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Antibacterial activity of lactobacilli strains isolated from *Laban Rayeb* "Egyptian traditional fermented milk" against *Pseudomonas fluorescens and Escherichia coli in vitro*

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ABSTRACT

Lactobacilli are significant to dairy industry due to their involvement in the production of several fermented dairy products. The present study was conducted to assess the antibacterial activity of the of lactobacilli strains (*Lactobacillus pentosus* RMLB1, *Lactobacillus acidophilus* RMLB9, *Lactobacillus plantarum* RMLB20, *Lactobacillus pentosus* RMLB22 and *Lactobacillus salivarius* RMLB32) isolated from the Egyptian traditional fermented milk "*Laban Rayeb*". The lactobacilli strains were evaluated using the agar-well diffusion assay against pathogenic strains of *Pseudomonas fluorescens* and *Escherichia coli*. Furthermore, the ability of these strains to adhere to human intestinal mucous was examined. The results indicated that all the LAB strains showed a variable antibacterial activity against *P. fluorescens* and *E. coli*. *L. acidophilus* RMLB9 and *L. salivarius* RMLB32 showed the highest inhibitory effect against both pathogens. Moreover, all strains adhered *in vitro* to intestinal mucous with variable extents. The highest adhesive level, compared to the reference strain of *Bifidobacterium bifidum,* was showed by *L. plantarum* RMLB20 followed by *L. salivarius* RMLB32 and *L. acidophilus* RMLB9. The obtained results highlight that the Egyptian traditional fermented milk "Laban Rayeb" could be a source for potential probiotic bacteria possessing good inhibitory activity against *P. fluorescens* and *E. coli*.

Keywords: *Lactobacillus bacteria., Antibacterial activity probiotic potential, Laban Rayeb.*

INTRODUCTION

Lactic Acid Bacteria (LAB) including Lactobacillus spp. are essential to the dairy industry as they are widely used in preparation of variety of fermented dairy products and used as probiotics, due to their health-promoting effects (Puniya *et al.*, 2008).

Dairy products containing-probiotic consumption has been shown to be useful in overcoming multiple clinical conditions such as antibiotic-associated diarrhea, infantile diarrhea, infections of *Helicobacter pylori*, *Clostridium difficile* colitis and inflammatory bowel disease (Reid *et al.*, 2003). In addition, probiotics have other positive impacts, that include improving lactose intolerance, increasing nutrients utilization, serum cholesterol level lowering (Guo *et al.*, 2010).

There are various traditional fermented foods and beverages produced and consumed locally around the world (Marshall and Mejia 2011). *Laban rayeb* is an example of a known Egyptian traditional fermented milk product, which is a type of curdled skim milk that either drunk fresh or used to make Kariesh cheese (El-Gendy, 1983).

As fermented food products are mostly connected with beneficial microbes, research to find strains with probiotic potential from traditionally fermented products is growing rapidly (Angchok *et al.*, 2009; Angmo and Bhalla, 2014). In addition, probiotic LAB are being used as a preventive treatment alternative through excerpting antibacterial activity (Lin *et al.*,2009). The antibacterial effect of lactobacilli is mostly related to the production of organic acids, such as lactic, acetic and propionic acids, and sometimes hydrogen peroxide, bacteriocins, and antimicrobial peptides (Cortes-Zavaleta *et al.*, 2014; Gemechu 2015).

Pseudomonas spp. are predominant milk-associated psychrotrophic bacteria and one of the most important bacterial groups in the dairy industry (Wiedmann *et al.*, 2000). Among them *Pseudomonas fluorescens* which is a member of the fluorescent pseudomonad group and (unlike *P. aeruginosa*) has generally been regarded to be of low virulence and an infrequent cause of human infection. However, it has been reported to cause infections in immunocompromised patients (Hsueh *et al.*, 1998) and outbreak of *P. fluorescens* bacteraemia in cancer outpatients was reported by the Centers for Disease Control and Prevention (CDC) (Gershman *et al.*, 2008).

However, most of *Escherichia coli* are commensals, some *E. coli* strains are pathogenic and can cause a variety of diseases (Ombarak *et al.*, 2016).

Elimination of pathogens through microbial antagonism to ensure the hygienic quality and safety of food is a promising tool for biological preservation food (Jordan *et al.* 2014). Limited data are available about the antibacterial potential of lactobacilli from traditional fermented food. Therefore, the objectives of this study were to investigate the antibacterial activity and adhesion ability of LAB isolated from the known Egyptian traditional fermented milk product "*Laban Rayeb*".

MATERIALS AND METHODS

1. Bacterial strains and culture conditions

Five *Lactobacillus* strains, *Lactobacillus pentosus* RMLB1, *Lactobacillus acidophilus* RMLB9, *Lactobacillus plantarum* RMLB20, *Lactobacillus pentosus* RMLB22 and *Lactobacillus salivarius* RMLB32, that were previously isolated from *Laban Rayeb* "Egyptian traditional fermented milk" (Mahrous *et al.* unpublished) (Table 1), and the reference probiotic strain *Bifidobacterium bifidum* (ATCC 11147) were used in this study.

