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Observations on *Lactobacillus* Spp. In the Genital Tract of Buffalo-Cows with Emphasis on its *In Vitro* Probiotic Activity

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Abstract: *Lactobacilli* are among the predominant microorganisms in the vaginal tract of some homoeothermic animals. The current investigation was designed to throw line on *Lactobacillus* in the genital tract of buffaloes-cows. Vaginal and blood samples were collected from animals reared at Lower Egypt for a period of 2 years. Vaginal swabs were collected from buffalo-cows either showed normal ovarian activity or suffering from ovarian inactivity. Swabs were collected from each animal in either Tryptic soy broth for the routine bacteriological examination or De Man, Rogaso, Sharpe, MRS) for isolation of *Lactobacillus. In vitro* sensitivity and probiotic tests against the most predominant isolates isolated from buffalo cows, *Y. enterocolitic, C. diversus*, *E. coli*, *Micrococcus* spp., *E. Faecalis, S. aureus* and *Bacillus* spp. were carried out). Results showed that *Lactobacillus* was isolated from the vagina of normal cyclic buffalo-cows with a high incidence, 90.91%) as compared to in animals suffering from ovarian inactivity, 81.82%). Vaginal pH and the recovered isolates were recorded in relation to ovarian activity. Ciprofloxacin and ofloxacin are the most effective antibiotics followed by to tobramycin, gentamicin and oxytetracycline. The resistant rate of isolates to some antibiotics was recorded. *In vitro* use of *Lactobacillus* isolated from the genital tract of normal buffalo-cows as probiotic against the most predominant isolates from buffalo cows suffering from ovarian inactivity revealed that *L. acidophilus* is the most effective strain followed by *L. casei rhamnosus.* It could be concluded that *Lactobacillus* is one of the normal bacterial floras of the vagina of buffalo-cows, *L. acidophilus* and *L. casei rhamnosus* were the most predominant isolates and showed high probiotic effect against the most predominant bacteria isolated from cases suffering from ovarian inactivity.

Key words: Buffaloes · genital tract · *Lactobacillus* · antibiotics · probiotics

INTRODUCTION

Nowadays buffaloes represent an integral part of the agricultural economy in many developing countries. However, this species is mainly reared in small holder farms and suffer from a lot of stressful conditions such as malnutrition, bad hygiene, parasitic infestation and pollution [1]. Also, this species tends to have relatively slow rate of reproduction and more reproductive problems such as late maturity, seasonal breeding, silent heat, inactive ovaries, uterine infection and long calving interval [1-4].

As any system in the body, the genital tract has its own normal bacterial floras which play an important role in its protection against infection upset and enhance the host ability to compete pathogens [5-7]. However, the presences of some bacterial species in the uterus disturb its function and delay the post partum uterine involution [3-8]. Moreover, this uterine bacterial infection or bacterial products perturb ovarian function [9-11]. In the sametime, it was proved that some of bacterial strains isolated from the genital tract such as *Lactobacillus* spp. have probiotic activity, which contributes to health restoration and maintenance [12].

Owing to the obvious lack of literature concerning the microbiological aspect of reproductive system in buffaloes, the current investigation was designed to throw line on beneficial bacteria, *Lactobacillus*) in the genital tract of buffaloes.

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MATERIALS AND METHODS

The current work was carried out on female buffaloes reared at Lower Egypt during the period from July 2004 to June 2006 as a part of the National Research Center Project No-7120106.

Animals: A total number of 258 mature buffalo- cows reared in small holder farms was used for collecting samples of blood and vaginal swabs.

Experimental design: For each animal, case history, the general health condition and owner complain were recorded. Animals were subjected to vaginal and rectal examinations as outlined by [13]. Blood samples were collected from jugular vein of examined buffaloes and serum samples were separated for assaying progesterone levels by ELISA micro- well method [14]. Progesterone level, (Data were not shown in this paper) was used to confirm the reproductive status to avoid misdiagnosis [15].

