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Full Length Research Paper

Antagonistic activity of probiotic lactobacilli against *Staphylococcus aureus* isolated from bovine mastitis

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Staphylococcus aureus **is one of the major pathogens which cause Bovine Mastitis (BM). Probiotic lactobacilli have the great potential to produce antimicrobial compounds that inhibit and control pathogenic bacteria. Antagonistic activity of probiotic lactobacilli (** ***L. acidophilus*** **DSM 20079,** ***L. plantarum*** **ATCC 8014,** ***L. casei*** **ATCC 39392 and** ***L. reuteri*** **ATCC 23272) against** ***S. aureus*** **isolated from bovine mastitis (BM** ***S. aureus*****) and standard** ***S. aureus*** **ATCC 25923 was the objective of this study. Antagonistic effect of probiotic lactobacilli was investigated by modified double layer method, well diffusion method, co -culturing assay and co-aggregation method. Among four lactobacilli,** ***L. plantarum*** **showed the greatest inhibitory activity. In modified double layer method the zone of inhibition of BM** ***S. aureus*** **and standard** ***S. aureus*** **ATCC 25923 by** ***L. plantarum*** **was 44 and 40 mm, respectively. Cell Free Supernatant (CFS) of probiotic lactobacilli in well diffusion method had inhibitory effect. Inhibition zone of BM** ***S. aureus*** **(13 mm) and standard** ***S. aureus*** **ATCC 25923 (9 mm) by** ***L. plantarum*** **was achieved. Co-culturing of** ***L. plantarum*** **with these two bacteria resulted in 87 and 77% inhibition growth of BM** ***S. aureus*** **and standard** ***S. ureus*** **ATCC 25923, respectively after 12 h. Co-aggregation between** ***L. plantarum*** **with two mentioned** ***S. aureus*** **was obtained 88.4 and 76%, respectively. According to these data,** ***L. plantarum*** **and its antimicrobial compounds can be one of the selective choices to control the BM** ***S. aureus.***

Key words: Antagonistic effect, probiotic lactobacilli, bovine mastitis, *Staphylococcus aureus.*

INTRODUCTION

In an economical point of view, bovine mastitis (inflammation of the mammary gland) is an important disease in dairy industry (Bradley, 2002; Viguier et al., 2009; DeRong et al., 2010). One of the major contagious pathogens which cause bovine mastitis is *Staphylococcus aureus*. This bacterium is adapted to survival within mammary gland and by establishment of infection, stimulates the inflammatory response (Bradley, 2002). It produces many virulence factors, such as alpha and beta toxins, protein A, coagulase, etc., which help it to colonize and damage mammary gland (Palma et al., 1999; Shana et al., 2009). Although antibiotic therapy to control bovine mastitis is effective, it can be detrimental too, because of the emergence of antibiotic resistant human pathogens (Craven 1987; Van vee and Margolles, 1999; Naheed et al., 2006). So an effective treatment by other substances than antibiotics becomes an urgent need (Pyrola, 2002).

Probiotic lactobacilli with a variety of applications are now the best choice to treat many infectious diseases of human and also have a great potential to control bovine mastitis (Green et al., 1991; Tagg and Dierksen 2003). These bacteria are well known as having many properties which make them beneficial to control pathogenic microorganisms. These include, the ability to adhere to cell, reduce pathogenic bacteria adherents, co-aggregate, produce organic acids, hydrogen peroxide,

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bacteriocin and etc., be safe and nonpathogenic, which antagonize pathogenic microorganisms (Gergor, 1999; Mami et al., 2008). The aim of this study is to assess antagonistic activity of the probiotic lactobacilli culture and their Cell Free Super- natant (CFS) against *S. aureus* from bovine mastitis (BM *S. aureus*) in comparison with standard *S. aureus* ATCC 25923.

MATERIALS AND METHODS

Bacteria strains and media

Probiotic lactobacilli (*L. acidophilus* DSM 20079, *L. plantarum* ATCC 8014, *L. casei* ATCC 39392 and *L. reuteri* ATCC 23272) and standard *S. aureus* ATCC 25923 were purchased from Persian Type Culture Collection. Isolated *S. aureus* from bovine mastitis was provided from Biotechnological Research Center, Azad University, Shahrekord branch. Deman Rgosa and sharp (MRS) broth and agar (Himadia), Brain Heart Infusion broth (BHI) (Himadia), Baird Parker agar (BP) (Himadia), Triptic Soy Broth (TSB) (Merck), Muller Hinton agar (MHA) (Merck) were used in this research.

