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Research Letter

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In vitro activity of commercial probiotic *Lactobacillus* strains against uropathogenic *Escherichia coli*

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One sentence summary: *In vitro*, selected *Lactobacillus* spp. have an effect on UPEC by acidifying their environment, possibly aiding the restoration of the urogenital tract to a healthy state.

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ABSTRACT

Urinary tract infection (UTI) is one of the most prevalent infections in humans. In ≥80% of cases, the etiologic agents are strains of uropathogenic *Escherichia coli* (UPEC), which commonly reside in the gastrointestinal tract. Lactobacilli have been shown to prevent UTI reoccurrence by restoring the urogenital microbiota when administered vaginally or orally. The goal of this study was to determine if commercial probiotic *Lactobacillus* spp. reduce or clear UPEC *in vitro*. Results show that it is likely that lactobacilli may, in addition to restoring a healthy urogenital microbiota through acidification of their environment, also displace adhering UPEC and cause a reduction of infection.

Keywords: UPEC; UTI; *Lactobacillus* spp.; cell culture; bacterial coculture; cell-free culture supernatants

INTRODUCTION

The urinary tract is constantly challenged by microbial invasion (Ali *et al.* 2009). This results in urinary tract infection (UTI) being one of the most prevalent infections in humans. The lifetime risk for acquiring a symptomatic UTI is about 50% in women and 12% in men, with a rate of recurrence after six months of about 40% (Sivick and Mobley 2010). UTI incidence peaks in individuals in their early 20s and after age 85 (Foxman 1990). If left untreated, bacteria may ascend the urinary tract and establish a secondary infection in the kidneys (acute pyelonephritis). In ≥80% of cases, the etiologic agents for UTIs are strains of uropathogenic *Escherichia coli* (UPEC), which commonly reside in the GI tract (Sivick and Mobley 2010). It has been recognized that UPEC can invade host uroepithelial tissue, contributing significantly to the pathogenesis of UTIs by escaping a great number of antibiotics (Eto, Sundsbak and Mulvey 2006). Internalized UPEC can persist in quiescence for long periods without causing clinical symptoms (Mulvey, Schilling and Hultgren 2001).

For the treatment and prevention of UPEC-related UTIs including recurring infections, the use of low dose once daily or post-coital antimicrobials have been a cornerstone (Guay 2009). However, even with urine concentrations of antibiotics far exceeding minimal inhibitory concentrations, UPEC reservoirs in tissues were not eradicated effectively (Blango and Mulvey 2010). Therefore, some alternative non-antimicrobial based therapeutic approaches such as probiotics that may inhibit bacterial adherence and colonization may be of benefit.

The use of probiotics and fermented milk products against UTI has previously been assessed (Kontiokari *et al.* 2003). However, while there is evidence that lactobacilli have an effect on UTI, their mechanism of action has thus far not been elucidated, with most studies relying on circumstantial evidence (Bruce and

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Reid 1988; Saxelin, Pessi and Salminen 1995; Reid and Burton 2002). In all, biosurfactants, bacteriocins, lactic acid and hydrogen peroxide from *Lactobacillus* sp. seem to be inhibitory for UPEC growth, while adversely affecting fimbrial structure and adhesion and upregulating immunogenic membrane proteins (Hagan and Mobley 2007; Cadieux *et al.* 2009).

The goal of this study was to assess the *in vitro* antimicrobial/bacteriostatic activity of selected commercial *Lactobacillus* strains, against UPEC.

MATERIALS AND METHODS

Organisms

Lactobacillus johnsonii strains NCC533 and NCC2917, *L. rhamnosus* NCC4007 and *L. acidophilus* NCC2463, provided by the Nestlé culture collection (Nestec Ltd., Lausanne, Switzerland). Uroseptic *E. coli* strain CFT073 (O6:K2:H1, ATCC700928) was isolated from the blood and urine of a woman with acute pyelonephritis (Mobley *et al.* 1990). The strain was purchased from the American Type Culture Collection (LGC Standards, Molsheim Cedex, France). The UPEC strain UTI89 (O18:K1:H7) was isolated from a patient with an acute cystitis (Mulvey, Schilling and Hultgren 2001). The strain was kindly provided by Prof. Urs Jenal from the Biocenter of the University of Basel (Basel, Switzerland). For cell culture assays, the human bladder cancer cell line UM-UC-3 (ATCC CRL-1749) was purchased from the American Type Culture Collection (LGC Standards, Molsheim Cedex, France). Bacterial culture media and components were purchased from Oxoid (Basingstoke, UK) while cell culture media, cell culture components and chemicals were purchased from Sigma (Buchs, Switzerland) unless stated otherwise.

