

Original articles

P.A. CADIEUX^{1,2,3}, J.P. BURTON⁴, E. DEVILLARD⁵, G. REID^{1,2,3}

LACTOBACILLUS BY-PRODUCTS INHIBIT THE GROWTH AND VIRULENCE OF UROPATHOGENIC *ESCHERICHIA COLI*

¹Canadian Research and Development Centre for Probiotics, Lawson Health Research Institute, London, Canada; ²Department of Microbiology and Immunology, University of Western Ontario (UWO), London, Canada; ³Department of Surgery (Division of Urology), University of Western Ontario (UWO), London, Canada; ⁴BLIS Technologies Ltd. Centre for Innovation, University of Otago, Dunedin, New Zealand; ⁵Microbiology and Ruminant Nutrition, ADISSEO France S.A.S., Commeny, France

Uropathogenic strains of *Escherichia coli* (UPEC) can colonize the vagina and cause infections within the entire urogenital tract, including those associated with urinary tract devices. Lactobacilli typically dominate the vaginal microbiota in healthy women, and studies have shown that they can inhibit UPEC growth and vaginal colonization. However, little is known regarding the mechanisms behind these effects. Using a luciferase-based reporter construct, gradients of lactic acid, hydrogen peroxide and spent culture supernatants (SCS) from urogenital probiotic strains *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 were examined for their effects on growth and virulence factor expression in UPEC isolate C1212. In a dose- and pH- dependent manner, lactic acid, hydrogen peroxide and *Lactobacillus* SCS all strongly inhibited UPEC C1212 growth and increased the promoter activity of outer membrane proteins (OMPs) A and X, two porins normally upregulated in response to UPEC membrane stress. Lactic acid and the culture supernatants also downregulated the promoter activity of the major subunits of type 1 and P fimbriae, 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 downregulating proteins critical for host attachment.

Key words: *probiotics, Lactobacillus, uropathogenic strains of Escherichia coli, virulence, lactic acid, H₂O₂, fimbriae, outer membrane proteins*

INTRODUCTION

Escherichia coli is the most commonly isolated organism from the urinary tract, causing >70% of uncomplicated and 25-50% of complicated urinary tract infections (UTI) (1). Furthermore, as well as causing vaginal infections such as aerobic vaginitis, *E. coli* can be isolated from the vaginal microbiota of up to 20% of women at any one time (2). 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 contact. This is supported by data showing that strains causing the vast majority of UTI originate from the patient's own fecal flora (3, 4). *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 facultatively anaerobic metabolism. However, only a select number of strains can successfully survive, colonize and cause infection within the urogenital tract. These strains have been

termed uropathogenic *E. coli* (UPEC) and shown to express 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 (5). These properties can be encoded as single genes as well as entire operons, and their expression is important for the development and maintenance of both initial and recurrent UTI (6).

In a normal, healthy vagina the pH is generally below 4.5, which largely restricts the types of organisms that can survive. Understandably, acidophilic species of bacteria, such as lactobacilli, typically predominate. Indeed, they are the most commonly isolated organisms from the vaginal microbiota of healthy women and numerous studies have demonstrated that in addition to competing for nutrients, space and host receptors within the vagina, lactobacilli produce substances detrimental to the growth, survival and colonization of uropathogens including UPEC (7, 8). These substances include organic acids, biosurfactants, hydrogen peroxide and bacteriocins, and have been shown to function through pH effects, receptor interference and direct killing. Based upon these findings, lactobacilli have demonstrated significant promise as prophylactic and therapeutic agents against UPEC infection in the urogenital tract.

However, much remains unknown regarding the specific mechanisms involved in this inhibition.