Vaginal swabs were collected from buffalo-cows either showed normal ovarian activity or suffering from ovarian inactivity. Vulva was thoroughly dry cleaned using tissue paper and swabs were collected under aseptic conditions from the anterior vagina of all groups using the rectovaginal technique [13]. Two swabs were collected from each animal and either immersed in Tryptic soy broth for the routine bacteriological examination, details were given by [7] or De Man, Rogaso, Sharpe, MRS) media for examination of *Lactobacillus sp.* The vaginal pH was measured at the dorsal commeasures of the posterior vagina using narrow range pH paper, Wattman, BDH, UK). Vaginal swabs taken in M.R.S broth were incubated at 37°C and 5% CO_2 tension for 24 hrs, then subcultures were streaked from the enriched broth onto M.R.S. agar plates using layer plate method for anaerobic incubation [16]. Plates were incubated at 37°C for 48 hrs [17]. The suspected colonies were picked separately, inoculated into tubes containing M.R.S. broth and incubated at 37°C for 24 hrs under 5% CO_2 tension. A drop from each broth sample was examined microscopically, then subcultued in M.R.S. broth for subsequent biochemical identification [18]. Isolates were identified morphologically, culturally and biochemically [19-20].

***In vitro* sensitivity and probiotic tests:** *In vitro* sensitivity and probiotic tests against the most predominant isolates isolated from buffalo cows, *Y.enterocolitic, C.diversus*, *E. coli*, *Micrococcus* spp., *E. faecalis, S. aureus* and *Bacillus spp.* were carried out.

***Antibiotic sensitivity* assay:** Antibiotic sensitivity test of isolates was applied using disc diffusion technique [21] and sub-culture of the isolates. Results, (Table 1) were interpreted according to the manufacture company [16].

***Lactobacillus* as probiotic, Well diffusion assay:** Selected cultures from the present bacterial isolates were plated on Mueller Hinton agar plates and wells were drilled out using Pasture pipettes, 50 µl aliquots of cell free cultures supernatant in fresh M.R.S. broth were suspended in the agar wells. Plates were incubated for 48 to 72 hrs under microaerophillic conditions at 37°C and the diameters of inhibition zones around wells were measured. Results were expressed as a mean diameter and standard error [23].

Table 1: Interpretation of the results according to [16-22]

			Diameter of zone of inhibition, mm)		
Antibiotic discs	Symbol	Conc., µg/ disc)	Sensitive	Intermediate	Resistant
Augmentin					
When testing Staphylococcus	AMC	30	20 or more	-	19 or less
When testing other bacteria			18 or more	14-17	13 or less
Cefotaxime	CTX	30	23 or more	15-22	14 or less
Ciprofloxacin	CIP	5	21 or more	16-20	15 or less
Erythromycin	E	15	23 or more	14-22	13 or less
Gentamicin	GM	10	15 or more	13-14	12 or less
Ofloxacin	OFX	5	15 or more	13-14	12 or less
Oxytetracycline	Te	30	19 or more	15-18	14 or less
Tobramycin	TOB	10	15 or more	13-14	12 or less

Statistical analysis: Statistical analysis was carried out using "Student t" test and Analysis of Variance as outlined by [24]. Statistical analysis for the inhibition zones was performed with Fisher's exact test, Graph Pad Software).

RESULTS

Isolation and identification of *Lactobacillus* species: *Lactobacillus* was isolated from the vagina of normal cyclic buffalo-cows with an incidence of 90.91%. The recovered isolates were *L. casei rhamnosus*, 45.45%) at a vaginal pH of 6.65, *L. acidophlus*, 36.36%) at a pH of 6.06 and *L. helveticus*, 9.09%) at a pH of 6.50. Low incidence of isolation of *Lactobacillus* was recorded in animals suffering from ovarian inactivity, 81.82%) with respective incidence and vaginal pH of 27.27% and 6.75 for *L. casei rhamnosus,* 18.18% and 6.25 for *L. acidophilus,* 9.09% and 7.00 for *L. amylophilus,* 9.09% and 6.50 for *L. sharpeae* and *L.yamanashiensis* and 9.09% and 7.50 for *L. delbruechii delbruechii*, Table 2).