Coculture of UPEC and *Lactobacillus* spp.

All organisms were grown in fresh LAPT overnight at 37°C as it equally supports the growth of *Lactobacillus* spp. and UPEC. Cultures were diluted to 1×10^8 cfu mL^{-1} in sterile LAPT (1.5% Bacto Peptone, 1% Bacto Tryptone, 1% yeast extract, 1% glucose and 0.1% Tween 80). Next, 5×10^8 cfu of UPEC and 1×10^8 cfu of *Lactobacillus* spp. were added to fresh LAPT to give a final volume of 10 mL. One mL samples of the inocula and 1 mL samples of coculture were taken after 6 and 24 h to evaluate growth. Samples were spread in dilutions of 10^{-1}–10^{-8} on nutrient agar (NA) and MRS plates and incubated overnight at 37°C (NA aerobically, MRS anaerobically) for colony enumeration. To confirm colony ID, a standard Gram stain of unique colonies on MRS was performed before counting.

Production of *Lactobacillus* spp. cell-free culture supernatant

Lactobacillus spp. were grown overnight (37°C) on De Man-Rogosa-Sharpe agar (MRS; Difco, Becton Dickinson, Basel, Switzerland) and then subcultured overnight (37°C) on modified MRS broth (mMRS; 1% Peptone #3, 0.5% yeast extract, 0.1% Tween 80, 0.2% $[NH_4]_2C_6H_6O_7$, 0.5% CH_3COONa, 0.01% $MgSO_4$, 0.005% $MnSO_4$, 2% K_2HPO_4 and 60 μM $FeSO_4$–$7H_2O$ and 0.5% glucose; Cadieux *et al.* 2009). Next, cells were diluted to 1×10^5 cfu mL^{-1} in 100 mL of fresh mMRS and grown statically for 24 h at 37°C. Then, cells were pelleted by centrifugation at 10 000 g (20 min, 4°C) and the supernatant was collected. For one batch, the supernatant was adjusted to pH 7.0 using 1N NaOH while for another batch, the supernatant was kept at its native pH (4.0–4.4). Supernatants (cell-free culture supernatants, CFCS) were filter-sterilized (0.22 μm) and fortified using 20% 4× mMRS to avoid the effect of nutrient depletion when used to grow UPEC. This fortification minimally raised the pH of the supernatants to pH 4.7–5.5. In order to assess H_2O_2 production of *Lactobacillus* spp. and its influence on UPEC growth, *Lactobacillus* spp. were grown overnight (37°C) on LAPT. Next, cells were diluted to 1×10^5 cfu mL^{-1} in 100 mL of fresh LAPT and grown statically for 24 h at 37°C. Then, cells were transferred to a sterile 500 mL culture flask and incubated at 37°C for 2 h while shaking at 150 rpm. Cells were then pelleted by centrifugation at 4000 g (10 min, 4°C), and the supernatant was collected. The supernatant was pH neutralized using 1N NaOH, filter-sterilized (0.22 μm) and fortified using 20% 4× LAPT to avoid the effect of nutrient depletion when used to grow UPEC. Concentrations of H_2O_2 were measured before addition of UPEC and after 24 h using a Quantifix Peroxide 25 dipstick (Macherey-Nagel, Düren, Germany). All CFCS were stored at −20°C until use.

UPEC growth in cell-free culture supernatants

Two hundred thirty micro litres of fortified CFCS was added to 96-well Greiner flat bottom microtitre plates (Sigma, Buchs, Switzerland). Overnight cultures (37°C) of UPEC were diluted to 1×10^7 cfu mL^{-1} in PBS (pH 7.0) before 20 μL of culture was added to the microtitre plates giving a final volume of 250 μL. Optical density measurements at 600 nm were taken every 15 min for 12 h while incubating at 37°C on a BMG Labtech SpectroStar Omega (BMG LABTECH GmbH Ortenberg, Germany). Prior to each measurement, the plate was shaken for 10 s.

