INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 5, No. 10, p. 29-36, 2014

RESEARCH PAPER OPEN ACCESS

Inhibitory effect of *Lactobacilli* (isolated from Tarkhineh Dough) on *E. coli* and *Lis. monocytogenes* colonization

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Key words: *Lactobacilli*, Tarkhineh Dough, GIT Colonization, *E.coli*, *Lis.monocytogen*.

http://dx.doi.org/10.12692/ijb/5.10.29-36 Article published on November 26, 2014

Abstract

Lactobacilli are the most widely used probiotics that can inhibit the attachment of pathogenic bacteria through colonization and adhesion to epithelial cells or producing inhibitory compounds. In this study, we aimed to evaluate adhesion ability of *Lactobacillus* isolated from Tarkhineh Dough to Caco-2 cells in presence or absence of food-born pathogenic bacteria like *E.coli* and *Lis.monocytogenes*. In addition, we studied inhibitory effects of these isolates on above pathogenic bacteria. 16 strains of *Lactobacillus* isolates were grown under anaerobic conditions at 37°C for 24h. The Caco-2 cells were used in assay of *Lactobacillus* isolates adhesion. The inhibitory effects of these strains against *E.coli* and *Lis. monocytogenes,* were evaluated by diffusion method through measuring the ability of Caco-2 cells for adhesion or production of inhibitory compounds**.** TD16 showed the strongest attachment among the other isolates with more than 2×10^4 CFU/Well. In a competitive inhibition assay, TD3, TD4, TD12, and TD16 strains indicated more than 50% inhibitory effect on adhesion ability of *E.coli* and *Lis. monocytogenes* to Caco-2 cells. TD7 and TD14 revealed similar effects on attachment of *Lis. monocytogenes*. Moreover, the antagonist effects of *Lactobacillus* isolates which analysed through well diffusion method showed that TD5 had the strongest inhibitory effect on *Lis. monocytogenes* while TD3 and TD6 had the highest inhibitory effect on *E. coli*. Production of antimicrobial compounds like bacteriocin may be related to the inhibitory effects of *Lactobacillus* isolates against pathogenic bacteria, so application of probiotic strains such as *Lactobacilli* can be one of the therapeutic approaches in bacterial infections.

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Introduction

Probiotics are defined as live microorganisms that make a beneficial effect on the host by improving its intestinal microbial balance(Ingrassia *et al.*, 2005, Yli-Knuuttila *et al.*, 2006). They are the main members of *Lactobacilli* and *bifidiobacteria* family. In order to reach Gastero-Intestinal Tract (GIT) and exert their health function, they should be able to overcome biological barriers including acid in stomach and bile in intestine (Coeuret *et al.*, 2004).

Evaluation of adhesive features is regarded as an important step in screening new probiotic bacteria (Bogovic Matijasic *et al.*, 2003). The ability of attachment to intestinal cells is considered as a critical feature for survival and GIT colonization of bacteria (Jankowska *et al.*, 2008). Enterocyte-like Caco-2 cells (Pinto *et al.*, 1983, Chauviere *et al.*, 1992), as an intestinal cell model, has been used to examine the function of *Lactobacillus* and *bifidobacterium* for invasion or adhesion to pathogenic bacteria (Jacobsen *et al.*, 1999). It has been shown that lactic acid bacteria had inhibitory activity toward the growth of pathogenic bacteria including *Lis. monocytogenes*, *E.coli*, *salmonella* and other food-born pathogenic bacteria. This inhibitory activity could be due to producing inhibitory compounds such as organic acids, hydrogen peroxide and bacteriocin or even through competing for attachment (Jacobsen *et al.*, 1999, Bernet-Camard *et al.*, 1997). Previous studies revealed that lactic acid bacteria could inhibit pathogen attachment by reducing their colonization to a great extent and consequently could prevent infection (Jacobsen *et al.*, 1999). In the present study, the Caco-2 cells were used to investigate the adhesive characteristics of 16 strains of *Lactobacilli* isolated from traditional dairy products of Iran named as Tarkhineh Dough. Tarkhineh Dough is a kind of traditional dairy product in west rural areas of Iran. It is made from ewe's or cow's milk or even mixture of them. This dough was used as starter to make Tarkhineh from mushed wheat. The Iranian consumers prefer to use Tharkhineh not only due to its excellent natural taste and flavor but also because of therapeutic effects especially in treatment of infectious diseases. Hence, due to broad evaluations, such products are potentially good candidates for isolating new strains of probiotics and they can be considered as natural functional foods. The aim of this study is understanding the inhibitory effect of *Lactobacilli* on food borne pathogens. For this purpose, we evaluated 16 strains of the bacteria isolated from Tarkhineh Dough for GIT colonization on Caco-2 cells.

