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IN VITRO EFFECTS OF VARIOUS PROBIOTIC PRODUCTS ON GROWTH AND BIOFILM FORMATION OF CLINICAL UPEC STRAINS

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Abstract: Uropathogenic *Escherichia coli* (UPEC) is the most prevalent pathogen causing urinary tract infections (UTIs). UPECs have various virulence factors such as adhesins, biofilm forming and toxin producing etc., to survive in urinary tract. Under certain circumstances probiotics are preferred for prevention and treatment of UTIs. In this study, we aimed to investigate the *in-vitro* effects of four different *Lactobacillus* spp. cell-free supernatants on growth and biofilm formation inhibition in clinically isolated UPEC strains. Growths of 50 UPEC strains were determined in 96-well microplate and measured in a spectrophotometer after four hours incubation at 37°C. Biofilm formation was detected by crystal violet staining method on three UPEC strains. Statistical analysis of growth and biofilm formation experiments were performed by Kruskal-Wallis and one-way ANOVA Tukey's multiple-comparison tests, respectively. All tested cell-free supernatans of lactobacilli inhibited growths (p<0. 0001) and biofilm formation (p<0.05) of UPECs. All results were found to be statistically significant. As a conclusion, our findings supported previous studies which reported the high efficiency of these four *Lactobacillus* spp. in the prevention of UTIs.

Keywords: *Lactobacillus*, growth, biofilm formation, UPEC, probiotics.

1. Introduction

Uropathogenic Escherichia coli (UPEC) have various virulence factors such as fimbrial adhesins, biofilm formation, siderophore, toxins, cytotoxic necrotizing factor-1, bacteriocins, endonuclease activity, and outer membrane protease. They are known as the leading pathogens causing urinary tract infections (UTI) (Mandal, 2001; Ruiz et al., 2002; Miyazaki et al., 2002; Bower et al., 2005; Sabaté et al., 2006; Yamamoto, 2007; Uzun et al., 2015). Biofilm formation capacity of UPEC strains is also an important advantage for the persistence and recurrence in infections caused by them. Besides, biofilm formation protects from host immunity and antimicrobial components (Freestone, 2013). Urinary tract infections (UTI) are seen about 40% in women, 12% in men for their life time and often recur within 6 to 12 months nearly in 25% of infected women (Kulkarni et al., 2009; Sivick

and Mobley, 2010; Li et al., 2010). Recurrent infections could be due to unsuccessful treatments which is related to antibiotic resistance and invasive infections (Hunstad and Justice, 2010; Andersen et al., 2012). Therefore some alternative strategies such as probiotics are useful and more cost effective for their treatment (Geerlings et al., 2014; Beerepoot et al., 2012; Delley et al., 2015; Lee et al., 2016).

Probiotics are known as live microorganisms which beneficially regulate host health (FAO/WHO, 2001). It is well known that the beneficial effects of lactobacilli depend on secreting several strong antimicrobial compounds such as organic acids, benzoic acid, acetic acid, formic acid different types of bacteriocins, bacteriocin-like inhibitory substances and hydrogen peroxide (Lash et al., 2005; Kim and Kim, 2009).

Lactobacillus spp. and *Bifidobacterium* spp. are the most administered bacteria, especially for prevention and control of oral, gastrointestinal and urogenital system diseases (Saavedra 2000; Tomas et al., 2003; Morelli et al., 2004; Servin, 2004; Morais, 2006; Segarra-Newnham, 2007; Guarino et al., 2009; Miyazaki et al., 2010; Guandalini, 2011; Wagner and Johnson, 2012; Behnsen et al., 2013; Turroni et al., 2014; Wu et al., 2015). For example, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* are known to inhibit biofilm formation of pathogens (Miyazaki et al., 2010; Wagner and Johnson, 2012; Wu et al., 2015). Cadieux et al., (2009) mentioned the antagonist effects of urogenital lactobacilli for UPECs and explained that their lethal effects do not occur only directly, but they can provide stress conditions for bacteria.

In the present study, we aimed to evaluate the in-vitro effects of four different lactobacilli cell-free supernatants on growth and biofilm formation in UPEC strains isolated from UTI patients.