In this study, we examined the effects of two probiotic strains of lactobacilli and their by-products on UPEC growth and VF expression. Since attachment and survival in the highly acidic environment of the vagina are critical to UPEC colonization and infection within the urogenital tract, we selected VFs associated with adherence and acid tolerance for study. Specifically, we selected the major subunits of type 1 and p fimbriae and the outer membrane porins OmpA and OmpX. Type 1 and p fimbriae were selected as they are the two most common and well-studied UPEC attachment organelles, associated with host-cell adherence and invasion, cytotoxicity and inflammation (9). In addition to their known adhesive properties within the urinary tract, both have been shown to be important in UPEC binding to vaginal epithelial cells (10). 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 (Omp) such as the porins OmpA and OmpX have been shown to play an important role in these processes by controlling the movement of compounds across the outer membrane as well as offering structural support (11, 12). In addition to their upregulated expression in response to various environmental stresses (13), both have been shown to mediate host-cell attachment and the avoidance of host defences. UPEC C1212 was selected for investigation as it expresses all four factors under study and the *Lactobacillus* strains selected were *L. rhamnosus* GR-1 and *L. reuteri* RC-14, two strains with proven probiotic effects in the female urogenital tract (14, 15).

MATERIALS AND METHODS

Bacterial strains and media

E. coli C1212, a clinical UTI isolate, was maintained using Luria-Bertani (LB) media or brain heart infusion media supplemented with 0.5% yeast extract (BHYE). *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 were isolated from the vagina and distal urethra and vagina, respectively, of healthy women and were maintained using de Man, Rogosa and Sharpe (MRS) media. For experiments involving *E. coli* growth inhibition and virulence factor expression, as well as the generation of *Lactobacillus* spent culture supernatants (SCS), a modified MRS was created (mMRS)(1% [w/v] proteose peptone #3, 0.5% [w/v] yeast extract, 0.1% Tween® 80, 0.2% $[\text{NH}_4]_2\text{C}_6\text{H}_6\text{O}_7$, 0.5% CH_3COONa , 0.01% MgSO_4 , 0.005% MnSO_4 , 2% K_2HPO_4 , and 60

$\mu\text{M FeSO}_4\cdot 7\text{H}_2\text{O}$, 0.5% glucose) that promoted strong growth of both *E. coli* and lactobacilli. All culturing and experiments were conducted at 37°C.

Preparation of *Lactobacillus* by-products and heat-killed organisms

For SCS preparation, lactobacilli were cultured on mMRS agar, subcultured overnight (O/N (16-18 h)) in mMRS broth, diluted into 100 mL fresh mMRS at 1×10^5 CFU/mL and grown statically for 24 hours. Cells were pelleted by centrifugation (10000 g, 20 min, 4°C) and the SCS collected, pH adjusted, filter sterilized and stored at -20°C. To prevent nutrient depletion in the *Lactobacillus* SCS from causing unwanted growth or promoter effects, SCS and the controls were fortified with 4X mMRS to 20%, resulting in 80% SCS and ~1X mMRS nutrient and salt concentrations. Biosurfactants were isolated as previously described. To prepare heat-killed lactobacilli, cells from 100 mL O/N culture were pelleted, washed in H_2O , resuspended in 20 mL 1X PBS (pH 7.0) and incubated at 90°C for 15 min.

Preparation of competent *E. coli*

Competent *E. coli* DH5 α and C1212 were prepared by diluting an O/N culture 20 X into 100 mL LB and incubating to 0.05 OD₆₀₀ (2.5×10^7 CFU/mL). Cultures were chilled on ice for 5-10 min, pelleted (1500 g, 15 min, 4°C) and resuspended in 40 mL cold transformation buffer I (TfBI) (30 mM KOAc, 100 mM RbCl₂, 10 mM CaCl₂, 50 mM MnCl₂, 15% glycerol (v/v) (pH 5.8)) for 15 min on ice. Cells were pelleted, resuspended in 4 mL cold TfBI (10 mM MOPS, 75 mM CaCl₂, 10 mM RbCl₂, 15% glycerol (v/v) (pH 6.5))(heat shock) or 15% glycerol (electroporation), aliquoted and stored at -80°C.

Generation of UPEC virulence factor promoter constructs

The promoters of *fimA*, *papA*, *ompX* and *ompA* were PCR amplified using C1212 gDNA and primers containing *EcoRI* and *XmaI* restriction sites for directional cloning (Table 1). Products were restriction digested with 2 U/ μg *EcoRI* and *XmaI* (New England Biolabs, Pickering, Can) as per the manufacturer and purified using Qiagen's PCR purification kit (Mississauga, Can). Vector pSB2034 was digested as above, treated with shrimp alkaline phosphatase (SAP)(NEB)(37°C, 1 hour) and the SAP inactivated (65°C, 20 min). One hundred ng of each promoter fragment was ligated to 1 μg pSB2034 using T4 DNA ligase (0.5 U) (Invitrogen, Oakville, Can) at 20°C for 2 hours then 4°C O/N. Two microlitres of each 10 μL ligation was transformed into

Table 1. Oligonucleotide primers used for the amplification of the promoter regions for the UPEC virulence factors selected for study.

