COMPARISON OF THE ANTAGONISTIC EFFECTS OF \textit{LACTOCOCCUS LACTIS} PTCC 1403 AND ISOLATED VAGINAL \textit{LACTOBACILLI} AGAINST UTERINE AND UDDER PATHOGENS OF DAIRY COWS

P. Dini\textsuperscript{1}, P. Mottaghian\textsuperscript{2}, O. Ataie\textsuperscript{1}, M. Farhoodi\textsuperscript{1}

\textsuperscript{1}Department of Clinical Sciences, Faculty of Veterinary Medicine, Islamic Azad University, Karaj Branch, Karaj, Iran and \textsuperscript{2}Department of Clinical Sciences, Faculty of Veterinary Medicine, Tehran University, Tehran, Iran

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\textbf{ABSTRACT:} Antibiotics are used widely for the treatment of udder and uterine infections. Although this is an effective treatment, there are serious economic and public health concerns with this approach due to high cost and increase in antibiotic resistance. Consequently, there are continuous investigations on possible alternative remedies, like use of probiotics as a therapeutic or prophylactic tool. In this study, the antagonistic effect of \textit{Lactococcus lactis} PTCC 1403, a widely used probiotic in dairy industry, has been compared with a vaginal isolates of lactic acid bacteria from healthy cows. All of the indicator pathogens were susceptible to both \textit{L. lactis} and isolated \textit{Lactobacilli}. Among them, \textit{Pseudomonas aeroginosa} and \textit{Arcanobacterium pyogenes} showed the most susceptibility and \textit{Clostridium perfringens} showed the least susceptibility. Results show that the antagonistic efficacies of \textit{L. lactis} and isolated \textit{Lactobacilli} against pathogens were approximately similar.

\textbf{KEY WORDS:} \textit{Lactococcus lactis}, Mastitis, Probiotic, Uterine infection, Vaginal \textit{Lactobacilli}

\textbf{Introduction} 
Infertility and mastitis are among the most important causes of culling dairy cattle in the dairy industry. Uterine infections are one of the main causes of infertility in postpartum cows (Rajala-Schultz and Grohn 1999) that result in reduction of the reproductive performance in the dairy herd (Lewis 1997). Likewise, Bovine mastitis is one of the most prevalent, persistent and costly diseases affecting dairy cattle. Treatment of these conditions is the most common reason for antibiotic administration on dairy farms.

Although use of antibiotics for treatment of uterine and mammary gland infections has been effective to some extent, it also has some disadvantages, including antibiotic residues in animal products, high expenses and even some times they worsen the situation. Therefore it is necessary to investigate alternative therapeutic or prophylactic methods.

Currently the administration of probiotics is considered as an alternative method for prevention and treatment of infections. The mechanisms involved in competitive exclusion (the process by which beneficial bacteria exclude harmful bacteria) are multifactorial and include regulatory forces exerted by the host, the diet and the microbes. Some of the major microbial factors include competition for attachment sites, low PH, low redox Potential, and elaboration of antimicrobial substances (e.g., VFA, lactic acid, and bacteriocins). Mechanisms involving the antagonistic activity of probiotic microorganisms in vitro include the alteration of PH values by production of compounds such as organic acids, as well as production of antimicrobial substances such as hydrogen peroxide and especially bacteriocins.

Probiotic microorganisms consist mainly of members of \textit{Lactic acid bacteria} (LAB) and also selected species of yeasts (Yateem et al., 2008). The genus \textit{Lactobacillus} is the largest group among the lactic acid bacteria. The human vaginal microbiota is a complex community of microorganisms in which lactic acid bacteria (LAB) play a fundamental role (Sobel 1999). The presence–absence of LAB and particularly lactobacilli is considered a health-disease indicator and its predominant role in this ecological niche is now well recognized (Reid and Bruce 2006). \textit{Lactobacilli} are also present in the vaginal microflora of healthy cows. They can prevent pathogen colonization by mechanisms such as the production of antagonistic substances as lactic acid, H\textsubscript{2}O\textsubscript{2}, or bacteriocins.
The genus *Lactococcus* is one of the members of lactic acid bacteria. *Lactococcus lactis* and its subspecies are widely used in the industrial production of fermented dairy products; however, their probiotic properties have not been identified in contrast to other commonly used members of LAB such as *Lactobacillus* spp. and *Bifidobacterium* spp. (Kimoto et al., 2004). In two recent studies, food grade Lactococcus lactis has been compared with conventional antibiotics for the treatment of bovine mastitis (Klostermann et al., 2008 and Crispie et al., 2008). The results of these trials suggest that live culture treatment with *L. lactis* may be as efficacious as common antibiotic treatments in some instances of clinical mastitis in two separate field trials.