Acid production by *Lactobacillus* spp.

Overnight cultures of *Lactobacillus* spp. were diluted to 1×10^8 cfu mL^{-1} in sterile LAPT and added to fresh LAPT to give a final volume of 10 mL. In order to assess the production of volatile organic acids by *Lactobacillus* spp., 1 mL aliquots of culture was taken at inoculation (T0), after 6 h (T6) and after 24 h (T24) growth at 37°C and filtered using a 0.22 μm syringe filter. The filtrate was run on an HPLC Agilent series 1100 HPLC (Agilent, Basel, Switzerland) using a Cation H+ pre-column (BioRad, Cressier, Switzerland) and Aminex HP×-87H column (BioRad) at a flow rate of 0.6 ml min^{-1} (25 min, 35°C) and an isocratic mobile phase of 5 mM H_2SO_4. Citric acid, lactic acid, acetic acid, succinic acid, pyruvic acid, propionic acid and butyric acid were detected by refractive index and UV (210 nm) spectrometry.

Cell culture adherence–invasion assay

The human bladder cancer cell line UM-UC-3 (ATCC CRL-1749) was cultured to confluence in 6-well cell culture plates (Corning) using Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. UPEC and *Lactobacillus* spp. cultures were grown on NA and MRS overnight at 37°C (UPEC aerobically, *Lactobacillus* spp. anaerobically). UPEC and *Lactobacillus* spp. cultures were diluted to 1×10^8 cfu mL^{-1} in Dulbecco's modified Eagle medium supplemented with 10% FBS. Cell cultures were washed 1× with $CaCl_2$ or $MgCl_2$ free PBS and inoculated with 5 mL of Dulbecco's modified Eagle medium supplemented with 10% FBS containing 1×10^8 cfu mL^{-1} UPEC and 1×10^8 cfu mL^{-1} *Lactobacillus* spp. Plates were centrifuged at 600 × g for 2.5 min to synchronize the infection process. One millilitre of the UPEC and *Lactobacillus* spp. inoculum was

