Probiotic Activities of Lab in Endometritis: Probiotic Activities of Three Lactic Acid Bacteria Strains Isolated from Healthy Bovine Cervix: In Vitro Adherence to Immortalized Endometrial Epithelial Cells and Antimicrobial Properties

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Abstract: The ability of three Lactic Acid Bacteria (LAB), i.e., Lactobacillus acidophilus, Enterococcus faecalis and Enterococcus cecorum that isolated from cervical mucus in healthy cattle to adhere to immortalized Endometrial Epithelial Cells (EEC) and to affect the adhesion of two main endometritis-associated pathogens, Escherichia coli and Staphylococcus aureus were investigated. All probiotic strains exhibited strong adhesion levels to the cell lines and the adhesion levels of L. acidophilus was significantly higher as compared to that of E. faecalis or E. cecorum (p<0.05). In coculture assays, a significant decrease in the number of adhesion pathogens was observed, using either preincubation, postincubation or coculation of the pathogens with these LAB. L. acidophilus exhibited strong antagonistic activity against E. coli (p<0.01), nevertheless, E. Faecalis and E. cecorum showed strong inhibitory effect on S. aureus (p<0.01). In conclusion, the ability of LAB tested to inhibit the adhesion of endometritis-associated pathogens is highly specific and depends on both the probiotic and pathogen strains.

Key words: Immortalized endometrial epithelial cell, lactic acid bacteria, adherence, antimicrobial property, endometritis-associated pathogen, China

INTRODUCTION

Uterine infections are one of the most important disorders in postpartum cows (Azawi, 2008). These infections cause high economic losses due to prolonged days open and prolonged intercalving intervals, resulting in involuntary culling (Foldi et al., 2006; Azawi et al., 2007).

In the postpartum cow, the pathogenic bacteria that are frequently isolated from cases of endometritis are Escherichia coli, Staphylococcus sp. and Streptococcus sp. (Zerbe et al., 2001; Petit et al., 2009). The conventional treatments for uterine infection recommended the use of antibiotics as systemic route of administration in cows with toxic puerperal metritis (Amirid et al., 2003; Sheldon et al., 2004) but these treatments are associated with the increasing frequency of drug-resistant pathogenic bacteria and residues in animal products. Alternative therapeutic agents need to be sought and it has been suggested that the administration of Lactic Acid Bacteria (LAB) can restore ecological balance in the vagina by controlling the infectivity of pathogenic microbes (Abad and Safdar, 2009). Possible mechanisms of the health benefits provided by probiotics include immune exclusion, competition for adhesion sites and production of antimicrobial agents such as bacteriocins (Foligne et al., 2010; Guo et al., 2010). Antagonistic effect against pathogens and temporary colonization of the gastrointestinal and urogenital tract have been demonstrated for some probiotics (Kon et al., 2008; Johnson et al., 2009; Ruiz et al., 2009). However, the effect of probiotics during postpartum uterine infection in vitro has not been thoroughly investigated for the limited information of highly suitable endometrial epithelial cell vitro model. Previous studies have indicated that mucous adhesion properties are more dependent on the probiotics strain than on the host (Tuomola et al., 1999; Rinkinen et al., 2003). In this study, immortalized hircine Endometrial Epithelial Cells (EEC) was used as vitro model and the adherence of various strains of LAB as well as pathogenic bacteria to EEC was compared. The abilities of these LAB strains to inhibit the adhesion of endometritis-associated bacterial pathogens were also examined.

MATERIALS AND METHODS

Bacterial strains and culture conditions: Three lactic acid bacteria, including Lactobacillus acidophilus YL305,
Enterococcus faecalis YL303, Enterococcus cecorum YL306 were originally derived from cervical mucus in cattle that had no history of metritis infection. The LAB strains were cultured in De Man, Rogosa, Sharpe (MRS) broth at 37°C under anaerobic conditions. Bacterial pathogens used were Escherichia coli C83917 and Staphylococcus aureus C56008 which were purchased from the China Veterinary Culture Collection Center (Beijing, China). They were clinical isolated from cervical mucus in postpartum metritis cattle and were grown in Luria-Bertani (LB) medium at 37°C under aerobic condition. All strains were stored in -80°C with 20% sterile glycerol until needed.