The objective of this study was to evaluate and compare the antagonistic effects of *Lactococcus lactis* PTCC 1403 and isolated vaginal lactic acid bacteria against the most prevalent pathogens of uterine and mammary gland infections of dairy cows. In addition, in this study, using the method by Perea Velez et al. (2007) to classify antagonistic effects of LAB, a simple method was suggested to statistically compare the antagonistic effects of probiotic microorganisms.

**MATERIAL AND METHODS**

**Probiotic microorganism and culture conditions**

*Lactobacillus* strains were isolated from bovine vaginal samples of 28 healthy Holstein dairy cows during their estrus phase and were enriched in Brain Heart Infusion (BHI) broth for 48 hour in 37°C and then the culture were centrifuged and the sediment were incubated on MRS agar at 37°C in a 5% CO₂ Atmosphere for 5 days. Identification to genus level was performed by morphological characteristics, Gram staining, catalase reaction, NO₃ reduction and indol production.

*Lactococcus lactis* PTCC 1403 were obtained from Persian Type Culture Collection (PTCC) reference laboratory. The strain was inoculated in Brain Heart Infusion broth and incubated for 48 hours at 30°C under microaerophilic conditions. Due to the short duration of the experiment, the strains were preserved using serial cultures.

**Pathogenic microorganisms and culture conditions**

Pathogenic strains were isolated from mastitic milk and uterine infection specimens submitted to the laboratory and were identified using standard phenotypic methods. The isolated pathogenic strains used as indicators in agar spot test are listed as follows: *Staphylococcus aureus*, *Streptococcus agalactiae*, *streptococcus dysgalactiae*, *Streptococcus uberis*, *Arcanobacterium pyogenes*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Clostridium perfringens*.

All of the pathogenic strains except *E. coli* were inoculated in BHI agar. *E. coli* was inoculated in Nutrient agar. Afterwards, the plates were incubated either anaerobically or aerobically, depending on the growth characteristics of each indicator pathogenic strain at 37°C.

**Antimicrobial Activity Assay**

For detection of antimicrobial activity, an agar spot test was used (Jacobsen et al., 1999). Fresh cultures of *Lactococcus lactis* and isolated *Lactobacilli* were spotted (2.5 µl) on the surface of MRS agar (15 ml in 8 cm diameter plates) and incubated anaerobically for 24 hours at 30°C to develop the spots. Next, the plate surface was covered with 10 ml of BHI agar inoculated with the indicator bacteria (1 × 10⁷ cfu ml⁻¹). The plates were incubated either anaerobically or aerobically depending on the growth characteristics of each indicator pathogenic strain at 37°C. After 24 to 48 (for *Streptococcus spp.*) hours of incubation, the average diameters of inhibition zones were determined. The inhibition zones were classified as negative (-) for no visible inhibition, weak positive (+) for 0.5 to 6 mm, moderate positive (+++) for 7 to 12 mm and strong positive (++++) for more than 12 mm (Perea Velez et al., 2007). Each test was performed five times with different isolates of each pathogen obtained from new cases of infections.

**Statistical analysis**

The statistical analysis was performed by SPSS 16.0 software. Obtained data were analyzed using the Chi square test to compare antagonistic efficacy of vaginal *Lactobacilli* and *Lactococcus lactis* PTCC 1403. In addition, the two samples T test was used to compare the means of measured inhibition zones. P<0.05 was regarded as significant.

**RESULTS**

Comparing the average inhibition zones of the five cultures for each pathogen, all of the indicator pathogens were susceptible to both *L. lactis* PTCC 1403 and Isolated LAB. *Pseudomonas aeruginosa* (an average inhibition zone of 15.2 and 14 mm, respectively) and *Arcanobacterium pyogenes* (an average inhibition zone of 14.8 and 12.6 mm, respectively) showed the most susceptibility and *Clostridium perfringens* showed the least susceptibility to both *L. lactis* PTCC 1403 and isolated Lactobacilli (an average inhibition zone of 14.8 and 12.6 mm, respectively). Furthermore, one strain of *Clostridium perfringens*, one strain of *Streptococcus uberis* and one strain of *Streptococcus dysgalactiae* were totally resistant to the isolated Lactobacilli and did not show any measurable inhibition zones. Obtained data are summarized in tables 1 and 2.