Primer Name	Amplified Sequence	Primer Sequence (5'-3')	Length (bp)	Product Size (bp)
FimAPro-For	<i>fimA</i> promoter	CAGAATTCGTTTTGCCGGATTATG	24	520
FimAPro-Rev		TACCCGGGCAGTCGTTCTGTAC	22	
PapAPro-For	<i>papA</i> promoter	TAGAATTCATGCTCTCTGTTATCAACG	27	339
PapAPro-Rev		TACCCGGGAAATACTCTTCACG	22	
OmpAPro-For	<i>ompA</i> promoter	AAGAATTCTCACGCCACGAGACA	23	347
OmpAPro-Rev		TACCCGGGATCTGCTCAATATTAACCTC	27	
OmpXPro-For	<i>ompX</i> promoter	GCGAATTCTCCACAAATATCTAAACC	27	321
OmpXPro-Rev		TACCCGGGAAACTCTTCGCG	20	

competent *E. coli* DH5 α via 30 second 42°C heat shock and selected on LB plates containing 100 μ g/mL ampicillin (LB+amp). Colonies were screened by plasmid isolation, restriction analysis and sequencing. Verified constructs were electroporated into competent *E. coli* C1212 (2 kV,600 Ω , 25 μ F) and selected on LB+amp agar as above.

Growth and reporter activity assays

C1212 promoter construct transformants were analyzed for growth and promoter activity during 24 hours exposure to lactobacilli and/or their by-products. Cultures were inoculated to 1x10⁵ CFUs/mL in triplicate in 96-well luminometer and/or tissue culture plates (200 μ L/well). Plates were incubated at 37°C in a Fluoroskan/Ascent FL luminometer or Multiskan Ascent spectrophotometer (Thermo Labsystems, Helsinki, Finland) and luminescence or OD_{600nm} quantified every two hours. Luminescence was assessed as relative light units (RLU)/well per 10 seconds. Promoter activity was expressed per bacterial cell.

Statistical analyses

Analyses were conducted *via* Student *t*-tests (Microsoft Excel, Microsoft Canada) and results displayed as the average of at least 3 experiments. P values <0.05 were significant.

RESULTS

Biosurfactant and heat-killed cells collected from both *Lactobacillus* strains were among the various by-products examined for effects on UPEC C1212. It is important to note that neither one of these factors affected C1212 growth or virulence factor expression under any condition tested.

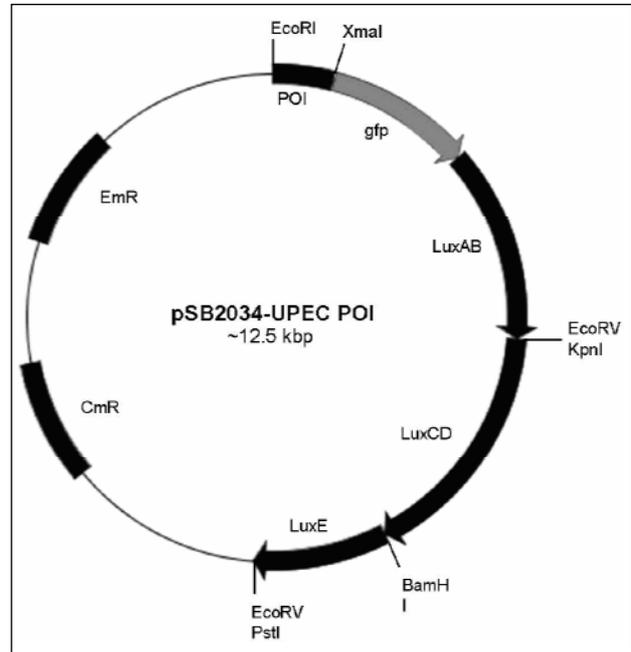


Fig. 1. Vector map for pSB2034 highlighting the restriction site where each UPEC virulence factor promoter of interest (POI) was cloned into.

