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

1S.I. Abd El-Moez, 2W.M. Ahmed, 3J.A. El-Jakee and 4F.R. El-Seedy

1Department of Microbiology and Immunology, National Research Center, Cairo Egypt
2Department of Animal Reproduction & AI, National Research Center, Cairo Egypt
3Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt
4Department of Microbiology, Faculty of Veterinary Medicine, Beni Suef University, Beni Suef, Egypt

Abstract: Lactobacilli are among the predominant microorganisms in the vaginal tract of some homoeothermic animals. The current investigation was designed to throw light 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, Rogosa, Sharpe, MRS) for isolation of Lactobacillus. In vitro sensitivity and probiotic tests against the most predominant isolates isolated from buffalo cows, Y. enterocolitica, 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 buffaloes-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 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 buffaloes-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 flora 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 flora 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 light on beneficial bacteria, Lactobacillus in the genital tract of buffaloes.

Corresponding Author: Dr. Sherein I. Abd El-Moez, Department of Microbiology and Immunology, National Research Center, Cairo, Egypt
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 complaint 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, Rogas, Sharpe, MRS) media for examination of Lactobacillus sp. The vaginal pH was measured at the dorsal commences of the posterior vagina using narrow range pH paper, Wattman, BDH, UK). Vaginal swabs taken in MRS broth were incubated at 37°C and 5% CO₂ 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% CO₂ tension. A drop from each broth sample was examined microscopically, then subcultured 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.enterococolitic, C.diversus, E. coli, Micrococus 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 Pasteur 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 microaerophile 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]

<table>
<thead>
<tr>
<th>Antibiotic discs</th>
<th>Symbol</th>
<th>Conc., μg/disc</th>
<th>Sensitive</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Augmentin</td>
<td>AMC</td>
<td>30</td>
<td>20 or more</td>
<td>-</td>
<td>19 or less</td>
</tr>
<tr>
<td>When testing Staphylococcus</td>
<td>CTX</td>
<td>30</td>
<td>25 or more</td>
<td>15-22</td>
<td>14 or less</td>
</tr>
<tr>
<td>When testing other bacteria</td>
<td>CIP</td>
<td>5</td>
<td>21 or more</td>
<td>16-20</td>
<td>15 or less</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>E</td>
<td>15</td>
<td>23 or more</td>
<td>14-22</td>
<td>13 or less</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>GM</td>
<td>10</td>
<td>15 or more</td>
<td>13-14</td>
<td>12 or less</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>OFX</td>
<td>5</td>
<td>15 or more</td>
<td>13-14</td>
<td>12 or less</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>Te</td>
<td>30</td>
<td>19 or more</td>
<td>15-18</td>
<td>14 or less</td>
</tr>
<tr>
<td>Tetracyclin</td>
<td>TOB</td>
<td>10</td>
<td>15 or more</td>
<td>13-14</td>
<td>12 or less</td>
</tr>
</tbody>
</table>
**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. acidophilus*, 36.36% at a pH of 6.66 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 *Lymanashienis* and 9.09% and 7.50 for *L. delbruechi delbruechi* (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, gentamycin 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. aureus* 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>
<thead>
<tr>
<th>Lactobacillus strains</th>
<th>Normal cyclic buffalo-cows (11)</th>
<th>Buffalo-cows suffering from ovarian inactivity (11)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>Incidence, %</td>
</tr>
<tr>
<td><em>L. acidophilus</em></td>
<td>4</td>
<td>36.36</td>
</tr>
<tr>
<td><em>L. casei rhamnosus</em></td>
<td>5</td>
<td>45.45</td>
</tr>
<tr>
<td><em>L. helveticus</em></td>
<td>1</td>
<td>9.09</td>
</tr>
<tr>
<td><em>L. amylophilus</em></td>
<td>-</td>
<td>--</td>
</tr>
<tr>
<td><em>L. sharpeae</em></td>
<td>-</td>
<td>--</td>
</tr>
<tr>
<td><em>L. delbruechi delbruechi</em></td>
<td>-</td>
<td>--</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10</strong></td>
<td><strong>90.91</strong></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>CIP5</th>
<th>CTX5</th>
<th>GM5</th>
<th>ES5</th>
<th>TE5</th>
<th>AMC5</th>
<th>TOB5</th>
<th>OFX5</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Y. enterocolitica (10)</em></td>
<td>100%S</td>
<td>100%R</td>
<td>100%S</td>
<td>50%I</td>
<td>50%S</td>
<td>50%I</td>
<td>50%R</td>
<td>100%S</td>
</tr>
<tr>
<td><em>C. diversus (10)</em></td>
<td>100%S</td>
<td>100%R</td>
<td>100%S</td>
<td>40%S</td>
<td>50%R</td>
<td>100%S</td>
<td>20%S</td>
<td>100%S</td>
</tr>
<tr>
<td><em>E. coli (10)</em></td>
<td>100%S</td>
<td>100%S</td>
<td>50%S</td>
<td>60%S</td>
<td>40%I</td>
<td>100%R</td>
<td>20%S</td>
<td>100%S</td>
</tr>
<tr>
<td><em>Micrococcus spp (10)</em></td>
<td>100%S</td>
<td>100%R</td>
<td>100%S</td>
<td>100%S</td>
<td>100%S</td>
<td>100%S</td>
<td>100%S</td>
<td>100%S</td>
</tr>
<tr>
<td><em>E. faecalis (10)</em></td>
<td>100%S</td>
<td>100%S</td>
<td>50%S</td>
<td>50%S</td>
<td>50%S</td>
<td>50%S</td>
<td>50%S</td>
<td>100%S</td>
</tr>
<tr>
<td><em>S. aureus (10)</em></td>
<td>100%S</td>
<td>100%S</td>
<td>50%S</td>
<td>50%S</td>
<td>50%S</td>
<td>50%S</td>
<td>50%S</td>
<td>100%S</td>
</tr>
<tr>
<td><em>Bacillus spp. (10)</em></td>
<td>100%S</td>
<td>100%S</td>
<td>100%S</td>
<td>100%S</td>
<td>100%S</td>
<td>100%S</td>
<td>100%S</td>
<td>100%S</td>
</tr>
</tbody>
</table>

