Lactobacillus acidophilus and its Inhibitory Effect on Pyelonephritis Associated Pili and Outer Membrane Protein Expression in Escherichia coli

Mohammad Reza Fazeli¹, Mehdi Moazzami Goudarzi² and Abbas Akhavan Sepahi³

¹Department of Drug and Food Control, Pharmaceutical Quality Assurance Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.
²Department of microbiology, Faculty of basic science, Islamic Azad University, Science and Research branch, Tehran, Iran. (Corresponding author).
³Department of microbiology, Faculty of basic science, Islamic Azad University, North of Tehran Branch, Tehran, Iran.

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Uropathogenic strains of Escherichia coli (UPEC) can colonize the vagina and kidney and cause infections within the entire urogenital tract. Novel therapeutic approaches are needed to combat the urinary tract infection in women. In our study, first a unique and simple reporter construct for pap and OmpA promoter activity constructed which included luciferase coding sequence of Lampyris turkestanicus on pET28a vector, replacing existing T7 promoter separately. Bioluminescence activity of both recombinant reporter strains assessed when exposed to Lactobacillus acidophilus biomass and culture supernatant respectively. In a dose dependent manner, culture supernatant of L. acidophilus strongly inhibited recombinant reporter UPEC growth and increased the promoter activity of outer membrane proteins (OMPs) A. porins normally up regulated in response to UPEC membrane stress. Supernatants also down regulated the expression of pap operon, critical adherence factors within the urogenital tract. Our findings indicate that compounds secreted by lactobacilli likely protect the urogenital tract from UPEC colonization and infection by inhibiting growth, inducing stress and down regulating proteins critical for host attachment. The present study and others demonstrated that the production of pap fimbriae and outer membrane proteins in UPEC correlates with environmental stimulus and potentially affected by probiotics.

Key words: Lactobacillus acidophilus, pyelonephritis associated pili.

Urinary tract infection (UTI) is the most widespread infection in women worldwide after intestinal infection¹. Asymptomatic UTI affected more than 50% of women during the life²⁴. This implies that an asymptomatic E. coli reservoir likely exists in a substantial portion of women and that the local vaginal environment plays an important role in preventing urogenital tract infections from developing. Overall, the high prevalence of E. coli in vaginal colonization and subsequent UTI is primarily due to the large numbers constantly shed in the feces, promoting frequent urogenital contact⁵⁸. E. coli inherently exhibits a number of attributes for surviving varying environmental conditions including their short generation time, ability to metabolize a wide variety of carbon sources and facultative anaerobic
metabolism. However, only a select number of strains can successfully survive, colonize and cause infection within the urogenital tract. Possible sequel include pyelonephritis which can lead to renal scarring and sepsis\textsuperscript{10-11}. uropathogenic \textit{E. coli} (UPEC) exhibits a set of specific virulence factors (VFs) involved in host cell attachment and invasion, biofilm formation, host-cell cytotoxicity, iron acquisition, evading host defenses and increased antibiotic resistance\textsuperscript{12}. A number of virulence determinants facilitate the ability of Uropathogenic \textit{E. coli} to colonize the urinary tract and exert cytopathic effects, including type 1 fimbriae\textsuperscript{13}, P fimbriae\textsuperscript{14}, Dr adhesions\textsuperscript{15}, hemolysin\textsuperscript{16,17}, cytotoxic necrotizing factor 1\textsuperscript{18}, flagella\textsuperscript{19}, capsule polysaccharide\textsuperscript{20}, lipopolysaccharide O antigen\textsuperscript{21}, and TonB-dependent iron transport systems\textsuperscript{22}. During UTI outer membrane proteins of Uropathogenic \textit{E. coli} like porins (OmpA, OmpC, OmpX, NmpC, and LamB) and Outer membrane assembly factors, overexpressed\textsuperscript{23}.

Lactobacilli, typically compose the predominant population in the vaginal microbial community. They are the most commonly isolated organisms from the vaginal microbiota of healthy women\textsuperscript{24,25}. Lactobacilli can produce some secondary metabolites to overcome competitor populations in vaginal community. These metabolites include organic acids, biosurfactants, hydrogen peroxide and bacteriocins.