Media selection and influence of Lactobacillus by-products on UPEC growth

Comparing a number of bacterial media, the two lactobacilli only grew well in MRS and BHYE. Unfortunately, UPEC grow poorly in MRS and did not appear to express type 1 or p fimbriae

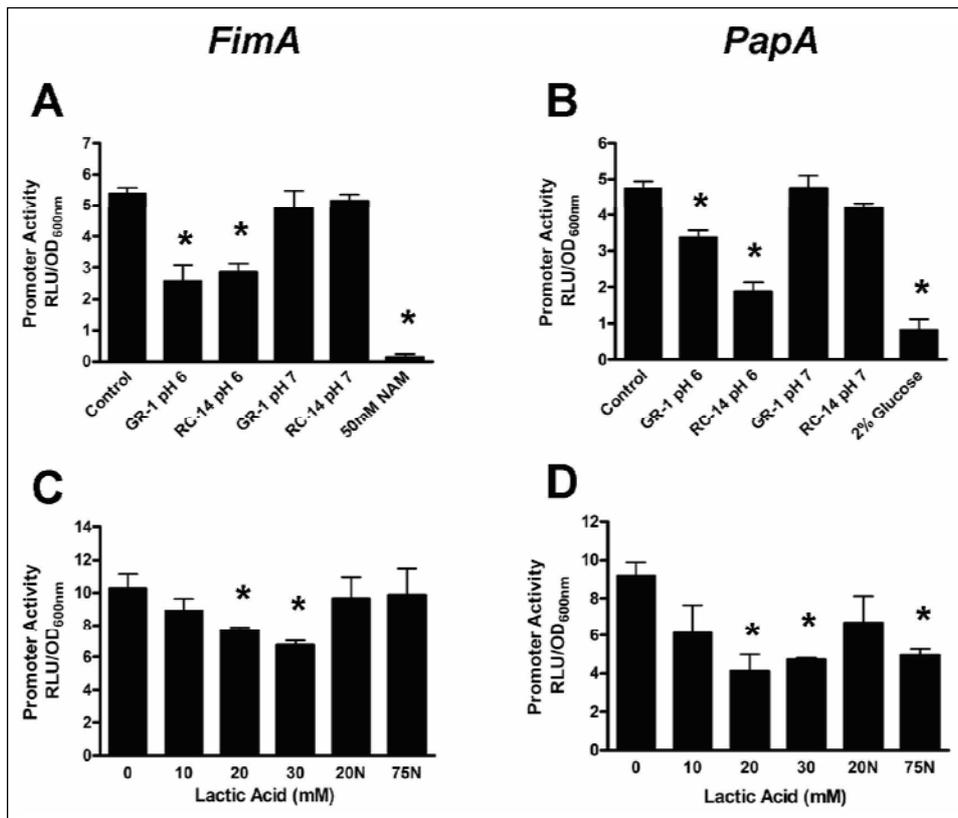


Fig. 2. Effects of *Lactobacillus* SCS and lactic acid on the activity of the major subunit promoters for type 1 (*fimA*) and p (*papA*) fimbriae. RLU/OD_{600nm}-Relative light units/optical density at 600 nanometers.