Antibiotic sensitivity test against the predominant isolates: Antibiotic sensitivity tests against some broad spectrum antibiotics revealed that ciprofloxacin and ofloxacin are the most effective antibiotics with sensitivity of 100%, followed by tobramycin, gentamicin and oxytetracycline, Table 3). On the other hand, the resistant rate of *Y. enterocolitica, C. diversus*, *E. coli, E. faecalis* and *S. aureus* isolates to erythromycin varied between 100- 20%. While, it varied between 80-50% for *E. coli, E. faecalis* and *S. auerus* to oxytetracycline, Table 3).

***In vitro* use of *Lactobacillus* as a probiotic:** *In vitro* use of *Lactobacillus* isolated from the genital tract of normal buffalo-cows as probiotic against the most predominant

Table 2: Isolation of *Lactobacillus* strains from the genital tract of normal cyclic buffalo-cows

	Normal cyclic buffalo-cows (11)			Buffalo-cows suffering from ovarian inactivity (11)		
Lactobacillus strains	No	Incidence, %	Vaginal pH	No	Incidence, %	Vaginal pH
L. acidophilus	4	36.36	6.06	2	18.18	6.25
L.casei rhamnosus	5	45.45	6.65	3	27.27	6.75
L.helveticus	1	9.09	6.50	-	--	--
L.amylophilus	-	--	--	1	9.09	7.00
L.sharpeae	-	--	--	1	9.09	6.50
L.yamanashiensis	-	--	--	1	9.09	6.50
L.delbruechii delbruechii	-	--	--	1	9.09	7.50
Total	10	90.91	6.58	9	81.82	6.75

No = number of positive samples. % = was calculated according to number of examined samples [11]

Table 3: Antibiotic sensitivity for the most predominant isolates obtained from the genital tract of buffalo-cows

Bacterial isolates	CIP_5	CTX_{30}	GM_{10}	E_{15}	Te_{30}	AMC_{30}	TOB_{10}	OFX_5
Y.enterocolitica(10)*	100%S	100%R	100%S	50% I 50% R	50% S 50% I	50% I 50% R	100%S	100%S
C.diversus (10)*	100%S	100%R	100%S	40% S 40% I 20% R	100%S	20% S 20% I 60% R	100%S	100%S
E. coli (10)*	100%S	10% S 50% I 40% R	60% S 40% I	100%R	40% I 60% R	20% S 10% I 70% R	100%S	100%S
Micrococcus spp.(10)*	100%S	100%R	100%I	100% I	100%S	100%I	100%S	100%S
E. faecalis (10)*	100%S	100%S	50% S 50% I	100%R	50% S 50% I	100%S	100%S	100%S
S. aureus(10)*	100%S	90%S 10%I	80%S 10%I 10%R	30%I 70%R	20%I 80%R	20%I 80%R	90%I 10%R	100%S
Bacillus spp. (10)*	100%S	90%S 10%I	70%S 30%I	90%S 10%I	80%S 20%I	80%I 20%R	80%I 20%R	100%S

*= Figures between brackets indicate number of isolates.

CIP5: Ciprofloxacin, CTX_{30}: Cefotaxime, GM_{10}: Gentamicin, E_{15}: Erythromycin, Te_{30}: Oxytetracycline, AMC_{30} Amoxicillin 20mcg + Clavulanic acid 10mcg, Augmentin), TOB_{10}: Tobramycin, OFX_5: Ofloxacin.