In this study, we examined the effects of \textit{Lactobacillus acidophilus} and its by-products on UPEC growth and VF expression including OmpA and OmpX and pap operon. Due to key role of attachment and survival in the highly acidic environment of the vagina, we selected those virulence factors associated with attachment and acid compatibility. This study was performed to investigate how \textit{Lactobacillus acidophilus} and its by-products can affect the expression of pap fimbrial operon and the outer membrane porins OmpA and OmpX. The fimbrial operon includes \textit{pap} operon, containing 7 to 8 structural genes responsible for constructing pyelonephritis-associated pili\textsuperscript{26, 27}. P pili are associated with \textit{E. coli} strains capable of directly infecting upper urinary tract tissues\textsuperscript{28}. The \textit{pap} operon is controlled by a phase variation mechanism in which individual bacterium could express (ON) or repress fimbriae (OFF)\textsuperscript{28}. Phase variation in this operon is controlled at the transcriptional level by a complex epigenetic mechanism involving the formation of specific DNA methylation patterns, similar to that observed in certain eukaryotic systems\textsuperscript{29}. UPEC survival under environmental stresses such as increased acidity involves a number of factors aimed at maintaining membrane integrity and cytoplasm neutrality. Outer membrane proteins have been shown to play an important role in these processes by controlling the movement of compounds across the outer membrane\textsuperscript{30,31}.

**MATERIALS AND METHODS**

**Primers, plasmid, strains and mediums**

We used \textit{Escherichia coli} DH5\textalpha, as standard strain and UPEC isolate (from a patient with pyelonephritis) as a wild type and \textit{lactobacillus acidophilus} (isolated from vagina) to investigate its effect on gene expression. We employed Recombinant cloning vector pET28a, containing \textit{Lampyris turkestanicus} (Iranian firefly) luciferase coding sequence (GenBank accession #AY742225.1). This cloning vector carried restriction sites for \textit{BamHI} and Hindl\textII, flanking the luciferase coding sequence. All primers, strains, and plasmid employed here are listed in Table 1. UTI isolate, and DH5\textalpha was maintained using Luria-Bertani (LB) media. \textit{lactobacillus acidophilus} was inoculated in Man, Rogosa and Sharpe (MRS) media. Co-culture of lactobacillus and UPEC isolate performed in modified M9 (enriched M9 medium {EM9}) including yeast extract, because UPEC isolate grew poorly in MRS medium. Assessment for the effect of lactobacillus supernatant was performed in standard M9 medium.

**Amplification of pap and OmpA promoters**

First, \textit{pap} regulatory region (406 bp) was amplified. Primer \textit{PAPF1} containing \textit{BglII} recognition site, and \textit{PAPR1} carrying \textit{BamHI} recognition site were applied in PCR with the following conditions: Pre-denaturation at 94°C for 3 min, denaturation at 94°C for 1 min, annealing at 57°C for 1 min, extension at 72°C for 1 min. This procedure was repeated for 35 cycles. Eventually, final extension at 72°C for 3 min performed. After that \textit{OmpA} promoter was amplified with \textit{OmpAF1} and \textit{OmpAR1} primers, containing \textit{BglII} and \textit{BamHI} recognition sites. The PCR performed at the same
condition with the exception of annealing temperature that adjusted in 59°C.

Construction pap-luciferase and omp – luciferase vectors

First, pET28a vector, carrying luciferase coding sequence was double digested with BamHI and BglII. Then pap-PCR product was double digested with the same restriction enzymes. Both vector and PCR product were ligated together to construct luciferase controlled papon new vector, named pETpap28a (Figure 2). The ligation mixture contained following elements: 2 µl of double digested vector, 10 µl of double digested pap regulatory region PCR product, 1 µl of T4 DNA ligase, 5 µl of ddwater, and 2 µl of T4 ligase buffer, in a final volume of 20 µl at 16°Cforemost7 h. At the same condition cloning OmpA – luciferase vector (pETOmpA28a) constructed separately. After construction, each of recombinant vectors transformed into UPEC isolate to prepare reporter light emitted strains separately.