Materials and methods

Bacterial strains and growth conditions

In our experiment, we used 16 strains of *Lactobacillus spp* which were isolated previously from Tarkhineh dough by Tajabadi Ebrahimi *et al* and were kept at Microbial center of Islamic Azad University at -80°C in 25% sterile glycerol (10, 11).

E. coli(ATCC 2143) and *Lis. monocytogenes* (ATCC 345) as food born pathogenic bacteria were obtained from Persian Type Culture Collection (PTCC) of the Iranian Research Organization for Science and Technology in Tehran.

The *Lactobacillus* isolates were Grown in MRS broth medium (Fluka and Catalogue no. 69966, Sigma-Aldrich) under anaerobic conditions at 37°C for 24 h. The pathogenic indicator bacteria were cultured in brain and heart infusion broth (Merck, Germany) at 37°C for 24 h. For long-term storage, the bacteria were kept at -80°C in 25% sterile glycerol. Each strain was subcultured twice prior to the tests.

Intestinal cell culture

The Caco-2 cell culture used in cell adhesion assay was obtained from Pasteur Institute of Iran. The cells were cultured in Dulbecco's Modified Eagle's Minimal essential medium (DMEM) containing 25mM glucose, 20% (vol/vol) heat inactivated fetal calf serum and 1% non-essential amino acids. The cells were grown at 37 °C in 5% CO_2. In order to study their attachment, 1×10^5 of Caco-2 cells in one milliliter of cell medium were incubated in each chamber slide. After 24 hours, monolayers of cells were washed with Phosphate Buffer Saline (PBS) (pH7.2)(Lee *et al.*, 2000).

Adhesion assay on Caco-2 cells

Caco-2 monolayers were washed twice with sterile PBS before proceeding for adhesion assay. 300µl of phosphate buffer containing *Lactobacillus* isolates at concentration of 10^8 CFU/ml was added to each chamber and incubated at 37 °C in 5% CO_2. After 90 minutes of incubation with mild cell shaking, the monolayers were washed twice with PBS for excluding the non-attached bacteria (Lee *et al.*, 2000, Lee *et al.*, 2003).

Counting the cells attached to Caco-2 cells

300µl of trypsin was added to each chamber. After 30 minutes, 30µl of calf serum was added in order to neutralize the trypsin. Finally, the cells isolated from each chamber were mixed with 9.7ml PBS. After preparing dilution serials, cells were counted on MRS agar, Eosin-Methylene Blue (EMB) agar and *Listeria* selective agar to evaluate attached *Lactobacillus*, *E. coli* and *Lis. monocytogenes,* respectively.

Antagonist activity

In this study, inhibitory effects of selected strains on *E.coli* and *Lis. monocytogenes* were evaluated based on competing in attachment and producing inhibitory compounds.

Competitive inhibition assay

Caco-2 monolayers were washed twice with sterile PBS before proceeding for competitive inhibition assay. 300µl of PBS containing *Lactobacillus* isolates at concentration of 10^8 CFU/ml was added to each chamber and incubated for 90 min in the same condition as described previously. All chambers were washed twice with PBS and non-attached *Lactobacilli* were excluded. Subsequently, 300 µl of PBS containing either *E. coli* or *Lis. monocytogenes* at concentration of 10^8 CFU/ml was added to each chamber and incubated for 90 min. Afterward, the chambers were washed and the cells were dissociated from the chamber as described previously and counted on selective media. *E. coli* and *Lis. monocytogenes* were separately added in two different chambers to evaluate their attachment ability.

Producing inhibitory compounds

Lactobacillus isolates were assayed for evaluating the production of inhibitory compounds such as bacteriocin using well diffusion method as described by Tagg *et al.* (Tagg *et al.*, 1976). MRS media containing *Lactobacilli* was centrifuged at 7000 rpm for 15 min and the pH of supernatant was adjusted on 7.0 ± 0.1 by using 1mol/l of NaOH . The supernatant was then filtered via a 0.22µl pore-size filter. The supernatant was examined directly through well diffusion method. 25ml of soft MRS agar (0.7% w/v agar) with the indicator strains such as *E.coli* or *Lis.monocytogenes* was solidified at room temperature at the final concentration of 10^6 CFU/ml. Wells (5mm) were put in the plates containing solidified agar and filled by 100µl of the suitable supernatant. In order to diffuse supernatant, the plates were kept at 5°C for 2h and subsequently incubated in aerobic condition for 24 h at 37°C. The diameter of inhibitory zone was measured in the plates (Strompfová *et al.*, 2004, Schillinger and Lucke, 1989).