2. Materials and Methods

Bacterial strains, media and culture conditions

In the present study cell-free supernatants of *Lactobacillus rhamnosus* ATCC 53103, *Lactobacillus fermentum* ATCC 9338, *Lactobacillus acidophilus* ATCC 314, and *Lactobacillus plantarum* ATCC 14917 were tested for their effects on growth and biofilm formation in UPECs. They were chosen according to their high usage in commercial probiotics (Karska-Wysocki, 2010; Nigam et al., 2012); therefore we prepared cell-free supernatants. All strains were stored in −80 °C prior to the experiments. De Man, Rogosa and Sharpe (MRS) broth and (MRS) agar (Conda, Spain) were used for isolation of all lactobacilli. Cultures were performed in anaerobic atmosphere (10% H_2, 5% CO_2 and 85% N_2) at 37°C for 48 hours. 50 UPEC strains from our culture collection were included in the present study; they were previously isolated from symptomatic, acute, uncomplicated UTI patients and they were proven for their pathogenic abilities (Uzun et al., 2015). Bacteria were kept in −80°C and revived after 10 years via inoculating into Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA). Overnight cultures of UPECs and all lactobacilli were prepared by inoculation of single colonies into TSB and MRS broth, respectively. The overnight cultures of each *Lactobacillus* strains were centrifuged at 4000 rpm for 30 minutes at 4°C; strains and the supernatants were collected then filtered by using 0.2 µm filter.

Effects of cell-free supernatants of Lactobacillus spp. on growth of UPECs

Initial bacterial concentrations of UPECs were arranged to 10^7 CFU/mL. UPEC strains were cultured into TSB alone (as control) or TSB supplemented with different supernatants of *Lactobacillus* (100 µL cell-free supernatants+80 µL TSB+20 µL bacteria) and

incubated at 37°C. Growths were determined by measuring the changes via spectrophotometer in absorbance (OD) at 600 nm in four hours period. The samples were tested in duplicate and each experiment was performed twice.

Effects of cell-free supernatants of Lactobacillus spp. on biofilm formation of UPECs

The biofilm formation in three out of 50 UPEC strains (which were determined previously as biofilm forming strains) were observed with crystal violet staining method.The effects of lactobacilli cell-free supernatans on three biofilm positive UPEC strains were analyzed. *E. coli* ATCC 25922 and MRSA ATCC 43300 were used as positive controls. The strains were cultured in TSB-glucose (1%v/v) for 24 h at 37 °C and diluted 1/50 in fresh TSB-glucose, yielding a final concentration of approximately 10^7 CFU/mL. 100 µL from bacterial suspension and 100 µL from cell-free supernatants of *Lactobacillus* probiotic products were added to each well of a 96-well tissue culture microtiter plate, and then incubated for 24 hours at 37°C. TSB-glucose was used as a negative control. After incubation, the waste media was gently aspirated, and the wells were washed 3× with 250 µL Phosphate Buffered Saline (PBS) solution to remove any unattached bacteria and air-dried. Then, 200 µL 99% methanol was added to each well to fixate for 15 min, then it was aspirated. Wells were stained with 200 µL 0.1% crystal violet (in water) for 5 min. Excess stain was gently rinsed off with tap water, and the plates were air-dried. The stain was re-solubilized by adding 200 µL 95% ethanol. The optical density was measured at 450nm.For the purposes of comparative analysis of test results, we introduced classification of adherence capabilities of tested strains into four categories (OD ≤ ODc non-adherent, ODc< OD ≤ 2x OD weakly adherent, 2xODc < OD ≤ 4 x ODc moderately adherent, 4xODc <OD strongly adherent) as described previously (Christensen et al., 1985).

Statistical analysis

Growth alterations were detected by using Kruskal-Wallis test. The effects of probiotic supernatants of *Lactobacillus* on biofilm formations of UPECs were determined via one-way ANOVA Tukey's multiple-comparison test. All measurements were compared to control conditions (TSB). Multiple comparisons were made at a level of $P < 0.05$.

3. Results and discussions

Effects of probiotic supernatants of Lactobacillus spp. on growth of UPECs

The direct antagonism of compounds contained in *Lactobacillus* cell-free supernatants against UPECs was monitored by turbidmetric method. Supernatants of all four *Lactobacillus* species inhibited growth of UPECs with a high rate of 99% when compared to control (TSB) (**Fig. 1**).

These results were found to be statistically significant ($p<0.0001$). To satisfy our own curiosity, we analyzed the effect of neutralized pH supernatants on the growth of randomly selected five UPEC strains and we detected that, supernatants with pH:6.8 did not alter the growth of UPECs ($p>0.05$).