Bacterial growth

Promoter construct transformants were analyzed for growth and promoter activity during 24 hours exposure to lactobacilli and/or their by-products separately in EM9 and MM9 medium. In the case of culture supernatant (CS) To reach standard growth conditions, bacteria were cultured in a 100 ml Erlenmeyer containing 10 ml of M9 glycerol (M9 minimal medium containing 30 mM thiamine, 100 mM calcium chloride, 1 mM magnesium sulfate, and 0.2% glycerol as carbon source, pH 7). But to assess the effect of lactobacillus Cs, modified M9 medium was prepared with including 0.1% to 1.5% of lactobacillus Cs as serial dilution.

In the case of co-culture of reporter strains and lactobacillus acidophilus EM9 medium was selected. Cultures were inoculated to 1x10⁵ CFUs/ml in a shaking incubator as following conditions: 200 rpm, 37°C, and 18 to 24 h.

Measurement of bioluminescence activity

In this procedure equal volume (that is, 20 µl) of both overnight culture of reporter strain and luciferin substrate solution, was mixed in luminometer cuvette. Then light emission measured at 530 nm. Intensity of emitted light was measured according to bioluminescence unit: relative light unit per second (RLU/S). Substrate solution comprising: (i) 4mM ATP solution, (ii) 2 mM D luciferin, (iii) 5 mM MgSO₄ solution, (iv) 50 mM Tris-HCl. Eventually, pH of substrate solution was adjusted to 7.8 by Tris-HCl. The stock solution was kept frozen at -20°C. All the samples were assessed for light emission every 2 hours, just when they had same optical density.

RESULTS

After treatment of both recombinant strains in the condition which described above light emission was assessed. In the co-culture method pap promoter activity determined 2021 RLU/S when reporter UPEC strain exposed to lactobacillus

<table>
<thead>
<tr>
<th>Primers</th>
<th>Name</th>
<th>sequence*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ompA</td>
<td>ompA F₄: 5′-AAGATCTCTCAGCCACGAGACA</td>
<td>this study</td>
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<tr>
<td></td>
<td>ompAR₅: 5′-TACGATTAATCTGCTCAATATTAAC</td>
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<td>PAP F₅: 5′-TCAGATATTCATCATCTCAGT-3′</td>
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<td></td>
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<tr>
<td>Region</td>
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<td></td>
<td></td>
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<tr>
<td>Vector</td>
<td>pET28a</td>
<td>T7 promoter + Iranian firefly luciferase coding sequence</td>
<td>Novagen</td>
</tr>
<tr>
<td>Strains:</td>
<td>Name</td>
<td>Relevant genotype and property</td>
<td>Reference</td>
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<tr>
<td>DH5₅</td>
<td>Sup E₁, DLacU 169(F80 Lac Z DM15) hsdR17 Yec A₁ endA₁ gyr A96 thi-1 rel A₁</td>
<td>NIGEB</td>
<td></td>
</tr>
</tbody>
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Pyelonephritis patient isolate (wild type) pap’ LALEH hospital’
BglII and BamHI restriction sites are underlined.
§National Institute for Genetic Engineering and Biotechnology, Tehran, Iran.
*Department of microbiology, LALEH hospital, Tehran, Iran.

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Lactobacillus acidophilus in the EM9 medium. We used of EM9 including 2% of glucose as a classical inhibitor for pap promoter activity. Light emission of reporter strain in this medium concerning as negative control which detected 5208 RLU/s. But OmpA Promoter activity in the co-culture condition was clarified in 3520RLU/s. EM9 medium that adjust at pH: 7 used to down regulate OmpA promoter. Light emitted reporter strain considered as negative control and clarified in 312 RLU/s. OmpA promoter was able to emit in 3193 RLU/s when inoculated in EM9 alone.