Antimicrobial activity assays

Modified double layer method

Spot - on- lawn method (Tagg and Mc Given, 1971) which was called double layer method by (Maia, 2001) was used with somehow modification to evaluate the antagonistic activity. An overnight culture of each probiotic *Lactobacillus* in MRS broth at 37°C was prepared. 100 µl of each probiotic *Lactobacillus* culture (10 cfu ml-1) was spotted onto the surface of MRS agar and incubated for 24 h at 37°C. The plate of MRS agar containing lactobacilli spot was overlaid with melted Muller Hinton agar and allowed to solidify. 100 µl of (BM *S. aureus*) and Standard *S. aureus* ATCC 25923 (0.5 McFarland) individually inoculated by streaking the swab over the entire agar surface. The plates were incubated for 24 h at 37°C. The sensitivity of bacteria in the presence of each probiotic *Lactobacillus* spot was determined by measuring the clear zones around spot (Guessas et al., 2006).

Preparation of cell free supernatant

In order to prepare Cell Free Supernatant (CFS), each probiotic *Lactobacillus* was cultivated in MRS broth for 24 h at 37°C. CFS was obtained by centrifuging the culture (10000 rpm, 10 min) followed by filtration of the supernatant through a 0.2 µm pore size filter (Norroozi and Mirzaii 2004).

Well diffusion method

Inhibitory activity of CFS of probiotic lactobacilli was investigated by well diffusion method (Mami et al., 2008). An overnight culture of BM *S. aureus* and Standard *S. aureus* ATCC 25923 in TSB was prepared. These bacteria (10 cfu ml^{-1}) were inoculated by streaking the swab over the entire MHA surface. Wells sized (6 mm) were cut into the agar plate and 50 µl of each CFS was placed into each well. The plates were incubated for 24 h at 37°C and inhibition of growth was examined by clear zone surrounding each well.

Co-culture assay

Co-culture assay, another method for determination of antimicrobial effect of probiotic lactobacilli, was used (Lim, Sung-Me et al., 2009). BM *S. aureus* and standard *S. aureus* ATCC 25923 were grown in BHI broth for 24 h at 37°C. All lactobacilli were grown overnight in MRS broth at 37°C. All cultures were centrifuged at 10000 rpm for 10 min and washed twice with buffer phosphate saline (PBS). 1% of each *Lactobacillus* (10 cfu ml-1),BM *S. aureus* and standard *S. aureus* (10 cfu ml-1) were co-incubated in 50 ml BHI broth for 24 h at 37° . Initial and then at predetermined intervals (after 12 h), 1 ml of cell culture serially diluted and plated on Baird Parker agar for *S. aureus*. The controls were the monoculture of each bacterium. The plates were incubated overnight at 37°C and the number of bacteria was evaluated. The inhibition was calculated using the following equation:

$$\% \text{ Inhibition} = \frac{(\text{CFU/ml in control}) - (\text{CFU/ml in co-incubation culture})}{(\text{CFU/ml in control})} \times 100$$

Co-aggregation method

Co-aggregation of each probiotic *Lactobacillus* with BM *S. aureus* and standard *S. aureus* ATCC 25923 was determined with somehow modified method of Svetoslav et al., 2009. Probiotic lactobacilli were grown in MRS broth for 24 h at 37°C. An overnight culture of BM *S. aureus* and standard *S. aureus* ATCC 25923 in TSB were prepared. Cells were harvested (10000 rpm, 5 min, 20°C) and washed with PBS. Then cells were suspended in sterile saline and adjusted on optical density (OD) of 0.4 measured at 660 nm. Equal volumes (500 µl) of each were mixed and incubated for 4 h at 37°C.Then centrifuged (1600 rpm, 5 min, 20 °C) and the OD of supernatants were measured at 600 nm. Co-aggregation was calculated using the following equation:

$$\% \text{ Co-aggregation} = \frac{\text{Atot} - \text{As}}{\text{As}} \times 100$$

Where Atot represents the OD of strains right after mixing and As refers to the OD of supernatants after 4 h.