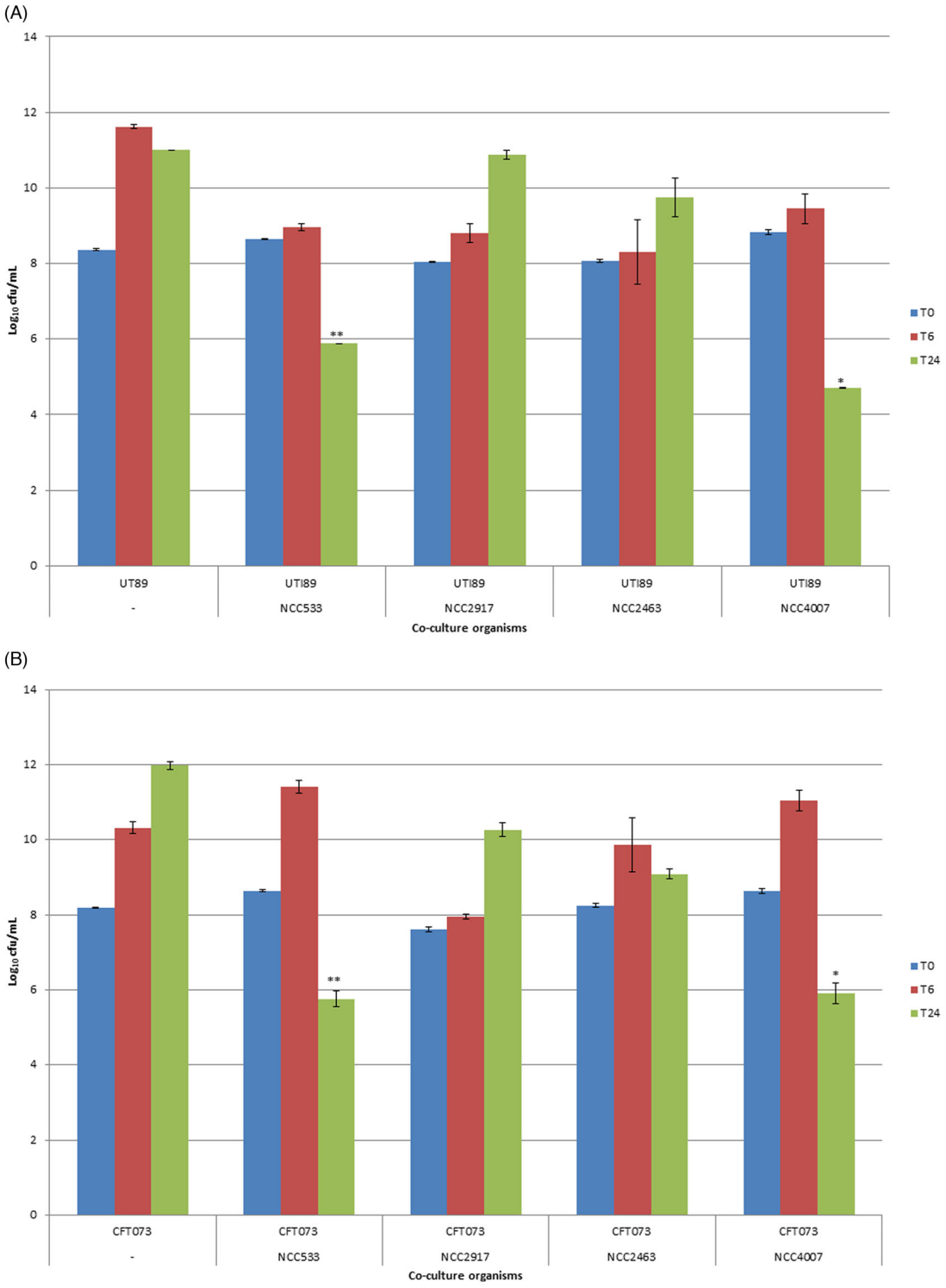

Figure 1. *In vitro* cocultures of UPEC and *Lactobacillus* spp. (*L. johnsonii* strains NCC533 and NCC2917, *L. rhamnosus* NCC4007 and *L. acidophilus* NCC2463) showing influence of *Lactobacillus* spp. on **(A)** growth of *E. coli* UTI89 and **(B)** growth of *E. coli* CFT073, +/− standard deviation (*$P < 0.05$, **$P < 0.001$, three replicates).

diluted and spread (10^{-2}–10^{-8}) onto NA or MRS, respectively, and incubated (UPEC aerobically, *Lactobacillus* spp. anaerobically) at both 37°C for 24 h. The infected epithelial cells were incubated at 37°C with 5% CO_2 for 2 h. To measure bacterial adherence, cells were first washed 4× with PBS to remove non-adherent or non-invasive microorganisms. Epithelial cells, along with any adherent bacteria, were lifted by incubation in 1× Trypsin-EDTA and plated (10^{-2}–10^{-8} dilutions) onto NA and MRS to enumerate adherent UPEC and *Lactobacillus* spp. Bacterial identities of unique colony types on each agar (after 24 h, 37°C incubation) were

