RESEARCH LETTER – Pathogens & Pathogenicity

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

Michèle Delley¹, Anne Bruttin¹, Michel Richard¹, Michael Affolter¹, Enea Rezzonico¹ and Wolfram M. Brück¹,²,∗

¹Nestec Ltd, Nestlé Research Center, PO Box 44, CH-1000 Lausanne 26, Switzerland and ²Institute of Life Technologies, University of Applied Sciences Western Switzerland, CH-1950 Sion, Switzerland

∗Corresponding author: Institute of Life Technologies, University of Applied Sciences Western Switzerland, HES-SO Valais-Wallis, Route du Rawyl 47, CH-1950 Sion, Switzerland. Tel: +41-(0)27-606-86-64; Fax: +41-(0)27-606 86 16; E-mail: wolfram.bruck@hevs.ch

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.

Editor: Séamus Fanning

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...
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 L. coli strain CFT002 (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 (ATCC 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 (ATCC Standards, Molsheim Cedex, France). Bacterial culture media and components were purchased from Oxoid (Basel, Switzerland) 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 × 10^5 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 to 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)_2CO_3, 0.5% CH_3COONa, 0.01% MgSO_4, 0.005% MnSO_4, 2% K_2HPO_4 and 60 μM FeSO_4•H_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 mMRS 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 Quantiflex 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 × 10^8 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 Osnabrück, 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 were 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 HPX-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 × 10^5 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 × 10^6 cfu mL^-1 UPEC and 1 × 10^5 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
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% CO₂ 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 pH 4.7–5.5, CFCS at pH 7.0 and CFCS at pH 7.0 with ≤2 mM H₂O₂), 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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Succinic (g L⁻¹)</th>
<th>Lactic (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCC 533 T = 0</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>NCC 533 T = 6</td>
<td>0.1</td>
<td>1.8</td>
</tr>
<tr>
<td>NCC 533 T = 24</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>NCC 2917 T = 0</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>NCC 2917 T = 6</td>
<td>0.1</td>
<td>1.4</td>
</tr>
<tr>
<td>NCC 2917 T = 24</td>
<td>0</td>
<td>5.6</td>
</tr>
<tr>
<td>NCC 2463 T = 0</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>NCC 2463 T = 24</td>
<td>0.1</td>
<td>1.3</td>
</tr>
<tr>
<td>NCC 2463 T = 6</td>
<td>0.1</td>
<td>4.7</td>
</tr>
<tr>
<td>NCC 4007 T = 0</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>NCC 4007 T = 6</td>
<td>0.1</td>
<td>2.9</td>
</tr>
<tr>
<td>NCC 4007 T = 24</td>
<td>0</td>
<td>6.4</td>
</tr>
<tr>
<td>LAPT N/A</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>

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

% of adhesive UPEC = Number of invasive cfu × 100
Number of adherent cfu

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 with PBS (without CaCl₂ or MgCl₂) and lysed using 1% Triton-X-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 stain and microscopy:

% of invasive UPEC = Number of invasive cfu × 100
Number of adherent cfu

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 hypothesizing ranging from no role at all to lactic acid being an important factor (Fayol-Messauidi 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 h of 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 was also a significant reduction of growth of E. coli CFT073 when comparing counts at 0 and 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 A₆₀₀nm 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 (pH 7.0), 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, H₂O₂-containing CFCS adjusted to pH 7.0 had no effect. Furthermore, indicator strips for hydrogen peroxide quantification showed a complete loss of H₂O₂ (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 H₂O₂-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, Ratkowski and Ross 1997).

Cell culture adherence–invasion assay

Cell culture of the human bladder cancer cell line UM-UC-3 coinnoculated 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 coinnoculated 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 coinoculated 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 × 10⁸ cfu mL⁻¹). While there was a general trend for reduction of invasiveness for both UTI89 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.
con incubated with UPEC strains CFT073 and UTI89. Here, L. johnsonii NCC533, L. johnsonii NCC2917 and L. rhamnosus NCC4007 re duced 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.

ACKNOWLEDGEMENTS

The authors would like to thank Anne-Cécile Pittet and Laure Marvin-Guy for their technical assistance during this project.

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.

REFERENCES

Atassi F, Servin AL. Individual and co-operative roles of lactic acid and hydrogen peroxide in the killing activity of ent eric strain Lactobacillus johnsonii NCC933 and vaginal strain Lactobacillus gasseri KS120.1 against enteric, uropathogenic and vaginosis-associated pathogens. FEMS Microbiol Lett 2010;304:29–38.
De Keersmaecker SCJ, Verhoeven TLA, Desair J, et al. Strong antimicrobial activity of Lactobacillus rhamnosus GG against Salmonella typhimurium is due to accumulation of lactic acid. FEMS Microbiol Lett 2006;259:89–96.