RESULTS AND DISCUSSION

According to Ryan et al. (1999), using non-antibiotic formulations to prevent bovine mastitis can reduce the need of using antibiotics in treatment of this disease, so the problem of the emergence of antibiotic resistance pathogens can to a great extent be solved. In this study, the antimicrobial activity of non-antibiotic compounds, such as probiotic lactobacilli, against one of the major causes of bovine mastitis, *S. aureus* was investigated.

One of the important roles of probiotic lactobacilli is the production of inhibitory compounds that antagonize pathogenic bacteria (Nemcova et al., 1997; Jacobsen et al., 1999). The inhibitory activity of probiotic lactobacilli (*L. acidophilus* DSM20079, *L. plantarum* ATCC 8014, *L. casei* ATCC 39392 and *L. reuteri* ATCC 23272) against BM *S. aureus* and standard *S. aureus* ATCC 25923 by modified double layer method is shown in Table 1. The best zone of inhibition of BM *S. aureus* and standard *S. aureus* was achieved in the presence of *L. plantarum*, 44 and 40 mm, respectively. This result was supported

Table 1. The inhibitory effect of probiotic lactobacilli against BM *S. aureus* and standard *S. aureus* ATCC 25923.

Bacteria culture	Inhibition zone diameter(mm)	
***Lactobacillus* strains**	**BM *S. aureus***	***S. aureus* (ATCC25923)**
L. acidopillus DSM 20079	40	38
L. plantarum ATCC 8014	44	40
L. casei ATCC 3939	39	35
L. reuteri ATCC 2327	35	32

Table 2. The inhibition zone of BM *S. aureus* and standard *S. aureus* ATCC 25923 by CFS of probiotic lactobacilli.

Cell free supernatant	Inhibition zone diameter (mm)	
***Lactobacillus* strains**	**BM *S. aureus***	***S. aureus* (ATCC25923)**
L. acidopillus DMS 20079	11	8
L. plantarum ATCC 8014	13	9
L. casei ATCC 39392	10	7
L. reuteri ATCC 23272	10	7

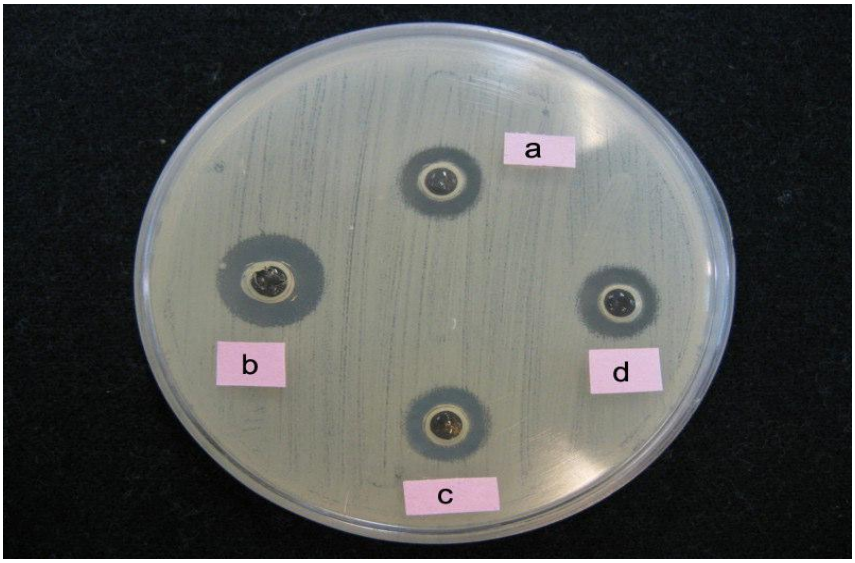

Figure 1. The inhibitory effect of CFS of probiotic lactobacilli against BM *S. aureus*. a) *L. reuteri*, b) *L. plantarum*, c) *L. acidophilus* and d) *L. casei.*

with the findings published by Mami et al. (2008) and in some extent was comparable with the findings of Bilg et al. (2005). According to Con and Gokalp (2000), this inhibitory effect was because of all or every metabolite such as lactic acid, acetic acid, diacetyl, bacteriocin, etc. which was produced during the assay period.