Table 4: *In vitro* use of *Lactobacillus* strains isolated from the genital tract of buffalo-cows as probiotic against the predominant isolates

Lactobacillus stains	Average zone of inhibition	*E.coli*, normal cyclic), (10)	*E.coli*, ovarian inactivity) (10)	Y.entero Colitica (10)	C.diversus (10)	E. faecalis (10)	S.auerus (10)	Bacillus spp. (10)
L. acidophilus	16mm	100.00%S	100.00%S	100.00%S	100.00%S	100.00%S	100.00%S	100.00%S
L. casei rhamnosus	14mm	80.00%S 20.00%R	80.00%S 20.00%R	70.00% S 30.00%R	100.00%S	70.00% S 30.00%R	80.00% S 20.00%R	80.00%S 20.00%R
L. helveticus	8mm	80.00%S 20.00%R	60.00%S 40.00%R	100.00%S	70.00% S 30.00%R	70.00% S 30.00%R	90.00% S 10.00%R	70.00% S 30.00%R
L. amylophilus	10mm	100.00%S	100.00%S	70.00% S 30.00%R	80.00%S 20.00%R	50.00% S 50.00%R	60.00% S 40.00%R	50.00% S 50.00%R
L. sharpeae	6mm	80.00%S 20.00%S	80.00%S 20.00%R	70.00% S 30.00%R	30.00% S 70.00%R	30.00% S 70.00%R	30.00% S 70.00%R	30.00% S 70.00%R
L. yamanashiensis	7mm	100.00%S	60.00%S 40.00%R	70.00% S 30.00%R	70.00% S 30.00%R	50.00% S 50.00%R	40.00% S 60.00%R	50.00% S 50.00%R
L. delbruechii delbruechii	6mm	100.00%S	80.00%S 20.00%R	70.00% S 30.00%R	70.00% S 30.00%R	30.00% S 70.00%R	20.00% S 80.00%R	30.00% S 70.00%R

S= sensitive, R= resistance

isolates from buffalo cows suffering from ovarian inactivity revealed that *L. acidophilus* is the most effective strain as all tested isolates showed 100% sensitivity against it followed by *L. casei rhamnosus as* shown in Table 4.

DISCUSSION

Every system in the body has its own normal bacterial floras which play an important role in its protection against infection upset and enhance the host ability to compete pathogens [5-6]. Pathogenic and nonpathogenic bacteria can enter the uterus, especially during the time of parturition and oestrus. Although, bacteria can multiply rapidly in the presence of such favourable conditions, the normal uterine defence mechanisms can counteract these bacterial invasions [25-26]. Uterine bacterial infections are important because they disrupt not only the function of the uterus, but also the ovary and the overarching higher control centres in the hypothalamus and pituitary [27].

Lactobacilli are among the predominant microorganisms in the vaginal tract of some homoeothermic animals. These microorganisms have probiotic actions to maintain the ecological equilibrium of the genital tract by protecting against pathogenic microorganisms. Nowadays there are many trends to use probiotics as an alternative to antibiotics and drugs that may have severe side effects [28].

In the current work, *Lactobacillus* was isolated in high incidence from the vagina of normal cyclic buffalo-cows as compared to animals suffering from ovarian inactivity. There is no available data on this topic in buffaloes, however, this species was previously isolated from vagina of saws [29] and does [30]. The relation between vaginal pH and the recovered strains is intriguing in light of the cyclical change in pH of the female lower genital tract [31]. However, *Lactobacilli,* function as endogenous microbicides through the production of lactic acid, which acidifies the vagina and hydrogen peroxide which reacts with myeloperoxidase to form reactive molecules toxic to human immunodeficiency virus, HIV) and other pathogens [32]. A disruption of this balance could be a factor that leads to infections, while the presence of *Lactobacilli* in the vagina seems to be essential to prevent overgrowth of other bacteria [33].