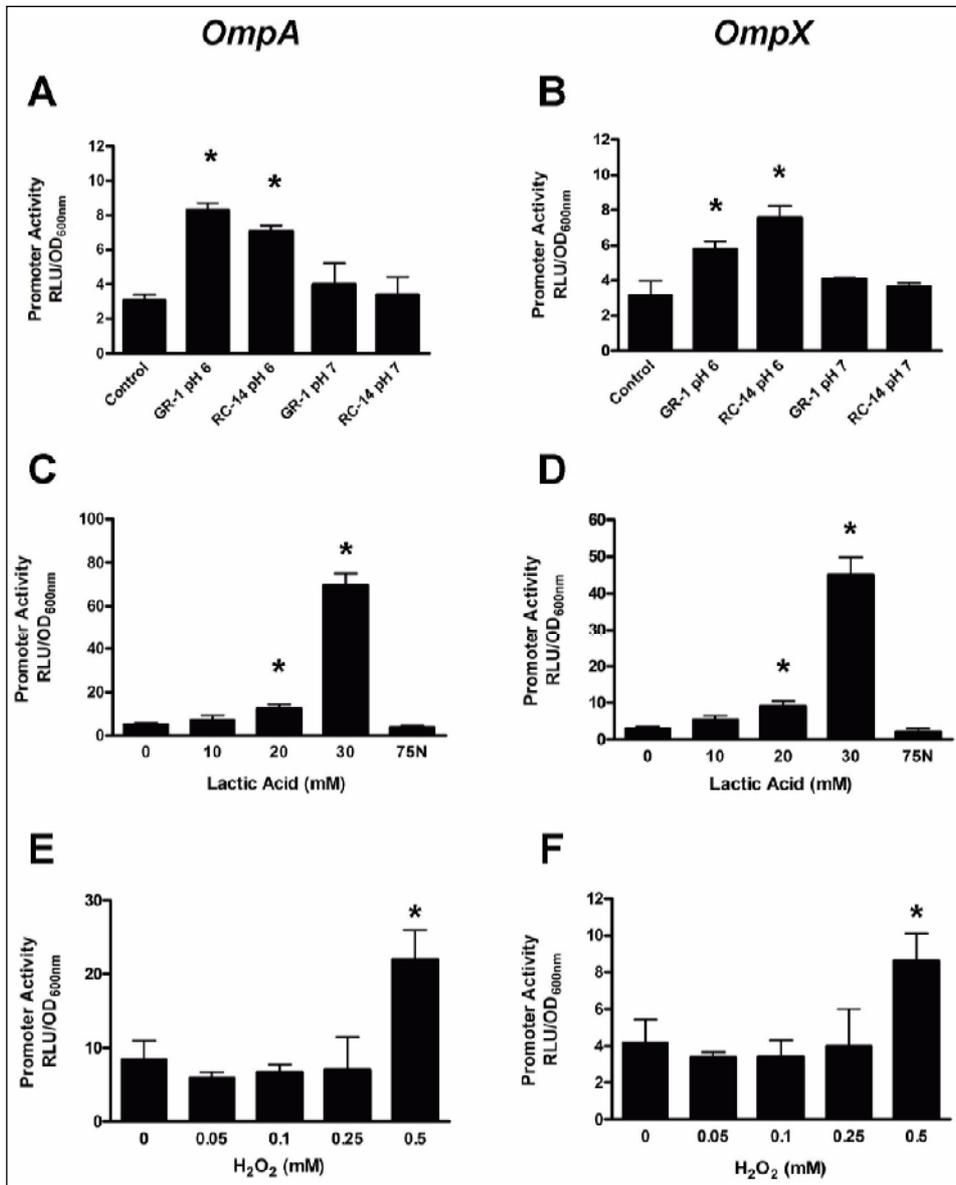


Fig. 3. Effects of *Lactobacillus* SCS, lactic acid and hydrogen peroxide on the activity of the promoters for outer membrane proteins A (*ompA*) and X (*ompX*).

as determined *via* hemagglutination (data not shown), two of the factors whose expression were under direct examination. Although UPEC grew well in BHYE and expressed both types of fimbriae, we were concerned that it was too rich a media to accurately assess the effects of the by-products in comparison to the normal urogenital tract environment. Thus, we developed a modified MRS media (mMRS) in which we prepared the most minimal media possible that would support the growth of both lactobacilli and UPEC, as well as induce fimbrial expression for investigation.

Since the inhibitory effects of *Lactobacillus* SCS, lactic acid and hydrogen peroxide on C1212 growth were pH and/or dose-dependent and were largely expected, we only summarize the results here. *Lactobacillus* SCS from 24 hour cultures of both probiotic strains completely inhibited C1212 growth (GR-1, pH 4.0; RC-14, pH 4.5). These effects were not only completely abrogated upon pH neutralization to 7, but actually led to a growth increase of ~20%. Lactic acid alone inhibited UPEC growth in a dose- and pH-dependent manner with no growth observed above 30 mM (media pH 5.2). In a similar fashion to the SCS findings, pH-neutralized (7.0) lactic acid either had no effect (≤ 30 mM) or actually increased growth (100 mM, >50%

increase). Finally, H₂O₂ reduced C1212 growth in a dose-dependent manner with complete inhibition occurring at concentrations above 0.5 mM. At 0.5 mM, the shape of the C1212 growth curve was maintained but shifted to the right 4 hours, showing a delay in the beginning of the exponential and stationary growth phases. However, final cell counts were similar compared to controls.