Regarding the percentages of susceptibility (78% versus 76%) as well as the mean measured inhibition zones (10.94 mm versus 9.82 mm), there were no significant (p>0.05) differences between the antagonistic effects of *L. lactis* PTCC 1403 as a known probiotic and the isolated LAB. No significant differences (p>0.05) were also seen between
the mean measured inhibition zones of similar pathogens in both groups.

**TABLE 1. Statistical Analysis of measured inhibition zones around *L. lactis* spot (all values in millimeters).** SE: Standard Error, SD: Standard Deviation.

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>MEAN ± SE</th>
<th>SD</th>
<th>MIN</th>
<th>MAX</th>
<th>CLASSIFICATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>15.20 ± 2.010</td>
<td>4.494</td>
<td>12</td>
<td>23</td>
<td>+++++</td>
</tr>
<tr>
<td>Arcanobacterium pyogenes</td>
<td>14.80 ± 2.131</td>
<td>4.764</td>
<td>10</td>
<td>21</td>
<td>+++++</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>13.40 ± 2.522</td>
<td>5.639</td>
<td>8</td>
<td>23</td>
<td>+++++</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>13.40 ± 1.720</td>
<td>3.847</td>
<td>10</td>
<td>20</td>
<td>+++</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>12.20 ± 1.281</td>
<td>2.864</td>
<td>9</td>
<td>16</td>
<td>+++</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>11.40 ± 1.806</td>
<td>4.037</td>
<td>7</td>
<td>17</td>
<td>+</td>
</tr>
<tr>
<td>Streptococcus dysgalactiae</td>
<td>9.60 ± 3.140</td>
<td>7.021</td>
<td>2</td>
<td>21</td>
<td>+</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>9.60 ± 1.435</td>
<td>3.209</td>
<td>6</td>
<td>14</td>
<td>+</td>
</tr>
<tr>
<td>Streptococcus uberis</td>
<td>6.20 ± 2.267</td>
<td>2.267</td>
<td>2</td>
<td>15</td>
<td>+</td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>3.60 ± 0.748</td>
<td>0.748</td>
<td>1</td>
<td>5</td>
<td>+</td>
</tr>
</tbody>
</table>

**TABLE 2. Statistical Analysis of measured inhibition zones around isolated *Lactobacilli* spot (all values in millimeters).** SE: Standard Error, SD: Standard Deviation

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>MEAN ± SE</th>
<th>SD</th>
<th>MIN</th>
<th>MAX</th>
<th>CLASSIFICATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>14.00 ± 2.025</td>
<td>4.527</td>
<td>7</td>
<td>18</td>
<td>+++++</td>
</tr>
<tr>
<td>Arcanobacterium pyogenes</td>
<td>12.60 ± 2.131</td>
<td>4.277</td>
<td>5</td>
<td>15</td>
<td>+++++</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>11.40 ± 1.600</td>
<td>3.577</td>
<td>6</td>
<td>15</td>
<td>+</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>10.80 ± 1.393</td>
<td>3.114</td>
<td>6</td>
<td>14</td>
<td>+</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>10.60 ± 2.064</td>
<td>4.615</td>
<td>7</td>
<td>17</td>
<td>+</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>9.60 ± 1.400</td>
<td>3.310</td>
<td>5</td>
<td>13</td>
<td>+</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>9.40 ± 1.965</td>
<td>4.393</td>
<td>2</td>
<td>13</td>
<td>+</td>
</tr>
<tr>
<td>Streptococcus dysgalactiae</td>
<td>9.20 ± 2.478</td>
<td>5.541</td>
<td>0</td>
<td>15</td>
<td>+</td>
</tr>
<tr>
<td>Streptococcus uberis</td>
<td>7.00 ± 1.817</td>
<td>4.062</td>
<td>0</td>
<td>10</td>
<td>+</td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>3.60 ± 0.927</td>
<td>2.073</td>
<td>0</td>
<td>5</td>
<td>+</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The competitive exclusion by Lactic Acid Bacteria is mostly due to the accumulation of main primary metabolites such as lactic and acetic acids, ethanol and carbon dioxide. Additionally, LAB are also capable of producing antimicrobial compounds such as formic and benzoic acids, hydrogen peroxide, diacetyl, acetoin and bacteriocins. The production levels and the proportions among these compounds depend on the strain, medium compounds, and physical parameters (Tannock, 2004). LAB has shown to possess inhibitory activities mostly towards gram-positive pathogens and closely related bacteria, due to the bactericidal effect of protease sensitive bacteriocins (Jack *et al.*, 1995). However, LAB was also able to control the growth of gram-negative pathogens including food borne pathogens by the production of organic acids, and hydrogen peroxide (Lu and Walker, 2001; Ito *et al.*, 2003).