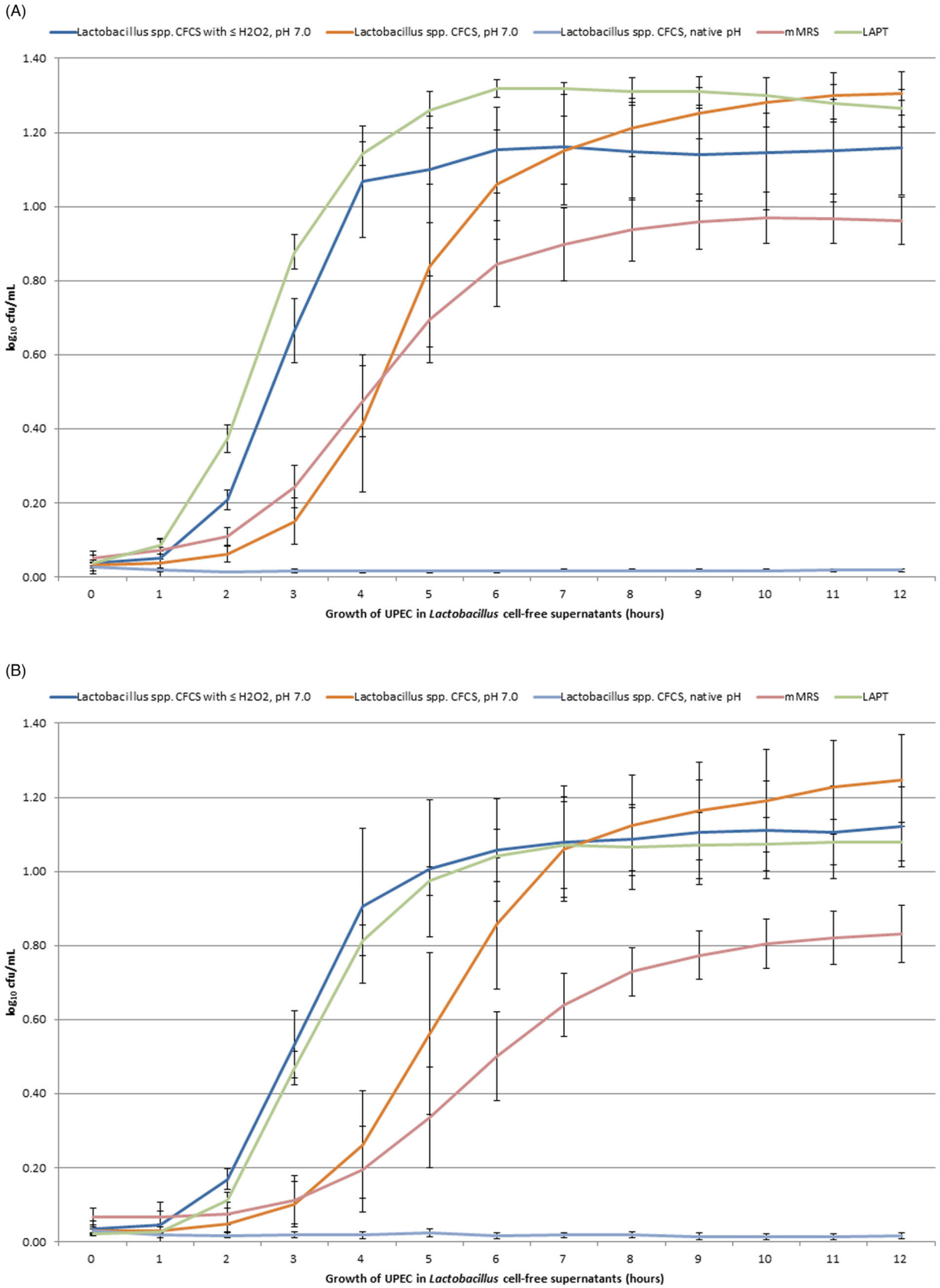

Figure 2. Growth of *E. coli* UTI89 **(A)** and *E. coli* CFT073 **(B)** on cell-free culture supernatants (CFCS; CFCS at native pH4.7–5.5, CFCS at pH7.0 and CFCS at pH7.0 with ≤2 mM H_2O_2), LAPT and mMRS (modified MRS) medium, +/− standard deviation (six replicates per group). UPEC growth on CFCS of *L. johnsonii* strains NCC533 and NCC2917, *L. rhamnosus* NCC4007 and *L. acidophilus* NCC2463 was averaged as no significant difference between groups was observed.

Table 1. Acid production of commercial probiotic *Lactobacillus* spp. (*L. johnsonii* strains NCC533 and NCC2917, *L. acidophilus* NCC2463 and *L. rhamnosus* NCC4007) is used in this study. Time points at inoculation ($T = 0$), after 6 h ($T = 6$) and after 24 h ($T = 24$) growth at 37°C.

Sample		Succinic ($gL^{-1}g/l$)	Lactic (gL^{-1})
NCC 533	$T = 0$	0.2	0.1
NCC 533	$T = 6$	0.1	1.8
NCC 533	$T = 24$	0	6.2
NCC 2917	$T = 0$	0.2	0
NCC 2917	$T = 6$	0.1	1.4
NCC 2917	$T = 24$	0	5.6
NCC 2463	$T = 0$	0.2	0
NCC 2463	$T = 24$	0.1	1.3
NCC 2463	$T = 6$	0.1	4.7
NCC 4007	$T = 0$	0.2	0
NCC 4007	$T = 6$	0.1	2.9
NCC 4007	$T = 24$	0	6.4
LAPT	N/A	0.2	0

confirmed as either UPEC or *Lactobacillus* spp. by Gram strain and microscopy:

$$\%\ \text{of adhesive UPEC} = \frac{\text{Number of invasive cfu}}{\text{Number of adherent cfu}} \times 100.$$