Influence of Lactobacillus by-products on UPEC virulence

Reporter vector pSB2034 was kindly donated by Dr. David Heinrichs at the University of Western Ontario (16). This plasmid contains the genes necessary for expression of both green fluorescent protein (GFP) and luciferase, downstream of a multiple cloning site into which the UPEC promoters of interest (POI) were cloned (Fig. 1). The promoters for the major subunits of type1 and p fimbriae, FimA and PapA respectively, as well as those of OMPs A and X, were selected and utilized to generate four reporter constructs. Promoter activity was assessed in the presence of *Lactobacillus* by-products by measuring luciferase production and growth over 24 hours and determining the level of expression per cell.

Lactobacillus SCS from both strains were pH adjusted to 6.0 to challenge C1212 while still permitting survival. For the type 1 promoter in mMRS alone, activity increased steadily up to 8 hours, dropped slightly at 10 hours and increased to a maximum at 12 hours before steadily decreasing through stationary phase. As a control, cells were cultured with 50 mM N-acetylneuraminic acid (NAM), a sialic acid known to inhibit type1 fimbrial expression. As expected, NAM greatly reduced type 1 promoter activity after 6 hours, completely abolishing activity from the 12 hour timepoint forward. Treatment with SCS from either probiotic strain inhibited activity significantly between 8 and 14 hours, highlighted by 52.4 (GR-1, $p=0.023$) and 47.1% (RC-14, $p=0.019$) reductions at 12 hours (Fig. 2a). However, similar to the growth assays, the effects were pH-dependent, as neutralized SCS caused no change. The findings were almost identical with respect to the *papA* promoter (Fig. 2b), where pH 6.0 SCS from both strains inhibited activity (GR-1 28.9%, $p=0.042$; RC-14 60.4%, $p=0.011$) along with the control (glucose) at the maximum expression timepoint (10 h) and neutralized SCS had no effect.

Interestingly, the effects of *Lactobacillus* SCS on the activity of the *ompA* and *ompX* promoters were in sharp contrast to those observed with the fimbrial promoters. In untreated mMRS media, the activity of both promoters steadily increased to a maximum during late log phase growth (8-10 h) after which point they steadily decreased to zero by 24 h. SCS (pH 6.0) from both lactobacilli upregulated the activity of both promoters, most significantly at 8 hours for *ompA* and 10 hours for *ompX* (Figs. 3 a,b). GR-1 SCS increased *ompA* promoter activity by 2.7 fold ($p=0.007$) and *ompX* promoter activity by 1.83 fold ($p=0.039$) at 8 and 10 hours, respectively. RC-14 SCS increased *ompA* and *ompX* promoter activities by 2.3 ($p=0.031$) and 2.38 ($p=0.009$) fold at the same timepoints, respectively. Similar to the fimbrial promoters, SCS effects on both Omp promoters were pH-dependent and completely abrogated upon neutralization.

Based upon the strong association between pH and promoter activity in all four reporter strains, it was decided to examine the effects of lactic acid itself on the promoters, with and without pH neutralization. Overall, the effects were similar to those observed using *Lactobacillus* SCS, with lactic acid reducing the activity of the *fimA* and *papA* promoters (Figs. 2c,d) while upregulating those of *ompA* and *ompX* (Figs. 3c,d) in a dose/pH-dependent fashion. The maximal effect occurred at 30 mM (pH 5.2) since growth was not observed at higher concentrations. Specifically, 30 mM lactic acid reduced *fimA* and *papA* promoter activities 47.7 ($p=0.017$) and 35.3% ($p=0.036$), respectively, and greatly increased those of *ompA* and *ompX*, 13.8 and 12.2 fold (both $p<0.001$), respectively. Interestingly, the *papA* promoter was the only one affected by neutralized lactic acid with 75 mM reducing activity 46.1% ($p=0.031$) (Fig. 2d).