Statistical analysis

Results are shown as Mean ± SEM (standard error of mean) evaluated by prism software.

Results

Adhesion of Lactobacillus isolates on Caco-2 cells

Lactobacillus isolates were differently adhered to Caco-2 cells. These isolates were counted on MRS media using dilution serials. TD1, TD9, TD10, TD11 and TD13 strains showed weak attachments (the number of colonies on MRS agar was < 2×10^3) to Caco-2 cells. TD2, TD3, TD4, TD6, TD7, and TD12 isolates revealed middle adhesion (the number of colonies on MRS agar was 2×10^3 to 2×10^4). TD5, TD14 and TD16 strains represented strong attachments (the number of colonies on MRS agar was ≥ 2×10^4). Among the studied strains, TD16 showed the strongest attachment (Figure 1).

Inhibitory effect of Lactobacillus isolates on E.coli and Lis. monocytogenes competitive inhibition

After preparing serials, the attachments of *E.coli* and

Lis. monocytogenes to Caco-2 cells were studied in presence of *Lactobacillis* isolates. In Competitive inhibition assay, TD3, TD4, TD12, and TD16 showed inhibitory effects (more than 50%) on attachment of *E.coli* to Caco-2 cells (Figure 2). On the other hand, TD3, TD4, TD7, TD12, TD14, TD16 showed inhibitory effects (more than 50%) on attachment of *Lis. monocytogenes* to Caco-2 cells (Figure 3).

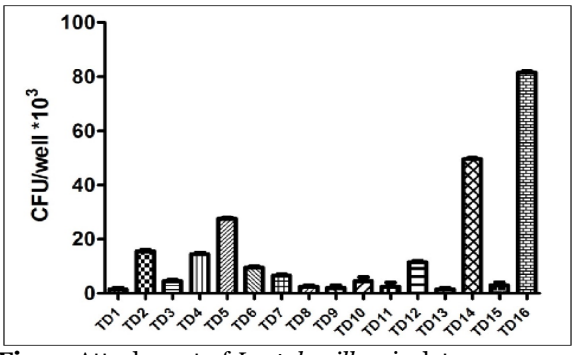

Fig. 1. Attachment of *Lactobacillus* isolates on *caco-2 cells*. Each value is mean ± SEM for three separate experiments.

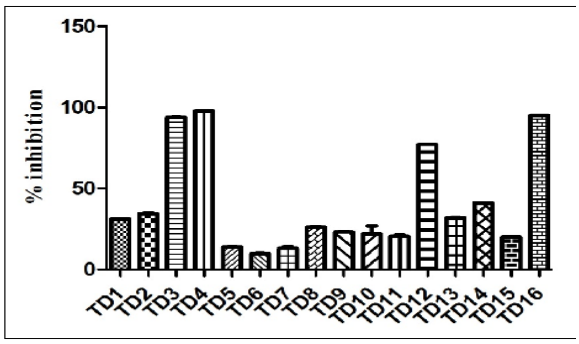

Fig. 2. Competitive inhibitory effects *of Lactobacillus* isolates *on E.coli.* Each value is mean ± SEM for three separate experiments.

Producing inhibitory compounds

The antagonist effects of *Lactobacillus* isolates on *E. coli* and *Lis. monocytogenes were* assayed. Results demonstrated that TD5 isolate had the highest antagonist effect with more than 10 mm inhibition zone and TD1, TD8, TD11 and TD14 isolates had the lowest antibacterial effects with less than 5mm inhibition zone against *Lis. monocytogenes* (Figure 4). Furthermore, TD3 and TD6 isolates had the strongest antagonist effects with more than 10 mm inhibition zone while TD11 and TD12 had the least antagonist effects with less than 5mm inhibition zone on *E. coli* (Figure 5).

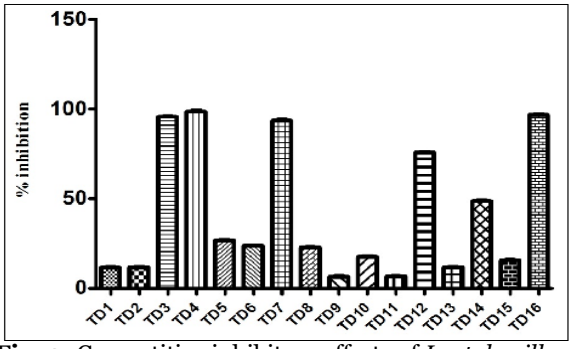

Fig. 3. Competitive inhibitory effects of *Lactobacillus* isolates on *Lis. monocytogenes*. Each value is mean ± SEM for three separate experiments. Each value is mean ± SEM for three separate experiments.

