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Recombinant Colicin producer *Lactobacillus acidophilus* and its Inhibitory Effect Against UPEC

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Lactobacillus **species are a predominant member of vaginal microflora and play a crucial role in prevention of a number of urogenital diseases. To increase antimicrobial potential of vaginal lactobacilli, Colicin E2 possessing specific activity against uropathogenic *Escherichia (E.) coli* was over-expressed in vaginal probiotic *lactobacillus(L.) acidophilus*. Recombinant *L. acidophilus* expressing Colicin E2 showed much higher inhibitory activity against uropathogenic *E. coli* than wild type in vitro. Efficacy of probiotic *L. acidophilus* expressing colicin E2 protein required for further in vivo evaluation. The present study in consistent with similar studies demonstrated that the production of colicin by vaginal probiotics may increase the preventive effects of lactobacilli against urinary tract infections.**

Key words: *Escherichia coli, Lactobacillus acidophilus*, urinary tract infections

Urinary tract infection (UTI), after intestinal infection, is the most widespread infection among women worldwide (Dielubanza EJ, 2011). Asymptomatic UTI affected more than 50% of women during their lifespan (Foxman B *et al*., 2000). This implies that an asymptomatic *Escherichia (E.) coli* reservoir likely exists in a substantial portion of women population and that the local vaginal environment plays an important role in preventing urogenital tract infections from developing. The high prevalence of *E. coli* in vaginal colonization and subsequent UTI is primarily due to their large numbers constantly shedding in feces and, promoting frequent urogenital contact. (Delzell and Lefevre, 2000). *E. coli* inherently exhibits different attributes that make it survives under varying environmental conditions including short generation time and ability to metabolize a wide variety of carbon sources and ability to perform facultative anaerobic metabolism. However, only a few numbers of strains can successfully survive, colonize and cause infection within the urogenital tract. A possible sequel includes pyelonephritis which can lead to renal scarring and sepsis (Jones, 1999). Uropathogenic *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, evasion of host defenses, and increased antibiotic resistance (Roberts *et al*.1994). A number of virulence determinants enhance the ability of UPEC to colonize the urinary tract and exert cytopathic effects including type 1 fimbriae (Goluszko *et al*., 1997), P fimbriae (Van den Bosch *et al*. ,1998), Dr adhesions (Welch, 1981),

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hemolysin (Rippere *et al*., 2001) cytotoxic necrotizing factor 1 (Bahrani *et al*.,2002), flagella (Schilling *et al*., 2001), capsule polysaccharide (Torres *et al*.2001), lipopolysaccharide O antigen (Hagan and Mobley, 2007), and TonB-dependent iron transport systems (Trautner and Hull, 2005). During UTI the outer membrane proteins of UPEC (Omp A, OmpC, OmpX, NmpC, and LamB) and outer membrane assembly factors are overexpressed (Cascales *et al*., 2007).

Lactobacilli are typically the predominant population in the vaginal microbial community. They are the most commonly isolated organisms from the vaginal microbiota of healthy women (Gupta *et al*.,1999). Lactobacilli can produce some secondary metabolites to overcome competitor populations in vaginal community. These metabolites include organic acids, biosurfactants, hydrogen peroxide and bacteriocins.

Colicin E2 (E2) is a protein antibiotic synthesized by certain strains of coliform bacteria that carry the ColE2 plasmid. It is classified under the E group of colicins along with colicins El and E3 (Dielubanza, 2011). Although the physical properties of E2 and E3 are similar (Nicolle, 2008) and both colicins share the same receptor (Dielubanza 2011), their apparent mode of action is different. It was first discovered that E2 causes degradation of DNA, while colicin E3 (E3) causes specific inhibition of protein synthesis in sensitive *E. coli* cells treated with these colicins (Neal, 2008). Subsequent work on the mode of action of E3 has demonstrated that ES inactivates ribosomes in the treated cells (Foxman, 1990) by causing the cleavage of a fragment from the 3' end of the 16S RNA (Ikaheimo *et al*., 1996). The same cleavage reaction has also been demonstrated in vitro using "purified" E3 protein and purified ribosomes (Delzell and Lefevre, 2000). This in vitro demonstration of E3 action has strongly indicated that E3 influences ribosomes directly rather than indirectly and that E3 itself is an RNase with a very stringent substrate specificity.

In contrast to ES, the mechanism of action of E2 has not been clear. Since the initial discovery of DNA degradation in E2-treated sensitive cells, several studies have confirmed and extended the original observations on DNA cleavage reactions (Zhanel, 2000 -13). However, other studies have suggested that the primary action of E2 is not on the cellular DNA, but on some other targets such as membranes (Van den Bosch *et al*., 1982) or tRNA (Welch *et al*., 1981).