*= Figures between brackets indicate number of isolates,
Table 4: In vitro use of Lactobacillus strains isolated from the genital tract of buffalo cows as probiotic against the predominant isolates

<table>
<thead>
<tr>
<th>Lactobacillus strains</th>
<th>Average zone of inhibition (mm)</th>
<th>E. coli, normal inactivity (%)</th>
<th>Y. enterol (10)</th>
<th>C. ileal (10)</th>
<th>C. jejuni (10)</th>
<th>E. coli, resistant (10)</th>
<th>S. aureus (10)</th>
<th>Bacillus spp. (10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. acidophilus</td>
<td>16 mm</td>
<td>100.00%S</td>
<td>100.00%S</td>
<td>100.00%S</td>
<td>100.00%S</td>
<td>100.00%S</td>
<td>100.00%S</td>
<td>100.00%S</td>
</tr>
<tr>
<td>L. casei rhamnosus</td>
<td>14 mm</td>
<td>80.00%K</td>
<td>80.00%K</td>
<td>70.00%K</td>
<td>70.00%K</td>
<td>100.00%K</td>
<td>70.00%K</td>
<td>80.00%K</td>
</tr>
<tr>
<td>L. helveticus</td>
<td>0 mm</td>
<td>80.00%K</td>
<td>60.00%K</td>
<td>100.00%S</td>
<td>70.00%K</td>
<td>70.00%K</td>
<td>90.00%K</td>
<td>70.00%K</td>
</tr>
<tr>
<td>L. amylophilus</td>
<td>10 mm</td>
<td>100.00%K</td>
<td>40.00%R</td>
<td>70.00%S</td>
<td>80.00%K</td>
<td>50.00%R</td>
<td>60.00%K</td>
<td>50.00%K</td>
</tr>
<tr>
<td>L. reuteri</td>
<td>6 mm</td>
<td>80.00%K</td>
<td>80.00%K</td>
<td>70.00%S</td>
<td>30.00%R</td>
<td>30.00%S</td>
<td>30.00%K</td>
<td>30.00%S</td>
</tr>
<tr>
<td>L. reuteri</td>
<td>4 mm</td>
<td>80.00%K</td>
<td>60.00%K</td>
<td>70.00%K</td>
<td>70.00%K</td>
<td>70.00%K</td>
<td>70.00%K</td>
<td>70.00%K</td>
</tr>
<tr>
<td>L. delbrueckii</td>
<td>6 mm</td>
<td>100.00%K</td>
<td>80.00%K</td>
<td>70.00%S</td>
<td>70.00%S</td>
<td>70.00%S</td>
<td>30.00%K</td>
<td>50.00%R</td>
</tr>
<tr>
<td>L. delbrueckii</td>
<td>6 mm</td>
<td>20.00%R</td>
<td>30.00%R</td>
<td>30.00%R</td>
<td>70.00%R</td>
<td>80.00%R</td>
<td>70.00%R</td>
<td>70.00%R</td>
</tr>
</tbody>
</table>

S=sensitive, R= resistant

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 flora 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 homoesthermic 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 buffaloes 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 sows [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, Lactobacillus, function as endogenous microbiocides 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 floridin 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 pyometra 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 chemotherapy 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. acidophilus 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 buffaloes 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. yamamashiensis. 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 methicillin 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. roentum 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 environment, 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.

REFERENCES


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