Effects of cell-free supernatants of Lactobacillus spp. on biofilm formations of UPECs

All three biofilm forming strains were classified as weakly adherent according to Christensen et al., (1985) criteria. All tested cell-free supernatants of lactobacilli were shown to inhibit biofilm formation in three UPEC strains in 24 hours significantly ($p<0.05$) (**Fig. 2**).

Globally a large number of people suffer from urinary tract infections which are mostly caused by uropathogenic *E. coli* (Hacker et al., 1999; Kulkarni et al., 2009; Li et al., 2010). It is well known that *Lactobacillus* strains have antibacterial effects with their secreted compounds such as bacteriocin or exopolycacharides and organic acids (Makino et al., 2006; Hagan and Mobley 2007; Nader-Macías et al., 2008; Cadieux et al., 2009; Martín and Suárez, 2010; Stoyancheva et al., 2014). In our study we aimed to detect the inhibitory effects of four different *Lactobacillus* spp. cell free supernatants on growth and biofilm formation because of their widely usage in dairy products, fruit drinks, chewing gums and tablets which are available on market (Karska-Wysocki, 2010; Nigam et al., 2012).

Cadieux et al., (2009) have documented that some *L. rhamnosus* and *L. reuteri* strains could affect UPECs surface membrane traits. Delley et al., (2015) have shown that *L. acidophilus*, *L. rhamnosus* and *L. johnsonii* strains' cell free supernatants inhibited some UPEC strains. Similarly Tomas et al., (2011) have shown the growth inhibition of UPECs in the presence of *L. acidophilus*. Ocana et al., (1999) have also observed that some *L. acidophilus* strains (CRL 1259, CRL 1307, CRL 1320 and CRL 1324) inhibited the growth of *E. coli*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Enterococcus faecalis*, *Klebsiella*, *N. gonorrhoeae* and *G. vaginalis*. Terraf et al., (2017) have detected that supernatants from *L. reuteri* and *L. rhamnosus* inhibited the growth of UPEC strains. Miyazaki et al., (2010) shown that supernatants of *L. casei* subspecies and *L. acidophilus* inhibited the growth of the EAggEC TN-2 strain

Many studies have suggested that in order to prevent growth of pathogenic microorganisms that cause urogenital infections, probiotic products can be used. Some researchers suggested that antagonistic effects (bacteriostatic or bactericidal) of *Lactobacillus* on growth of *E. coli* related to the presence of organic acids that are released during growth (Axe and Bailey, 1995; Diez-Gonzalez and Russell, 1997). In line with previous studies we detected that, supernatants of *L. acidophilus*, *L. plantarum*, *L. fermentum* and *L. rhamnosus* supernatants inhibited growth of UPECs.