Discussion

The ability of probiotic bacteria for attachment to intestinal cells is considered as an important parameter for colonization and provides potentially beneficial effects. The first objective of this study is the evaluation of the adhesion ability of the isolates for attachment to the Caco-2 cells. The ability of *Lactobacillus* strains to adhere to the Caco-2 cells strongly varies among different strains. It means that the adhesion ability of *Lactobacilli* is not a universal feature of *the bacteria*. Kleeman & Klaenhammer (1982) demonstrated di- and trivalent cations which act by providing an ionic bridge between epithelial cells and bacterial surfaces. They claimed that the attachment of *Lactobacilli* to Caco-2 cells was increased by Calcium cations (Kleeman and Klaenhammer, 1982). In several studies, the adhesion ability of *Lactobacillus* isolates was evaluated by staining method. In the current study, adhered cells were determined by counting colony forming units (CFU) on selective media regarding to low precision of staining method and microscopic counting (Bogovic Matijasic *et al*., 2003). Based on the results of this study, the adhesion ability varied among the isolates. According to reported studies on *Lactobacilli,* there were different adhesion affinities for applied isolates (Bogovic Matijasic *et al*., 2003, Sarem *et al*., 1996, Tuomola and Salminen, 1998). Among these isolates, TD16 had the strongest adhesion ability for attachment to the Caco-2 cells which carried on till the saturation of binding sites on the Caco-2 cells. In fact, important features for

adhesion ability of these strains were not only based on the number of binding sites but also the adhesion affinity of the isolates. The differences between these isolates were plausible, because it has been previously demonstrated that adhesion ability was not related to the bacteria but was actually dependent to specific strains (Bogovic Matijasic *et al.*, 2003). Moreover, the mechanism of bacterial attachment would be facilitated by an appropriate cell culture. Unfortunately intestinal cell culture is not readily available. Furthermore, the poor viability of these cells and various cell donors led to different results in bacterial attachment (Chauviere *et al.*, 1992, Tuomola and Salminen, 1998).

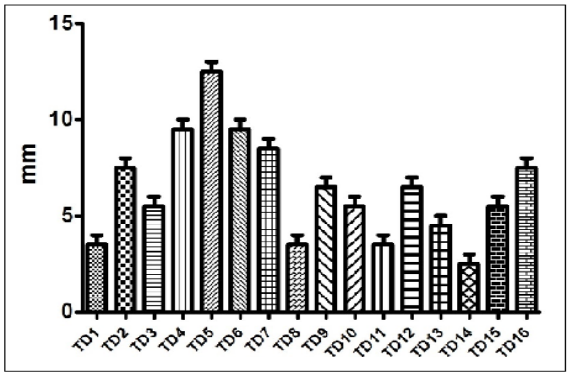

Fig. 4. Antagonist effect of *Lactobacillus* isolates on *Lis. monocytogenes* by well-diffusion method. Each value is mean ± SEM for three separate experiments.

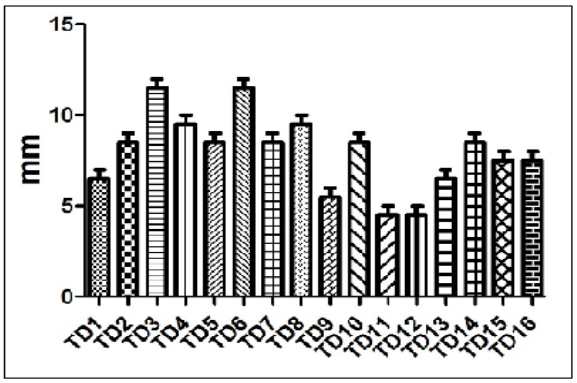

Fig. 5. Antagonist effect of *Lactobacillus* isolates on *E. coli* by well-diffusion method. Each value is mean ± SEM for three separate experiments.