The Supernatant of lactobacillus culture which used in continued mood would be able to affect the pap promoter activity. Interestingly reporter UPEC strain prepares 3050 RLU/s emission when inoculated in the standard M9 (but not MM9). We consider the behavior of pap activity in M9 medium as control. By the way pap promoter activity increased significantly in MM9 including 0.1% to 0.3% CS. And pap promoter activity decreased when 0.4% to 0.5% of CS was exposed. In the next MM9 mediums light emission was increased with the exception of 1%, 1.1% 1.3%1.5% (figure 2a).

OmpA promoter activity significantly improved by increased level of lactobacillus CS in MM9. Reporter recombinant strain which inoculated in Standard M9 medium considered as control. M9 conditions, stimulates the reporter strain to emit in 753RLU/S (figure 2b).

Fig. 1. Recombinant construct for pap and OmpA promoter activity monitoring

Fig. 2. The effect of serial concentration of culture supernatant on pap (A) and OmpA (B) promoter activity
DISCUSSION

The urogenital tract is constantly under assault by microorganisms. Usually these microorganisms originated from the surrounding environment specially the skin and feces. Nevertheless of Vaginal microbiota, there are only a select number of pathogenic bacteria that are able to readily colonize and cause UTI. Based upon this and their vaginal presence even in some healthy women, one would expect UPEC to also play a major role in vaginal infections. The inhibitory effect of Lactobacillus CS presumably related to lactic acid and hydrogen peroxide. Two potent inhibitors of UPEC growth that supports the protective role of lactobacilli against UPEC strains. Although lactic acid is a weak acid, it has been shown to exhibit potent antibacterial effects on numerous pathogens including UPEC [33], especially under nutrient limiting conditions such as those observed in the vagina.

Previous investigation has determined that 24 hour cultures of L. rhamnosusGR-1 and L. reuteriRC-14 produced approximately 45 and 35 mM lactic acid, respectively. Since the normal vaginal lactic acid content typically measures between 10-50 mM, our results support lactic acid and hydrogen peroxide. Two potent inhibitors of UPEC growth that supports the protective role of lactobacilli against UPEC strains. Although lactic acid is a weak acid, it has been shown to exhibit potent antibacterial effects on numerous pathogens including UPEC [33], especially under nutrient limiting conditions such as those observed in the vagina.

Here, we constructed a new reporter vector for simple and accurate monitoring of pap and OmpA promoters activity using luciferase. Our reporter constructs are consisted of only regulatory region lacking any structural and regulatory genes on pET28a. So the Expression of pap/OmpA luciferase construct was regulated directly by chromosomal regulatory proteins. Lactobacillus strains GR-1 and RC-14 have long been known to inhibit the adhesion of uropathogens [37,38], our results support this hypothesis that lactobacillus acidophilus also may play a critical role to inhibit the UPEC attachment to urinary tract. At the same time our results and some another works demonstrated that lactobacillus biomass or culture supernatant can stimulate OmpA expression. This is a usual bacterial response to environmental stresses. So probably some by- product of lactobacillus can down regulates and up regulates pap fimbrial and OmpA operons respectively. The presence of the lactobacilli appears to cause the UPEC to produce porins to try and maintain osmotic balance and stability in the membrane. Both OmpA and OmpX are highly immunogenic [39], and their up regulation may also induce antimicrobial immune responses in the host. The present study and others demonstrated that the production of pap fimbriae and outer membrane proteins in UPEC correlates with environmental stimulus. Although more in vivo studies are required for better understanding of pap and OmpA promoter activity, in vitro studies are also favorable for designing therapeutic strategies that targeting E. coli mediated urogenital tract infections. Finding the cross reactions between sensing systems in E. coli and trace elements in urinary tract, with a reporter system such as luciferase biosensor, will be favorable for finding and designing a new method for bacterial elimination from urogenital tract by probiotics.

Fig. 3. The effect of co-culture of reporter strain and lactobacillus acidophilus on pap (A) and OmpA (B) promoter activity

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REFERENCES