Antibacterial effect of cell free supernatant (CFS) of probiotic lactobacilli by well diffusion method is shown in Table 2 and Figure 1. A strong inhibition zone of BM *S. aureus* was obtained by *L. plantarum*, 13 mm. Standard *S. aureus* ATCC 25923 is nearly resistant to CFS of all lactobacilli, although *L. plantarum* had the most inhibitory effect against this bacterium, 9 mm. The findings of Paird et al. (1971) and Esaya et al. (2008) supported these results. According to Millettee (2006) antimicrobial effect of lacto-bacilli in co-culture with pathogenic bacteria is mainly due to production of organic acids which result in pH reduction, although they can produce some other substances as well. The inhibitory effect of probiotic lactobacilli in co-culture with BM *S. aureus* and standard

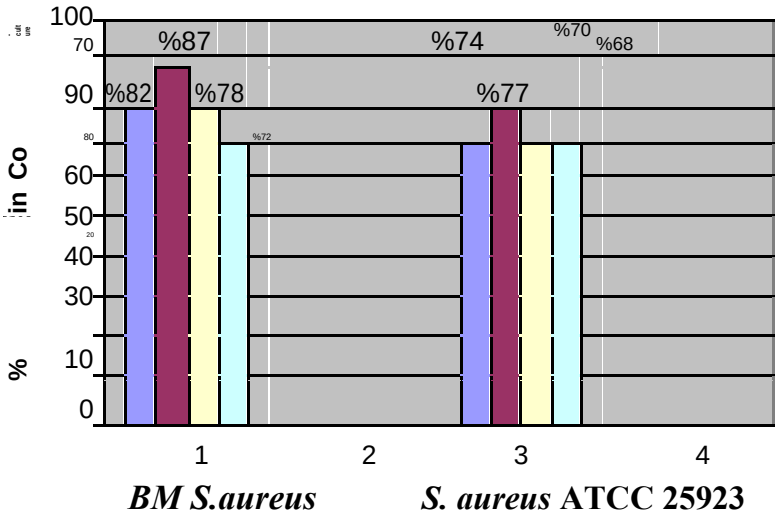

Chart 1. The inhibitory activity of co-culturing of probiotic lactobacilli with BM *S. aureus* and standard *S. aureus* ATCC 25923. *L. acidophilus*, *L. plantarum*, *L. casei* and *L. reuteri*.

Table 3. % Co-aggregation of probiotic lactobacilli with BM *S. aureus* and standard *S. aureus* ATCC 25923.

% Co-aggregation		
***Lactobacillus* strains**	**BM *S. aureus***	***S. aureus* (ATCC25923)**
L. acidopillus DSM 20079	81	72.4
L. plantarum ATCC 8014	88.4	76
L. casei ATCC 39392	80.1	68
L. reuteri ATCC 23272	79.9	65.4

S. aureus ATCC 25923 is shown in Chart 1. The best result was obtained when BM *S. aureus* and *S. aureus* ATCC 25923 co-incubated with *L. plantarum*, 87% and 77%, respectively. This result agrees with Bilge et al. (2005) who announced that antimicrobial sub-stances produced by *Lactobacillus* have a great potential for inhibiting the growth of pathogenic microorganisms. Co-aggregation assay is a reliable method to evaluate the close interaction between lactobacilli and pathogenic bacteria. Many surface proteins are found in lactobacilli which are predicted to promote binding to environmental surface like other bacteria surface. Co-aggregation may be beneficial to *Lactobacillus* that produces antimicrobial compounds, as it would force the cells into closer contact (Reid and McGroarty 1988). The result of co- aggregation of probiotic lactobacilli and BM *S. aureus* and standard *S. aureus* ATCC 25923 is shown in Table 3. Co-aggregation of *L. plantarum* with BM *S. aureus* and standard *S. aureus* 88.4% and 76% was the best result. As a conclusion, all used probiotic lactobacilli have an antagonistic activity against Staphylococcus aureus isolated from Bovine Mastitis and standard *S. aureus* ATCC 25923 but *L. plantarum* showed the great potential to inhibit mentioned pathogens. This bacterium can be one of the proper organisms to control bovine mastitis and a good choice for further investigations.

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