Different reports have confirmed that probiotics can inhibit pathogen attachment to the epithelium through occupying adhesion sites (Bernet-Camard *et al.*, 1997, Greene and Klaenhammer, 1994, Chauviere *et al.*, 1992, Coconnier *et al.*, 1993, Gopal *et al.*, 2001). Other inhibition mechanisms such as producing lactic acid, exopeptides, or exopolysaccharides can inhibit the attachment of pathogenic bacteria (Jankowska *et al.*, 2008). Isabelle *et al.* showed the *in vitro* inhibitory effects of *Lactobacillus casei* DN-114001 on adhesion to intestinal epithelial cells by invasive *E.coli* bacteria isolated from crohn disease (Ingrassia *et al.*, 2005). Based on the results of this study, pre-inoculation of Caco-2 cells with *Lactobacillus* isolates prior to infection with *E. coli* and *Lis. monocytogenes* led to a decrease in pathogen adhesions. Furthermore, it can be assumed that more inhibitory effects may be associated with the increased adhesion of *Lactobacillus* isolates. Adhered strains were able to exclude pathogenic bacteria at various levels. For example, TD14 and TD16 with the highest adhesion affinity to Caco-2 cells (more than 55% and 95% respectively) could hinder the attachment of pathogenic bacteria. However, the reduction in pathogen attachment by *Lactobacillus* isolates was not closely concerned with adhesive capabilities of *Lactobacilli*. For example, TD3, TD4 and TD12 inhibited *E. coli* and *Lis. monocytogenes* adhesion to epithelial cells to approximately 95%, 98% and 75% , respectively. On the other hand, data clearly demonstrated that each *Lactobacillus* could only compete with a limited range of pathogenic bacteria for binding to adhesion sites. For example, Yuan-Kun Lee *et al.* confirmed that L. casei GG was not capable of competing with the enteropathogenic Ecoli strain O157 (Yuan-Kun *et al.*, 2003). According to this study, despite of competitive inhibition of *Lis. monocytogenes* by TD7, this isolate could not inhibit E. coli adhesion. It is proved that adhesion was site specific. Competition for a specific receptor was due to steric hindrance. In other words, the binding of this strain prevented from attachment of *Lis. monocytogenes* to the receptor (Yuan-Kun *et al.*, 2003). The potential probiotic isolates were screened by well-diffusion method for evaluating their antagonist activity against *E. coli* and *Lis. monocytogenes*. Many studies have performed on the nature of antibacterial substances secreted by *Lactobacillus* isolates. Alicja jankowska *et al.* found

that supernatant of *Lactobacillus* medium containing compounds with molecular weight of 10-30 KD, caused the strongest inhibition on *Salmonella Enterica* growth. These results suggested the presence of some substances like peptides and proteins besides lactic acid in contents of supernatant (Jankowska *et al*., 2008). Production of antimicrobial compounds like bacteriocin may be related to the inhibitory effects of *Lactobacillus* isolates against pathogenic bacteria (Allison *et al*., 1994, Axelsson *et al*., 1993, Larsen *et al*., 1993, Parente and Ricciardi, 1999, Yamato *et al*., 2003, Zhu *et al*., 2000). Bacteriocins are proteinaceous antimicrobial substances produced by *Lactobacilli* and show bactericidal effect against other taxonomically related bacteria(Bernet-Camard *et al*., 1997). Other metabolic substances which are produced by *Lactobacillus* isolates such as lactic acid, peptides or exopolysaccharids may inhibit the attachment of pathogenic bacteria. Most of the inhibitory molecules have low molecular weight (less than 3 KD). In addition to potential bacteriocin production, sugar fermentation and reduction in pH due to the production of lactic acid seem to be important factors for inhibition of undesired microorganisms' growth (Jankowska *et al*., 2008). In this experiment, the inhibitory value for each strain was defined according to the inhibition zone. TD 12 had the least antagonist effect against *E. coli*. It showed more than 50% inhibitory effect against *E.coli* and *Lis. monocytogenes*. Hence, this strain is an excellent isolate for competitive inhibition rather than producing inhibitory compounds. Among the studied isolates, TD3 had the strongest antagonist effect on *E. coli*. Additionally, it revealed high competitive inhibition against *E.coli* and *Lis. monocytogenes*. Therefore, isolates such as TD3 with a high adhesion feature, strong ability of competitive inhibition, and more antibacterial effects were considered as the most effective inhibitory strains. The *Lactobacillus* isolates exert inhibitory effects on adhesion of pathogenic bacteria to intestinal epithelial cells by producing lactic acid and antimicrobial compounds such as bacteriocin. These features enable *Lactobacillus* isolates to be colonized in the GIT and compete with pathogenic bacteria. Thus, the use of these probiotics can have beneficial effects on bacterial infections and also can be a useful approach to select specific probiotics for therapeutic applications and treatment of those diseases which caused by pathogenic bacteria.

Acknowledgments

This paper extracted as part of the project which has been operated in Islamic Azad University, Tehran Central Branch, Iran. The authors are grateful for this support.

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