Cell lines and culture conditions: The endometrial epithelial cell lines from goat were kindly provided by Dr Y.P. Jin (Northwest A and F University, Yangling, China) immortalized by transfected the human Telomerase Reverse Transcriptase (hTERT) into EECs (Wu et al., 2010).

The morphological and immunochemical characteristics of the immortalized lines closely resembled those of their tissues of origin and primary cultures. The cells were cultured and maintained in Dulbecco’s Modified Eagle Medium (DMEM)-Ham F12 (GIBCO, Invitrogen Corporation, Grand Island, NY) supplemented with 5% fetal calf serum (FCS; Atlanta Biologicals, Lawrenceville, GA), antibiotics (100 U mL⁻¹ penicillin, 100 μg mL⁻¹ streptomycin; Gibco) at 37°C in a 5% CO₂ -95% air atmosphere.

In vitro adhesion assays on EEC: Adhesion of the bacterial was assayed by seeding cell lines in 24-well tissue culture plates at 2.5×10⁵ epithelial cells/well and allowing them to grow to complete confluence. Prior adhesion assays EEC monolayers were washed twice with DMEM without antibiotics (streptomycin and penicillin). Bacterial strains were cultivated in MRS broth under micro-aerophilic conditions at 37°C. Cells were harvested at the stationary phase after 18 h of incubation at an amount corresponding to approximately 10⁶ colony forming units (CFU) mL⁻¹ and washed three times in buffered saline PBS (pH 7.2). The volume of the culture with the required number of CFU was determined by plating serial dilutions of bacterial suspensions on MRS agar plates and incubating the plates in micro-aerophilic conditions at 37°C overnight. In the following procedure DMEM without antibiotics was used. Bacterial cells were re-suspended in DMEM and added onto the EEC monolayer at the concentration of approximately 10⁶ CFU well⁻¹. After 1 h of incubation carried out at 37°C under 5% CO₂ air atmosphere, suspensions were removed and the monolayers were washed three times with DMEM to release unbound bacteria. For qualitative analysis, the cell monolayers and the bacteria were methanol fixed and stained with Gram stain (Bonetti et al., 2003). For each monolayer on a glass coverslip, the numbers of adherent bacteria to the EEC were counted in 20 random microscopic areas according to the method of Gopal et al. (2001). Assays were carried out in triplicate.

Inhibition of pathogens adhesion to EEC: Three different procedures were used in order to differentiate exclusion, competition or displacement of the endometritis-associated bacterial pathogens by LAB strains used in this study. For exclusion tests, hircine EEC monolayers were cultured and washed as previously described and incubated with lactobacilli (10⁵ CFU well⁻¹) for 30 min. Afterward, nonadhering lactobacilli were removed, Escherichia coli C83917 or Staphylococcus aureus C56008 (10⁵ CFU well⁻¹) was added and incubation was continued for a further 30 min. For competition tests, lactobacilli and any of the pathogens and the endometrial cells were mixed and incubated for 1 h. For displacement tests, the pathogens and the endometrial epithelial cells were incubated together for 30 min after removal of nonadhering pathogens lactobacilli were added and incubation was continued for a further 30 min. Unbound bacteria were then washed and bound bacteria were released and lysed. Adherence of the pathogens was performed in the presence of 0.5% methyl mannos to avoid type-1-mediated adherence, a nonspecific interaction. The number of viable bacteria adhering to the EEC was determined by plating serial dilutions on MRS plates (for Lactobacillus) or LB plates (for Escherichia coli or Staphylococcus aureus). The inhibition of pathogens adhesion was quantified by comparison of CFU counts of adhered bacteria to the counts in the control experiments without lactobacilli. Inhibition was conducted in three independent experiments and each assay was performed in triplicate.

Statistical analysis: Data was analyzed by Kruskal-Wallis one way Analysis of Variance (ANOVA) using commercially available software (SPSS 16.0, Chicago, IL, USA). Results were logarithmically transformed to obtain geometric means. Comparisons of means were conducted using student’s t-test or a Mann-Whitney rank sum test. Results of all statistical analyses were considered significant only if p<0.05.