Antibiotics are important remedies in modern farm animal production. The use of these chemical agents should be based on an accurate diagnosis since there are increasing incidences of bacterial resistance to antibiotics in humans. This phenomenon was attributed to the use of anti-microbial drugs in food-producing animals. Also, there is a concern about possible residues in animal products [34]. Results of antibiotic sensitivity for the present predominant isolates against different broad spectrum antibiotics revealed that ciprofloxacin and ofloxacin were the most effective antibiotics against all tested isolates followed by tobramycin, gentamicin and oxytetracycline. Sheldon and Noakes [35] Concluded that floxidin is effective for the treatment of cases of retained placenta. It was found that *C. Pyogenes, E. coli, S. pyogenes, S. zymogenes, S. aureus* and *P. aeruginosa* isolated from cases of pyometras were sensitive to chloramphenicol, kanamycin, erythromycin, oxytetracyclin, streptomycin, ampicillin and amoxicillin [36]. Kang and Park [37] Recorded that all isolates were susceptible to gentamicin. The *E. coli* strains were highly susceptible to gentamicin followed by enrofloxacin, norfloxacin and nalidixic acid [38]. Using ten different antibiotics, [39] revealed that amikacin followed by norfloxacin, gentamicin, Kanamycin and chloramphenicol are the most effective antibiotics against majority of isolates while, penicillin and ampicillin are the least effective. *In vitro* drug sensitivity results showed that gentamicin was the most effective drug against the bacterial isolates while, erythromycin, penicillin ampicillin were totally ineffective [40].

In an attempt to develop a non chemotherapeutic means to restore and maintain a healthy urogenital tract, probiotic therapy using *Lactobacilli* has been considered and there is evidence to indicate that certain strains can be effective when inserted directly into the vagina or when ascending from the rectum after oral ingestion [41]. *Lactobacillus*-containing probiotic products have been proposed for the treatment of vaginal infection [42]. On the most, probiotic *L. rhamnosus* and *L. fermentum* have been shown in open studies to colonize the vagina following oral intake [43]. The current results revealed that *L. acidophlus* is the most effective strain as it showed 100% sensitivity against all tested isolates followed by *L. casei rhamnosus.* Among *E. coli* tested, 100% isolates were sensitive to *L. acidophilus* and *L. amylophilus*. 80% of *E. coli* isolates obtained from normal buffaloe-cows were sensitive to *L. casei rhamnosus, L. helveticus* and *L. sharpeae*. While, 80.00 % *E. coli* isolates obtained from buffalo-cows suffering from ovarian inactivity were sensitive to *L. casei rhamnosus, L. sharpeae* and *L. delbruechii delbruechii* and 60.00% were sensitive to *L. helveticus* and *L. yamanashiensis.*There is *in vitro* evidence that *Lactobacilli* can inhibit the growth and attachment of uropathogenic *E. coli* to uroepithelial cells, this has translated into reduced infection rates in animals and humans [12]. Also, *S. aureus* was sensitive to *L. acidophilus*, *L. Helveticus* and *L. casei rhamnosus*, All bacteriocin – producing *Lactobacilli* strongly inhibited the growth of the three clinical *S. aureus* isolates tested as indicators, two methicillin resistant and one methecillin susceptible) [44]. In mixed cultures of *Lactobacilli* and *S. aureus* under the optimal conditions of hydrogen peroxide production, the pathogen growth was inhibited [42]. The inhibition ability of selected strains in the form of cells free spent broth tested by [45] against common pathogens revealed that *Lactobacillus* produced maximum zone of inhibition against *S. typhi*, the strain NCDC 17 was consistently good in inhibiting all tested pathogens [46]. In a study on antibacterial properties of cell free filtrate from *L. casei* strains showed antibacterial activity against both Gram negative and Gram positive bacteria. *L. salivarius salivarius* CRL 1328 inhibited *E. faecalis*, depending on the initial inoculum of the pathogen [47]. Pretreatment with probiotics attenuates the effects of *C. roentium* infection in mice [48]. To date, the main anti- infective properties described for *Lactobacilli* are their ability to (i) adhere to surfaces and inhibit the adhesion of pathogens (ii) inhibit the growth of pathogens (iii) deplete nutrients otherwise available to pathogens and (iv) modulate the host immune response and microenvironment, such that risk of infection is reduced [49-51].

It could be concluded that *Lactobacillus* is one of the normal bacterial floras of the vagina of buffalo-cows, *L. acidophilus* and *L. casei rhamnosus* were the most predominant isolates and showed high probiotic effect against the most predominant bacteria isolated from cases suffering from ovarian inactivity. However, further urgent studies are needed to clarify the effect of *Lactobacillus* treatment on genital tract infection in buffalo-cows, especially those suffering from ovarian inactivity.

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