Bacterial invasion was measured using the gentamicin protection assay (Edwards and Massey 2011). After the 2 h of infection, cells were incubated with 100 μg mL^{-1} gentamicin for 30 min. Cells were then washed 4× with PBS (without $CaCl_2$ or $MgCl_2$) and lysed using 1 × Trypsin-EDTA including 0.25% (v/v) Triton ×-100 and plated (10^{-2}–10^{-8} dilutions) onto NA to enumerate invasive UPEC. Colonies (after 24 h, 37°C incubation) were identified as either UPEC or *Lactobacillus* spp. by Gram strain and microscopy:

$$\%\ \text{of invasive UPEC} = \frac{\text{Number of invasive cfu}}{\text{Number of adherent cfu}} \times 100.$$

RESULTS AND DISCUSSION

Coculture of UPEC and *Lactobacillus* spp.

In this study, we assessed the *in vitro* antimicrobial activity of five *Lactobacillus* strains against UPEC. There is evidence that *Lactobacillus* GR-1 upregulates host antimicrobial factors (Kirjavainen *et al.* 2008). However, other factors such as the acidic inhibition of growth, induction of stress in the outer membrane and modification of the environment to one that is less conducive to UPEC thriving seem equally plausible for this and other strains. In particular, the antimicrobial role of lactic acid produced by lactobacilli has been controversial in literature with hypothesis ranging from no role at all to lactic acid being an important factor (Fayol-Messaoudi *et al.* 2005; De Keersmaecker *et al.* 2006; Makras *et al.* 2006). Previous data suggest that *L. johnsonii* NCC533 reduced the viability of UPEC through a combined activity of hydrogen peroxide and lactic acid. Plate counts of UPEC on NA (Fig. 1) showed that after 24 h *L. johnsonii* NCC533 and *L. rhamnosus* NCC4007 significantly reduced UPEC counts to below inoculum levels. There was a significant reduction of growth of *E. coli* UTI89 when comparing counts at 0 and 24 hof coculture as well as when comparing counts at 6 and 24 h in cocultures containing *L. johnsonii* NCC533 ($P = 0.003$ and 0.0001, respectively). For *L. rhamnosus* NCC4007, the reduction in counts was only significant when comparing 6 and 24 h of culture ($P = 0.002$). There also was a significant reduction of growth of *E. coli* CFT073 when comparing counts at 0and 24 h of culture as well as when comparing counts at 6 and 24 h of culture with *L. johnsonii* NCC533 ($P = 0.004$ and 0.0001, respectively). For *L. rhamnosus* NCC4007, the reduction in counts was only significant when comparing 6 and 24 h of culture ($P = 0.002$). In contrast to previous reports, the acidification of the coculture medium may be the primary factor that causes this reduction (Atassi and Servin 2010). For other *Lactobacillus* species examined here, there was no significant effect on either UPEC strain. This may be due to various lactobacilli exerting differing activities on UPEC, which warrants more detailed characterizations of lactobacilli functions in the future. Similar has previously been described for lactobacilli protecting gut barrier functions from enterotoxigenic *E. coli* (Liu *et al.* 2015).

UPEC growth in cell-free culture supernatants

The growth of UTI89 and CFT073 on CFCS was examined spectrophotometrically over a period of 12 h at A_{600nm} in order to assess what cell-free factors may influence a reduction of UPEC cell counts (Fig. 2). Acidic cell-free culture supernatant (CFCS) (pH 4.7–5.5) from any *Lactobacillus* spp. completely inhibited the growth of both UPEC strains. When the CFCS was neutralized (pH7.0), both UPEC strains grew normally in CFCS of all *Lactobacillus* spp. even though the exponential phase was slightly shifted in time. Similar growth was detected in hydrogen peroxide-containing CFCS. The acidic hydrogen peroxide-free CFCS of *L. johnsonii* NCC533, and the other strains tested showed complete inhibition of UPEC strains CFT073 and UTI89. However, H_2O_2-containing CFCS adjusted to pH 7.0 had no effect. Furthermore, indicator strips for hydrogen peroxide quantification showed a complete loss of H_2O_2 (at concentrations produced by lactobacilli) over the time of the assay, presumably due to UPEC catalase activity. Other compounds that may be secreted by either *L. johnsonii* NCC533, *L. johnsonii* NCC2917, *L. rhamnosus* NCC4007 or *L. acidophilus* NCC2463 that may act in synergy with lactic acid as hypothesized by Niku-Paavola *et al.* (1999) also did not have an effect, as demonstrated in UPEC cultures with H_2O_2-free CFCS at pH7.0.