Since E2 has properties similar to E3, and E3 causes ribosomal inactivation in vitro, one might expect some kinds of E2 effects of on cellular DNA in vitro, if DNA is in fact the primary target of E2. Several attempts have been made to demonstrate such effects in vitro using purified E2. However, published results were either negative or too uncertain to allow a definitive conclusion regarding such direct effects.

The present study was first aimed at preparation of a colicin producer-recombinant strain of Iranian women vaginal isolated of *lactobacillus (L.) acidophilus*. In this recombinant bacterium, expression of colicin E2 is controlled by PAP promoter of UPEC. In this regard, the same condition supported PAP fimbrial and Colicin expression. Therefore, in the final recombinant *L. acidophilus*, Colicin will be expressed just in the same environmental stimuli as ones can induce the P fimbrial adhesions. Using the findings we will be able to reduce rate of returning UTI in women.

MATERIALS AND METHODS

Bacterial strains and plasmids used in this study are listed in Table. *L. acidophilus* is routinely cultured in MRS broth at 37 °C for 48 h. For the analysis of expression of Colicin E2, recombinant strains were grown in basal MRS medium supplemented with 2% Xylose. *E. coli DH5*±, and *E. coli* TNBM 1387 were routinely cultured in Luria bertani (LB) broth and agar at 37 °C for 16 to 24 h and Uropathogenic *E. coli* PTCC 1227 (Persian type culture collection) was routinely cultured in nutrient broth and agar 37 °C for 16 to 24 h.

DNA manipulation, construction of plasmids and transformation of *Lactobacillus*

E. coli competent cell preparation, transformation and PCR plasmid DNA were isolated using metabion plasmid miniprep Kit as per instruction. The DNA cloning and transformation procedures were followed as previously described (Aarnikunnas *et al*., 2003). The laboratory modified expression plasmid pET28a, a type of secretion expression vector containing slpA as secretion signal and having a cell wall anchor domain was

used. Nucleic acid manipulation and cloning procedures were performed according to standard procedures (Aarnikunnas *et al*., 2003). Colicin E2 gene fragment of about 2.01 kb encoding the Colicin E2 structural gene (ceaB) and Colicin E2 immunity gene ceiB were obtained from the plasmid pColE2-P9 (National Institute for genetic engineering and biotechnology, Iran)) by polymerase chain reaction(PCR) amplification with the primers:

5'- AGATCTTTCAGCCAAT GCGAT - 3' (forward) containing a *BamHI* site (underlined) and 5'- GGATCCTTCCCATGTTTAAATCCTGA- 3' (reverse) containing a *bglII* site (underlined). PCR conditions were as follows: 30 cycles of 30 s at 94º C, 30 s at 58 ºC, 2 min 20 s at 72 ºC after denaturing for 4 min at 94 ºC. The PCR product of Colicin E2 gene was cleaved with *BamHI* and *bglII* restriction endonuclease and inserted into the corresponding sites of pET28a digested by *BamHI* and *bglII*, respectively, giving rise to recombinant pET28a (Figures 1and 2).

To prepare the competent cell, *L. acidophilus* was carried through an overnight culture. Then, a 2% inoculum of an overnight culture was grown in MRS medium enriched with 1% glycine at 37 ºC until the OD660 of culture was 0.2 to 0.3. The cells were harvested and washed twice with cold washing buffer (5 mM sodium phosphate pH 7.4 and 1mM MgCl2). The cells were then re-suspended in 1% of the original culture volume in a cold electroporation buffer (1M sucrose, 3mM MgCl2). For electroporation, 45 µl of the cell suspension was mixed with 50 to 500 ng of plasmid DNA and subjected to 2.5 kV, 200- •, 25- µF electric pulse in a 0.2- cm cuvette. The transformation efficiency was calculated as the number of transformants per microgram of plasmid DNA.

Molecular Weight Determination

Recombinant *L. acidophilus* samples were grown in MRS medium at 37 ºC for 24 hr. Centrifuged (10,000 rpm, 30 min, and 4 °C) to collect supernatants. Then, ammonium sulfate was slowly added to the supernatants to 60 % saturation and stirred for 4 h at 4 °C and centrifuged (10,000 rpm, 30 min, 4 °C). The precipitate was re-suspended in 10 ml of 25 mM ammonium acetate buffer (pH 6.5) and desalted by dialysis using a 1,000 Da cutoff dialysis membrane against the same buffer. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS- PAGE) was used for further separation.

Antimicrobial Activity Assessment

Antimicrobial activity of supernatant was measured by well diffusion assay. Filtrates were neutralized with 5 N NaOH. Nutrient agar plates were flooded with pathogenic bacteria (0.1% of overnight grown UPEC strain), air dried, and then 6- mm diameter wells were punctured in each plate. The prepared supernatants were poured into respective wells (25¼L) and incubated for 24 h at 37°C. Wild type *L. acidophilus* used as negative control and *E. coli* TNBM 1387 was used as positive control.