The strains were grown anaerobically in de Man Rogosa Sharpe Broth (MRS) at 37°C overnight, and the reference probiotic strain *Bifidobacterium bifidum* (ATCC 11147) was grown in MRS with 0.05% cysteine.

To test the antibacterial activity of the isolates, broth culture of pathogenic strains of *Escherichia coli* and *Pseudomonas fluorescens*, previously isolated from milk at laboratory of Industrial Biotechnology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, were used. *E. coli* and *P. fluorescens*

2. Antibacterial activity of LAB

Antibacterial activity of the Lactobacilli isolates was tested against *P. fluorescens* and *E. coli* strains by agar-well diffusion method as described by (Setyawardani *et al.*, 2014). Briefly, bacterial cultures of *P. fluorescens* and *E. coli* were maintained and refreshed weekly. On a petri dish 1 mL diluted culture (10^6 CFU ml^{-1}) was poured and 15 to 20 mL of Muller Hinton agar (MHA) was poured over and

properly mixed and wells, 5 mm in diameter, were made after the medium hardened.

LAB Isolates culture were centrifuged at 15,000 x g, for 10 min, and washed twice with phosphate buffer saline (PBS; pH 7.2). The optical density of the culture suspensions at 600 nm (OD_{600} nm) was adjusted to 0.5 (approximately 10^8 CFU ml^{-1}). Then 50 µL were applied into each well and the plates were incubated at 37°C for 24 hours. The diameter of the inhibition zone surrounding each well was measured and recorded.

3. Adhesion to human mucus

The adhesion assay was carried out *in vitro* by using mucous extracted from healthy new-born (15-39 months of age) feces as a model for intestinal surface. Mucous and bacterial strains preparations were done as described previously (Juntunen *et al.*, 2001 and Vesterlund *et al.* 2005). For the *in vitro* adhesion assay Crystal violet method was followed as described by Vesterlund *et al.* (2005). Briefly, 100 µl of the test strains were added into thee wells of microtiter polystyrene plate coated with 150 µl of human intestinal mucous. Bacteria were allowed to adhere at 37°C for 1 h, then washed with 250 µl of PBS three times to remove the non-adherent bacteria. The adherent bacteria were fixed at 60°C for 20 min and stained with, 100 µl per well, crystal violet 0.1 % solution for 45 min. To remove excess stain, wells were washed with PBS three times. 100 µl of citrate buffer (20 $mmol^{-1}$; pH 4.3) was added to each well to release the stain bound to the bacteria. The absorbance values at 630 nm were determined using Universal Automated Microplate reader ELX800 after incubation at room temperature for 45 min.

Stained mucous without added bacteria was used as negative control while as positive control, mucous with added *Bifidobacterium bifidum* culture was used. Results of the samples absorbance values were recorded after subtracting negative control absorbance value.

4. Statistical analysis

A Student's t-test was used to examine significant differences ($P<0.05$) in cell growth and adhesion (ANOVA), and the Tukey and Kruskal-Wallis tests to determine the statistical differences between the groups (STATISTICA 6.0. software). The experiments were done three times in duplicate and the indicated values were the mean ± standard error.

Table 1. Biochemical and potential probiotic phenotypic characterization of LAB isolates used in this study

Isolate LAB	Gram Stain	Catalase	Growth at 10°C	Growth at 45 °C	Production of CO2	pH tolerance	Bile tolerance
L. pentosus RMLB1	Positive	Negative	+ [a]	+	- [b]	+	+
L. acidophilus RMLB9	Positive	Negative	+	+	-	+	+
L. plantarum RMLB20	Positive	Negative	-	+	-	+	+
L. pentosus RMLB22	Positive	Negative	+	+	-	+	± [c]
L. salivarius RMLB32	Positive	Negative	-	+	-	+	+

[a] *+ indicate viable* [b] *- indicate no production* [c] *± indicate fairly viable*

Results and Discussion

The antibacterial activity of the five LAB strains against pathogenic *P. fluorescens* and *E. coli* strains was screened by agar-well diffusion method, and all the examined strains showed antimicrobial activity as indicated by the obtained inhibition zones (Table 2). *L. salivarius* RMLB32 and *L. acidophilus* RMLB9 showed the highest inhibitory effect

among other LAB strains against both pathogens.

Our results agree with the findings of Setyawardani *et al.* (2014) and Aminnezhad *et al.* (2015), who also demonstrated antibacterial activity of lactobacilli against *E. coli* and *P. fluorescens*.