HPLC analysis of acids showed that lactic acid production increased in *Lactobacillus* spp. cultures over a 24 h period, ranging from 0 to 0.1 g L^{-1} at inoculation to 4.7–6.4 g L^{-1} after 24 h (Table 1). No other acids were detected in significant amounts. Besides a reduction of pH, possible mechanisms for the antimicrobial activity of lactic acid may be its function as a permeabilizer of Gram-negative bacterial outer membranes and its chelating properties (Alakomi *et al.* 2000). Lactic acid may capture iron which is essential for UPEC growth, causing inhibition (Presser, Ratkowsky and Ross 1997).

Cell culture adherence–invasion assay

Cell culture of the human bladder cancer cell line UM-UC-3 coinoculated with either UTI89 or CFT073 and *Lactobacillus* spp. showed large variations in adhesion and invasion properties between replicates (Fig. 3). When comparing the% adhesion of UTI89 to UM-UC-3 cells to the percentage of adhesion of UTI89 to UM-UC-3 cells when coinoculated with *Lactobacillus* spp., there

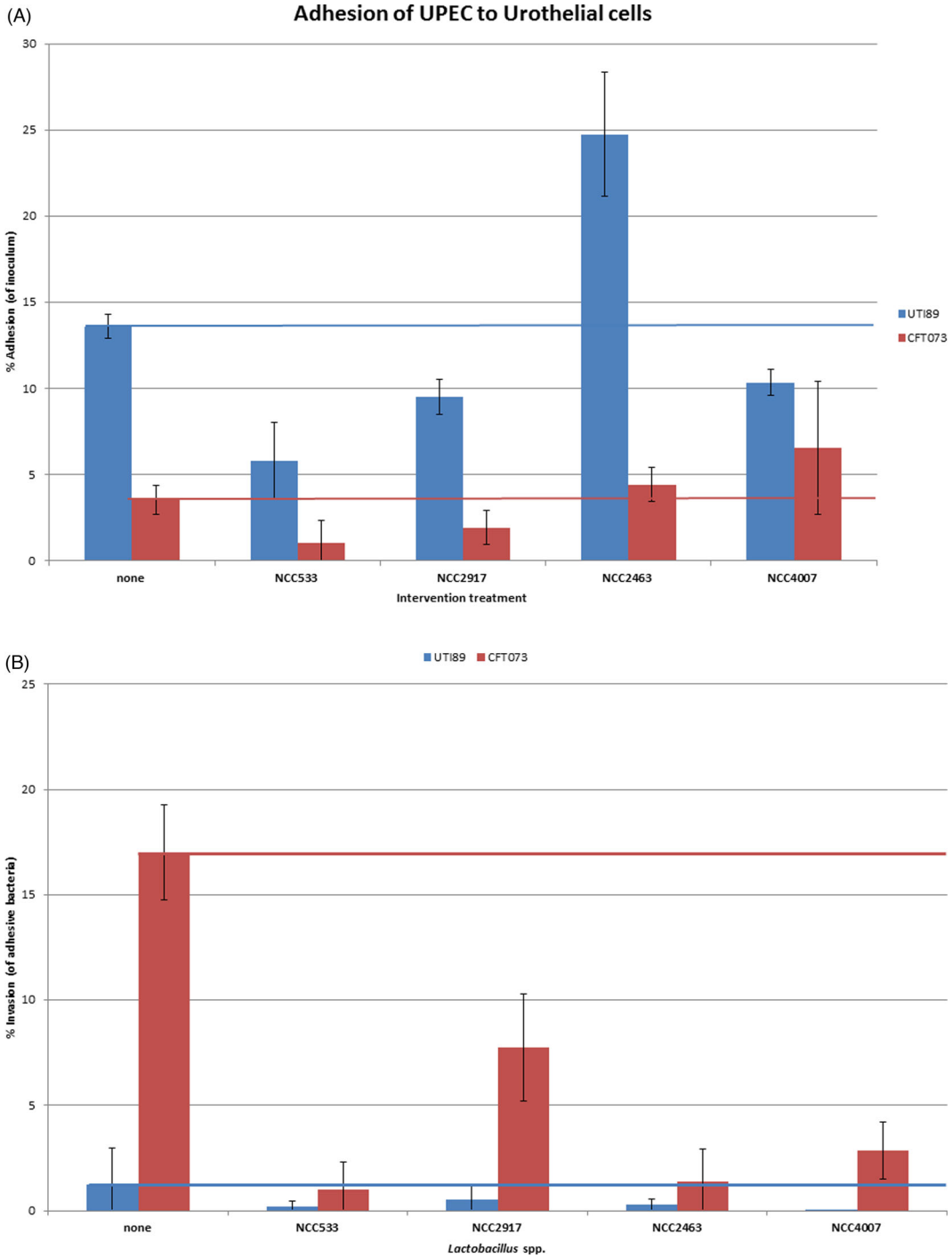

Figure 3. Percent adhesion **(A)** and invasion **(B)** of UTI89 and CFT073 in cell culture using the human bladder cancer cell line UM-UC-3 (ATCC CRL-1749) and *Lactobacillus* spp. (*L. johnsonii* strains NCC533 and NCC2917, *L. rhamnosus* NCC4007 and *L. acidophilus* NCC2463), +/− standard deviation (three replicates). Horizontal lines indicate the level of adhesion / infection of cells associated with UPEC only.

was a general trend of reduced adhesion of UTI89 when coinoculated with either *L. johnsonii* NCC533, *L. johnsonii* NCC2917 or *L. rhamnosus* NCC4007 while adhesion tended to increase when UPEC strains were coincubated with *L. acidophilus* NCC2463. This effect is possibly due to *L. acidophilus* NCC2463 not being able to displace UPEC from the surface of UM-UC-3 cells. For *E. coli* CFT073, a trend for reduction of adhesion was observed for *L. johnsonii* NCC533 and *L. johnsonii* NCC2917 only. In the absence of *Lactobacillus* strains, only 2% of adherent *E. coli* UTI89 were invasive while 17% of adherent *E. coli* CFT073 were invasive (100% = 1×10^8 cfu mL^{-1}). While there was a general trend for