RESULTS

Antimicrobial activity of Recombinant *L. acidophilus* expressing Colicin E2

Antimicrobial properties of transformed *L. acidophilus* showed higher zone of inhibition (66 mm) compared to wild type *L. acidophilus* (20 mm) were shown in Figure 3. However, there is no significant different in the inhibition zone showed by recombinant *L. acidophilus* and colicin producer *E. coli* (Figure 3).

Extracellular expression of Colicin E2

L. acidophilus transformants harboring recombinant pET28a were tested for extracellular expression in 500 ml of MRS broth. Transformant and control strains containing vector only were incubated with vigorous shaking (200 rpm), and cell growth was monitored by checking optical density at 600 nm. After 24 hrs of growth 52 kDa protein was observed seen in SDSPAGE analysis (Figure 4).

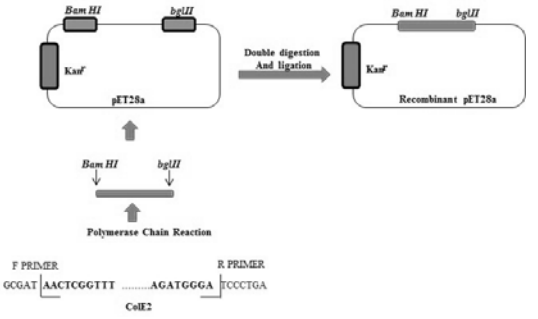
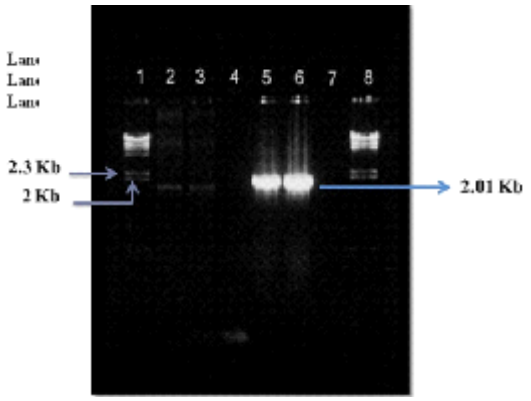


Fig. 1. colE2 gene Cloning procedure in pET28a to achieve new recombinant vector

Table 1. Vector and Strains used in this study

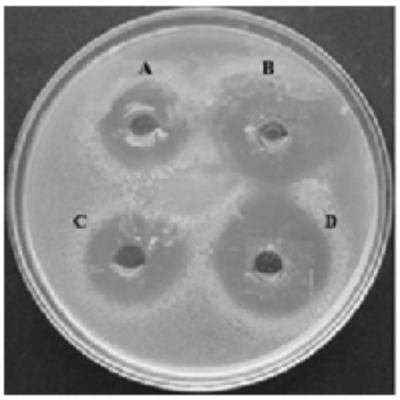
Vector: Name	Property	Reference
Modified pET28a	pap promoter	Novagen
Strains:		
Name	Relevant genotype and property	Reference
DH5α	Sup E_{44}ΔLacU 169(F80 Lac Z DM15) hsdR17 Yec A_1	
NIGEB§	endA1 gyr A96 thi-1 rel A_1	
Lactobacillus acidophilus	ATCC 4356	NIGEB
Lactobacillus acidophilu	wild type vaginal isolates	
LALEH HOSPITAL		
E. coli TNBM 1387	Recombinant strain witch is included *colE2* gene and produces colicin E2	IAUNTB[m]
E. coli PTCC 1227	UPEC	IAUNTB

§ National Institute for Genetic Engineering and Biotechnology, Tehran, Iran.
*Islamic Azad University- North of Tehran Branch



Lines 1 and 8: lader. Lines 2 and 3 falls positive control. Line 4: negative control.
Linles 5 and 6: colE2 gene carried out *BamHI* and *bglII* restriction sites.

Fig. 2. PCR amplification of colE2 gene



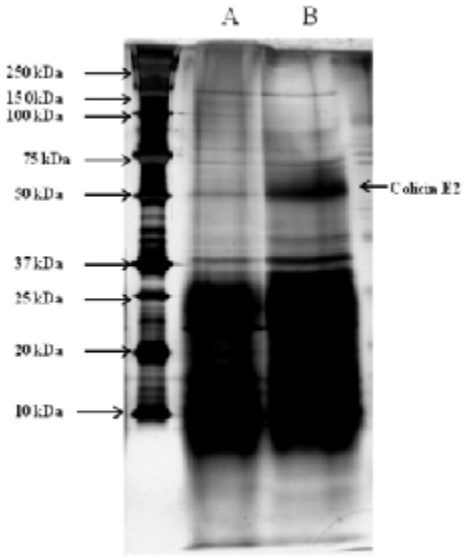
A &C) *Lactobacillus acidophilus* ATCC 4356 (non colicine producer)
B) Recombinant Lactobacillus acidophilus (colicine producer)
D) *Escherichia coli* TNBM 1387 (colicine producer)