RESULTS AND DISCUSSION

In the present study, immortalized endometrial epithelial cell monolayer grown on the glass was used as an in vitro model for investigation of three LAB (one
Lactobacillus and two Lactococcus) adhesive to the uterine epithelium. Besides lake of mucus production, this model offers a number of interesting features ideal for investigation bacterial adherence. Strong adhesion levels of all lactobacilli strains examined were exhibited in this study and these bacteria adhered to endometrial epithelial cells in the range of 38.5-70 bacteria 10^4 cell^-1. As shown in Fig. 1, the adhesion levels of L. acidophilus was significantly higher as compared to that of E. faecalis or E. cecorum (p<0.05).

This could also be related to the fact that different structures of bacteria are implicated in the interactions between bacteria strains and endometrial epithelial cells (Awall-Jaaskelainen and Palva, 2005; Mobilli et al., 2009). Microscopical observation of gram-stained preparation showed bacterial clusters of L. acidophilus or E. faecalis randomly dispersed on the cell surface (Fig. 2a, b). E. cecorum appeared to adhere individually or in pairs (Fig. 2c). Adherence of bacteria to the epithelial cells is an important prerequisite for colonization by microorganisms and virulence manifestations (Kon et al., 2008; Johnson et al., 2009). Inhibiting the adhesion of pathogenic bacteria to their receptor could decrease the uterine colonization and in consequence modify the process of pathogenicity (Varavutikunchai et al., 2006). In this study, three lactobacilli strains tested interfered with the adhesion process to endometrial epithelial cells of two pathogens E. coli and S. aureus.

The adherence of the two pathogens was significantly decreased by addition of probiotics strains (p<0.05 or p<0.01), regardless of whatever the probiotics strains was added before, during or after the incubation with the pathogen (Fig. 3). The presence of these probiotics may impede the access of pathogens to tissue receptors by steric hindrance and that may explain the decrease of adhesion of the pathogens in the presence of probiotics tested (Jankowska et al., 2008). It is important to note that although, the level of adhesion of L. acidophilus to endometrial epithelial cells was significantly higher than those of E. Faecalis and E. cecorum there was no significant difference of the adherence inhibition of two pathogenic bacteria to epithelial cells among these LAB examined. The mechanism(s) underlying this phenomenon may involve not only competition for eukaryotic cell receptors but also an action of production or secreted by lactobacilli antimicrobial compound (Abad and Sañá, 2009). Several studies have shown that the ability of selected strains of Lactobacillus to inhibit the adhesion of pathogenic bacteria is highly specific and depends on both the probiotic and pathogen strains (Jankowska et al., 2008; Lee et al., 2009; Zhang et al., 2010).

In the experimental system, L. acidophilus exhibited strong antagonistic activity against E. coli (p<0.01), nevertheless, E. Faecalis and E. cecorum showed strong inhibitory effect on S. aureus (p<0.01). This indicates the need of a case-by-case characterization of the probiotic

Fig. 1: Each adhesion assays was conducted in triplicate with cells from three successive passages. Adhesion assays were monitored after 1 h of incubation. The figure presents mean numbers±standard deviation of bacteria adhering per epithelial cell. Values with different superscripts letters are significantly different (p<0.05)

Fig. 2: Adhesion of bacteria strains to endometrial epithelial cell cultures observed using light microscopy after Gram-staining; (a) L. acidophilus YL305; (b) E. faecalis YL303 and (c) E. cecorum YL306
strains. It has to be taken under consideration the relationship between the inhibitory effect of LAB and the presence of specific adhesion molecules and receptors for which probiotic and pathogen are competing.

CONCLUSION

In this study, the lactic acid bacteria strains used in this study may protect the uterine epithelium through a serious of barriers and interference mechanisms. The extent to which these in vitro results correspond to in vitro conditions remains to be determined; clinical trials are needed to assess the benefits of probiotic organism consumption in the management of postpartum uterine infection.

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