Hydrogen peroxide production has previously been shown to play a role in pathogen inhibition by lactobacilli (17) and was demonstrated in this study to inhibit growth completely at concentrations above 0.5 mM. To determine what effects, if any, the compound had on the expression of our virulence factors of interest in this study, we exposed the four reporter strains to a gradient ranging from 0.05-0.5 mM. It is important to note that at 0.5 mM, promoter activity curves are shifted to the right in a similar fashion to the growth curves previously mentioned since it appears UPEC extends its lag growth phase to adjust to the presence of the compound. Thus, maximal promoter activity occurs 4 h after that observed in mMRS alone. With respect to the two fimbrial promoters, there were no effects at any concentration tested. However, the results were again quite different for the Omp promoters, as significant increases in maximum activity were observed for both at 0.5 mM (Figs. 3 e,f). *ompA* promoter activity was upregulated 2.69 fold ($p=0.017$) while that of the *ompX* promoter increased 2.05 fold ($p=0.029$).

DISCUSSION

While the urogenital tract is constantly under assault by microorganisms originating from the surrounding environment including the skin and feces, there are only a select number of pathogens that are able to readily colonize and cause infections there. UPEC are the most clinically relevant of these, causing the vast majority of UTI (1). 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 finding here that *Lactobacillus* SCS, lactic acid and hydrogen peroxide are potent inhibitors of UPEC growth, supports the protective role of lactobacilli against UPEC strains. The effects of SCS were likely due to lactic acid, since the results for both pH-balanced and unbalanced SCS mimicked those of lactic acid. Although lactic acid is a weak acid, it has been shown to exhibit potent antibacterial effects on numerous pathogens including UPEC (18), especially under nutrient limiting conditions such as those observed in the vagina. Herein we observed no growth in concentrations above 30 mM and an almost 70% growth reduction in as little as 10 mM. Furthermore, previous work by our group and others has determined that 24 hour cultures of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 produced approximately 45 and 35 mM lactic acid, respectively (19). Since the normal vaginal lactic acid content typically measures between 10-50 mM, our results support lactic acid playing a major role in UPEC inhibition by lactobacilli.

Notably, both pH-neutralized SCS and lactic acid actually stimulated growth under conditions similar to those found in mMRS media. This could have implications in the urogenital tract, especially when amine-producing pathogens such as *Prevotella bivia* are present (20). As amine production raises the vaginal pH, *Lactobacillus*-produced lactic acid may transform from an anti-bacterial compound to a carbon source for organisms like UPEC and bacterial vaginosis-associated *Prevotella*. Our findings may offer some explanation as to why vaginal infections sometimes occur despite *Lactobacillus* colonization.

Hydrogen peroxide is a potent antibacterial compound produced by many strains of lactobacilli (17) and has been shown to induce bacterial membrane stress (21). Our results support this finding as we observed the dose-dependent upregulation of both Omps in the presence of H₂O₂.

Lactobacillus strains GR-1 and RC-14 have long been known to inhibit the adhesion of uropathogens (22, 23). This has long been assumed to be a mechanism by which UPEC colonization is limited. The latest findings show that lactobacilli can induce stress on the outer membrane of UPEC thereby adversely affecting fimbriae structure and adhesion, and upregulating two outer membrane proteins OmpA and OmpX that play a role in stress response (13). 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 (24), and their upregulation may also induce antimicrobial immune responses in the host. This was not tested here, but in a separate study when *L. rhamnosus* GR-1 was administered vaginally, there was evidence of up-regulation of host anti-microbial factors (25).

In summary, urogenital lactobacilli can antagonize UPEC strains not necessarily by direct lethality, but through acidic inhibition of growth, induction of stress in the outer membrane, and by modifying the environment to one that is less conducive to UPEC thriving. This represents a further rationale for selecting probiotic lactobacilli that can prevent urogenital infections in women.

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Author's address: Dr. Gregor Reid, BSc Hons PhD MBA, Canadian R&D Centre for Probiotics, F2-116, Lawson Health Research Institute, 268 Grosvenor Street, London, ON Canada, N6A 4V2; E-mail: gregor@uwo.ca