Lactobacilli may engage several strategies that work co-operatively to show antibacterial activity. The antibacterial activity of LAB is mainly due to organic acid, hydrogen peroxide and bacteriocins that can inhibit both Gram-positive and Gram-negative bacteria (Bevilacqua *et al.*, 2003, Santos *et al.*, 2003). Santos *et al.* (2003) reported that antibacterial activity of LAB against pathogenic bacteria such as *E. coli* O157:H7 was due to aforementioned substances and/or inhibition of the pathogen adhesion. High inhibitory effect showed by *L. salivarius* RMLB32 and *L. acidophilus* RMLB9 may be attributed to higher production of these substances.

Table 2. Antibacterial inhibitory activity of LAB isolates inhibition against pathogenic *P. flurorescens* and *E. coli*

LAB Isolates	*P. flurorescens*	*E. coli*
	mm [a] (Mean ± SE)	
L. pentosus RMLB1	13 ±0.10	11.5±0.30
L. acidophilus RMLB9	20.5±1.01	21.4±1.10
L. plantarum RMLB20	11.5±0.20	12.3±0.03
L. pentosus RMLB22	12.5±0.26	10.9±0.03
L. salivarius RMLB32	21.8±1.03	22.4±0.02

[a] *Inhibition zones diameters presented as mean ± standard error*

In the areas of industry, environment and medicine, one of the main worries is bacterial adhesion. In many cases it is undesirable as it can lead to disturbance of the industrial processes or facilitate infection. However, it can also be a benefit as in the case when probiotics are used to promote intestinal health (Mattila-Sandholm *et al.*, 1999). Probiotic adhesion to the intestinal mucosa may prevent the binding of some pathogenic organisms (Bibiloni *et al.*, 1999).

In vitro, adhesion activity of the examined LAB strains showed that they could adhere to intestinal mucous with variable extents. The adhesion ability varied significantly ($P<0.05$) between the different strains and between the same strain types. The highest adhesive level, compared to the reference strain of *Bifidobacterium bifidum,* was showed by *L. plantarum* RMLB20 followed by *L. salivarius* RMLB32 and *L. acidophilus* RMLB9 (Figure 1).

The obtained results indicate that among strains and species in the genus *Lactobacillus* there are many differences in the adhesive characteristics and these results agreed with several literatures (Jacobsen *et al.*, 1999; Jonsson *et al.*, 2004; Collado *et al.* 2006, MacKenzie *et al.*,2009).

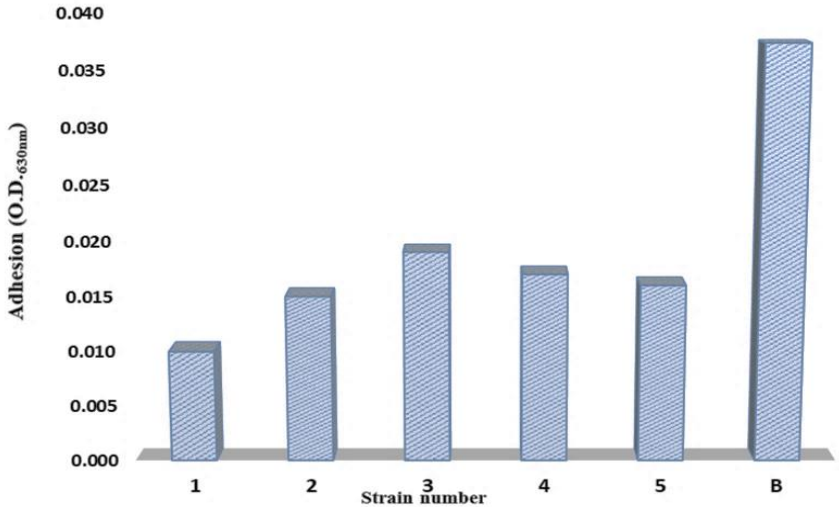

Figure. 1. Adhesion of LAB isolates to intestinal mucous. 1: *Lactobacillus pentosus* RMLB22; 2: *Lactobacillus pentosus* RMLB1; 3: *Lactobacillus plantarum* RMLB20; 4: *Lactobacillus salivarius* RMLB32, 5: *Lactobacillus acidophilus* RMLB9 and B: *Bifidobacterium bifidum.* Adhesion is expressed as the turbidity caused by crystal violet stain bound to the adhering bacteria as released by citrate buffer and subtracted from absorbance value of the negative control.

It has generally been assumed that adhesion to the GIT enhance the probiotic efficacy as it extends the period of the beneficial effects of probiotic organisms, such as immune system stimulation (Juntunen *et al.*, 2001). Furthermore, probiotic bacteria adhesion reduces the adhesion of pathogenic bacteria (Vélez *et al.*, 2010).

CONCLUSION

The obtained results highlight that the Egyptian traditional fermented milk "Laban Rayeb" could be a source for LAB isolates that could be used as natural antimicrobial agents. The tested *Lactobacilli* strains showed inhibition of undesirable bacteria and adherence properties. These results suggest them as a useful LAB in human and/or animal foods. However, further research is needed to carry out the *in vivo* effects of these strains.

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