,38], research on the use of this species as probiotics to improve the bovine reproductive health has been effective in terms of reduction in bacterial infection[39,40]. The genotypic methods used in this study enabled clear speciation of the isolated *Lactobacilli*[41]. This further highlights the power and specificity of molecular methods in speciating bacteria[41,42-44]. Earlier studies has proved that the application of culture-independent 16S rDNA sequencing technology is very effective in determining the vaginal bacterial diversity in livestock and to understand the role of vaginal microbiome in determining the uterine health and reproductive efficiency[44-46]. Inspite of the results obtained in this study with respect to restricted geographical location of the indegenous and cross bred cows, the knowledge of the spectrum of LAB from bovine vagina will help the researchers to develop LAB-based uterine probiotics for the improvement of bovine uterine health.

Conclusion

In conclusion, with the use of 16S rDNA technique a varied spectrum of Lactic acid bacteria was identified in the bovine vagina of indigenous and cross bred cows of India. The beneficial microbes such as *Lactobacillus*, *Enterococcus* and *Weissella* can be further characterized and formulated as an indigenous probiotic product that can be used to combat bovine uterine infections such as endometritis.

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Conflict of Interest

The authors declare no conflict of interest. The isolates obtained in this study was further characterized for its probiotic potential, the best performing probiotic isolates were further formulated as a product, field tested and is considered for commercialization.

Author's Contributions

VSV- Involved in sample collection, laboratory isolation and identification of the samples and drafting the manuscript. GDR contributed to the overall design and critical analysis of the results in this study. SR and SB supported in sample collection including the assessment of the stage of estrus of all the dairy cows. All the authors have read and agreed to communicate for the publication.

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