Fig. 3. Antimicrobial activity against UPEC

DISCUSSION

Various techniques have identified Lactobacillus species as the predominant microorganism present in vaginas of most healthy and fertile women (Redondo, 1990). Lactobacillus species have been studied as a potential probiotic for the prevention and treatment of urogenital disease in women (Falagas and Betsi, 2007). During menstruation, the vaginal pH becomes neutral, most likely due to the influx of menses blood, which has a pH range of 6.9 to 7.2 reducing Lactobacilli number in vagina and chances of infections like urinary tract infections and bacterial vaginosis.

Colicin E2 production by a non-pathogenic organism can be clinically important as a means to prevent catheter- associated urinary tract infection (Trautner and Hull 2005). Evans demonstrated the possibility of developing oral whole- cell vaccines against diarrhea caused by



A) Protein pattern of *lactobacillus acidophilus* (non colicin producer)
B) Protein pattern pf recombinant lactobacillus acidophilus (colicin producer)

Fig. 4. SDS-PAGE of protein pattern

enterotoxigenic *E.coli* through modifying the *E. coli* by the in situ destruction of chromosomal and plasmid DNAs by ColE2 (Evans *et al*., 1998). The colicin operon is carried on a plasmid and includes a structural gene (ceaB) encoding for the bacteriocin, an immunity gene (ceiB) that protects the producer cell from the toxin, and a lysis gene (celB) that leads to death of the producer cell and release of ColE2 into the surrounding medium (Cascales *et al*., 2007). Sensitivity of gram-negative microorganisms to ColE2 is conferred by the binding of the bacteriocin to an outer membrane receptor the TonB- dependent vitamin B12 transporter, BtuB (Cascales *et al*., 2007). After transport across the membrane, ColE2 acts as an endonuclease, degrading the DNA of the sensitive cell. During UTIs UPEC overexpresses surface protein BtuB which can act as receptor for binding Colicin E2.

One of the challenges of transforming ColE2 in Lactobacillus is the differences in the transport mechanisms of bacteriocins in gram-negative and gram - positive microorganisms (Cascales *et al*., 2007). In gram negative microorganisms, ColE2 is reportedly released into the surrounding medium after CelB- mediated lysis of the producer cell. Expression of celB modifies the cell envelope so that activates Omp LA, an outer membrane phospholipase A (Cascales *et al*., 2007). Mutation or deletion of the lysis protein has been shown to interfere with release, and in such cases, colicin remains in the cytoplasm (Cascales *et al*., 2007). In gram-positive microorganisms, secretion does not occur through cell lysis, and is not a lethal event for the cell. Secretion is dependent on a signal peptide, which typically contains conserved double- glycine regions, and is mediated by a bacteriocin- specific transport system or the sec- dependent export pathway (O'Sullivan *et al*., 2002). Although ColE2 does not contain a signal peptide to direct the secretion of the protein, it is predicted to contain 6 double glycine regions at the N terminus, which may function similarto a signal peptide. Therefore, ColE2 may be secreted by a gram - positive host without lysis of the producer cell. This feature is important if the genes encoding for colicin production are to be transferred to and expressed by a Lactobacillus (Wandersman, 1992). Our study demonstrated that genes associated with bacteriocin production from a gram- negative microorganism could be cloned, expressed, and secreted by a gram- positive microorganism with addition of a signal peptide. Our fidings showed that genes associated with ColE2 production (ceaB and ceiB) were transferred to *L. acidophilus*, probiotic isolate from vagina. The level of ColE2 production by the colicin- producing transformants of *L. acidophilus* was similar to that of *E. coli* TNBM 1387.

Secretion of ColE2 proteins into the surrounding medium by *E. coli* TNBM 1387 and the recombinat pET28a transformants occurred before cell leakage was observed. The mechanism proposed for secretion of ColE2 from *E. coli* involves release of the colicin caused by the lysis protein; CelB (Pugsley and Rosenbusch, 1981). Braun *et al*., in 1994 found inactivation of celB resulted in decreased release of colicin from the cells, compared with cells containing intact celB.

In addition, in the recombinant *L. acidophilus*, colicin has been expressed under controled of *PAP* operon promoter on modified pET28a. this probabely anabled us to combat UPEC vaginal microbial pathogenic population just whenever pap fimbrial adhesions (one of the most

importand virolence factors) start to express and at the same thime reduce probable undesirable effect/s of colicin against frindly vaginal microbiota in vivo.

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