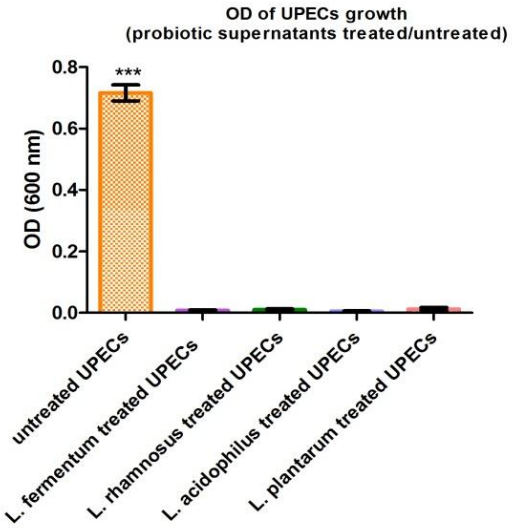

Fig. 1. Effects of different cell-free supernatants on growth in UPECs

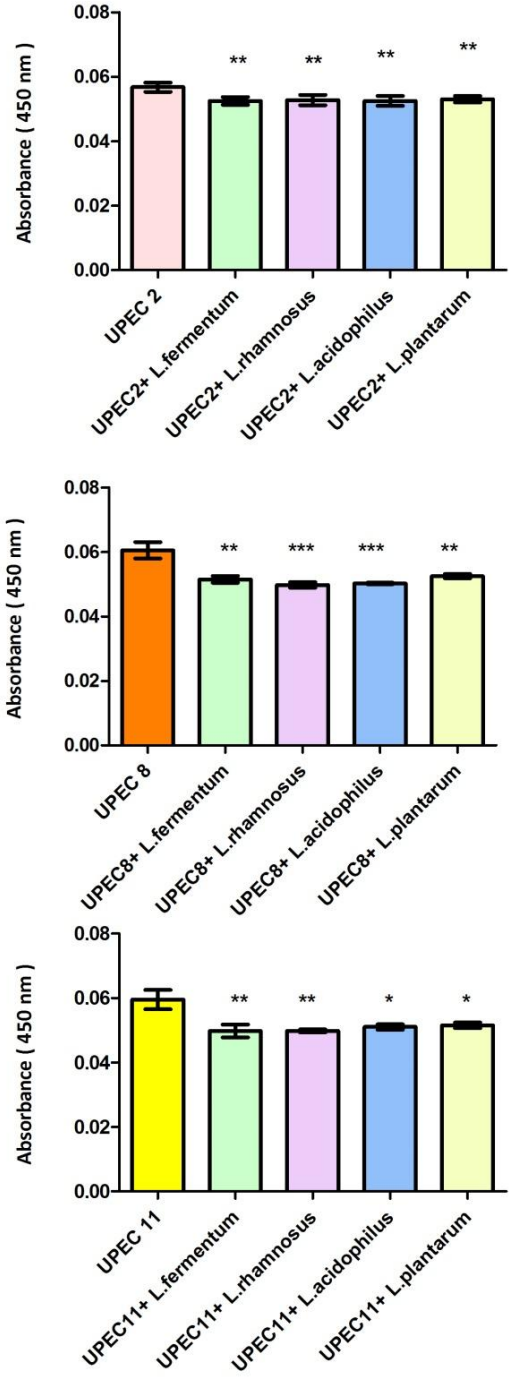

Fig. 2. Effects of different cell-free supernatants on biofilm formations in three different UPEC strains

Therefore we wanted to investigate whether the 99% of inhibition with probiotic supernatants depends on pH or not. Our results with a randomized selected five UPECs showed that, high level acidity leads the major inhibition on growth of pathogens. Evidence supports that the antagonist effects of *Lactobacillus* may be variable depending on exposure time, test microorganism, temperature as well as pH (high acidity) (Ogawa et al., 2001; Lash et al., 2005; Poppi et al., 2015).

The most known anti-biofilm activity is related to exopolysaccharides in *Lactobacillus* supernatants (Barken et al., 2008; Kim and

Kim., 2009; Wang et al., 2015). The first step of biofilm formation is adhesion to surface then multiplication of bacteria to compose extracellular polymeric matrix. Communication system which is known as quorum sensing (QS) plays an important role in adhesion and biofilm formation (Waters and Bassler, 2005; Bassler and Losick, 2006). QS provides cell to cell communication and it is important for bacterial survival and interactions in natural habitats. Previous studies have shown that there is a strong association between *Lactobacillus* supernatants and repression of the genes related with initial adhesion and chemotaxis (Balaban et al., 2007; Wang et al., 2015). It was suggested that, Lactobacilli supernatants could play role as molecules in reducing biofilm formation and quorum sensing related gene expressions (Balaban et al., 2007; Wang et al., 2015; Zamani et al., 2017). Sadri et al., (2016) have suggested that *L. acidophilus* inhibited adhesion of UPECs. Zamani et al., (2017) reported that *L. plantarum* isolated from a traditional cheese had anti-biofilm potential for *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *E. coli*. Vacheva et al., (2012) reported that *L. gasseri* Lb821, *L. plantarum* LbS11 had anti-biofilm effects on *E. coli* strains. On the other hand, Miyazaki et al., (2010) reported that the supernatants of *L. casei* and *L. rhamnosus* stimulated biofilm formation of enteroaggregative *Escherichia coli*. In our study, all the tested *Lactobacillus* supernatants were found to inhibit biofilm formation in UPEC strains in consistency with the results of many other studies (Kim and Kim., 2009; Vacheva et al., 2012; Aminnezhad et al., 2014; Zamani et al., 2017).

Conclusions

As conclusion in our study it was shown that, the growth of 50 different clinical UPEC strains were inhibited by *Lactobacillus* spp. with a rate of 99%. Besides, biofilm formations of three UPECs were also inhibited significantly in the presence of cell-free supernatant of four *Lactobacillus* strains tested. Therefore consistent with previous studies, our findings support that these four lactobacilli may be used to prevent the UTIs caused by UPEC strains, effectively.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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