In this study, the range of measured inhibition zone diameters in general was wide (1 mm minimum to 23 mm maximum for *L. lactis* and 0 mm minimum to 18 mm maximum for Isolated *Lactobacilli*) and also varied greatly for individual species of pathogens. These findings may suggest the importance of variations between strains of one species. Likewise the statement of Ito *et al.* (2003), both *L. lactis* PTCC 1403 and Isolated *Lactobacilli* produced relatively greater antagonistic effects against gram negative bacteria than gram positive bacteria. This finding was in contrast with the statement by Jack *et al.* (1995).

The results obtained by Yateem *et al.* (2008) showed that *Lactococcus lactis* ssp. *Lactis* isolated from camel milk produced inhibitory effects against other LAB (*Lactobacillus pentosus* and *Lactobacillus plantarum*) and also against strains of *Staphylococcus aureus*, *Escherichia coli* and *Salmonella spp*. Fraga *et al.* (2008) showed that lactic acid bacteria (*lactobacillus* and *enterococcus* strains) isolated from mares’ vagina produced antagonistic effects against *Staphylococcus aureus* as a representative of gram-positive bacteria and *Escherichia coli* as a representative of gram-negative bacteria. Similarly in our study, both of the probiotic microorganisms were able to inhibit the growth of both *Staphylococcus aureus* and *E. coli*. It is obvious that the similarities or differences between results obtained by various studies might be originated from similarities or differences between probiotic strains and also between indicator pathogenic strains.

**TABLE 3. Overall percentages of complete susceptibility, intermediate susceptibility and resistance of pathogenic strains to *L. lactis* and isolated *Lactobacilli***

<table>
<thead>
<tr>
<th>Antagonistic Activity</th>
<th>Susceptible Pathogens</th>
<th>Intermediate Susceptibility</th>
<th>RESISTANT PATHOGENS</th>
<th>TOTALLY RESISTANT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactococcus lactis PTCC 1403</td>
<td>34</td>
<td>44</td>
<td>22</td>
<td>-</td>
</tr>
<tr>
<td>Isolated Lactobacilli</td>
<td>34</td>
<td>42</td>
<td>18</td>
<td>6</td>
</tr>
</tbody>
</table>
In this study, antagonistic efficacy of isolated Lactobacilli and \textit{L. lactis} PTCC 1403 against indicator pathogens were compared to each other. Negative and weak positive antagonistic activities were considered as resistance; moderate positive was considered as intermediate susceptibility and strong positive antagonistic activity as complete susceptibility. Overall results show that 22\% and 24\% of the indicator pathogens were resistant to the antagonistic effects of \textit{L. lactis} and Isolated \textit{Lactobacillus}, respectively. Among resistant pathogens, 6\% was completely resistant to \textit{Lactobacillus} \textit{spp.} and no inhibition zones were recorded for them. 76\% (34\% + 42\%) of pathogens were susceptible to isolated \textit{Lactobacillus}: 78\% (34\% + 44\%) of pathogens were susceptible to \textit{L. lactis} PTCC 1403 (table 3). The percentages of overall susceptibility to the both the used probiotic microorganisms did not differ significantly (p<0.05). In addition, statistical difference between the mean measured inhibition zones of the total number of indicator pathogens of the both groups was calculated and no statistical difference (p>0.05) was shown. Likewise, no significant differences (p>0.05) were seen between the mean measured inhibition zones of similar pathogens in both groups. Eventually, despite the fact that antibacterial substances (bacteriocins), organic acids and other metabolites produced by these probiotics vary among probiotic microorganisms, it may be concluded that similar mechanisms that involved in the antagonistic action of these probiotics can cause the similarities of the results or based on the overall similarity between the susceptibility of indicator pathogens, there may be similar mechanisms involving in resistance of pathogens against the antagonistic effects of these probiotics. Therefore, further researches are necessary to clarify these mechanisms and evaluate the effect of various metabolites and antibacterial compounds against various pathogens. Likewise, it is necessary to more precisely investigate the mechanisms by which the pathogens may defense against the antagonistic effects of probiotic microorganisms.

REFERENCES