reduction of invasiveness for both UTI 89 and CFT073 coinoculated with *Lactobacillus* spp., results did not reach significance. An additional mechanism for UPEC inhibition may be the direct displacement of UPEC adhering to uroepithelial cells by lactobacilli. This would also cause a reduction in adherence and infection (Velraeds *et al.* 1999). This possible mechanism was demonstrated in the uro-epithelial cell culture model

coincubated with UPEC strains CFT073 and UTI89. Here, *L. johnsonii* NCC533, *L. johnsonii* NCC2917 and *L. rhamnosus* NCC4007 reduced the adhesion of both UPEC strains, even though the results were not significant and assay variation in this study were too great to make definite assumptions.

CONCLUSION

In conclusion, with increasing rates of antimicrobial resistance in important pathogens, there is a growing interest in the targeted application of lactobacilli against pathogens. UTI in particular has been a promising lead for *Lactobacillus* spp. therapy as it is mainly caused by one organism, UPEC. While cranberry juice has been a popular home-remedy, the most promising lead towards the treatment of UTI is the installation of *Lactobacillus* into the vagina to form a barrier from infection (Cadieux *et al.* 2009; Guay 2009). *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 have been the most promising candidates for this (Reid and Burton 2002). Commercial probiotic *Lactobacillus* spp., such as the ones examined here, may also provide protection from UTI not necessarily by direct killing of UPEC, but rather through growth inhibition and direct displacement of UPEC cells. A membrane permeabilization caused by produced lactic acid as described by Alakomi *et al.* (2000) may also be possible. Further *in vitro* studies followed by *in vivo* trials are needed for confirmation.

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Conflict of interest. The authors are or have been employees of the Nestlé Research Center, a commercial entity that aims to enhance the quality of consumers' lives through nutrition, health and wellness. Nestlé is active in research into prebiotics and probiotics.

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