

RESEARCH ARTICLE

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Effect of Utipro® (containing gelatin-xyloglucan) against *Escherichia coli* invasion of intestinal epithelial cells: results of an *in vitro* study

Barbara de Servi¹, Francesco Ranzini¹ & Nuria Piqué^{*2}

Aim: To evaluate whether Utipro®, a natural product approved to prevent urinary tract infections, protects intestinal epithelial cells from *Escherichia coli* adherence/intracellular invasion *in vitro*. **Materials & methods:** Caco-2 and CacoGoblet™ cells were treated with Utipro (1.5 to 10 mg/ml) or untreated (controls). *E. coli* adherence/intracellular invasion was evaluated by Trans-Epithelial Electrical Resistance, Lucifer Yellow assay and microbial counts. **Results:** Utipro was noncytotoxic. Utipro 5 and 10 mg/ml protected cell tight junctions (mean ± SD Trans-Epithelial Electrical Resistance [$\Omega \times \text{cm}^2$] 66.83 ± 0.29 and 71.33 ± 0.29 , respectively), and protected cells from *E. coli* intracellular invasion (mean ± SD reductions in total bacteria counts [Log_{10}] 0.9 ± 0.06 and 2.1 ± 0.56 , respectively). **Conclusion:** Results indicate that Utipro creates a protective physical barrier on intestinal epithelial cells *in vitro* which reduces the settling of *E. coli* reservoirs. These results constitute the first step in the demonstration of the efficacy of Utipro to prevent urinary tract infections. Further research is needed in *in vivo* models and clinical trials.

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Currently, urinary tract infections (UTIs) are among the most frequent community-acquired infections worldwide [1], mainly affecting women, but also patients with catheters, diabetes, immunodeficiency syndromes, underlying urologic abnormalities and children [2]. Although UTIs are usually mild, recurrent UTIs have detrimental effects on the quality of life of patients and on healthcare systems [2–7].

UTIs are mainly caused by Gram-negative bacteria, such as *Escherichia coli*, *Pseudomonas* spp., *Enterobacter* spp., *Klebsiella* spp. and *Serratia* spp., and by some Gram-positive pathogens, such as *Enterococcus* spp. and *Staphylococcus* spp. The most relevant uropathogen is *E. coli* which is responsible for 80% of UTIs in women [8]. The *E. coli* phylogenetic groups B2 and D prevail in women with recurrent UTIs. *E. coli* B2 finds a niche reservoir in fecal flora from UTI patients and healthy individuals [9,10], although the factors that may promote urinary tract colonization and bacterial virulence are not completely known [10]. The prevalence of fecal *E. coli* resistant to antibiotics in patients with recurrent UTIs is higher than in healthy individuals, thus increasing the risk of UTIs in these patients [11]. Currently, trimethoprim-sulfamethoxazole, nitrofurantoin and fosfomycin are first-line therapies for uncomplicated cystitis and fluoroquinolones and β -lactams are considered second-line options [3,12]. Clinical studies show that antimicrobial treatments achieve high percentages of cure after 3–7 days [12]. However, rates of drug and multidrug resistant uropathogens have increased in recent years, making the selection of antimicrobial treatment options for patients

KEYWORDS

• gelatin • intestinal epithelial cells • urinary tract infection • Utipro® • xyloglucan

¹VitroScreen Srl, Via Mosè Bianchi 103, 20149 Milano, Italy

²Department of Microbiology & Parasitology, Pharmacy Faculty, Universitat de Barcelona (UB), Diagonal Sud, Facultat de Farmàcia, Edifici A, Av Joan XXIII, 08028 Barcelona, Spain

*Author for correspondence: Tel.: +34 93 402 44 96; Fax: +34 93 402 44 98; npique@gmail.com

with recurrent UTIs more difficult [3,13]. In this scenario, treatment failure can negatively affect the quality of life of patients with recurrent UTIs and can also cause a non-negligible cost for the healthcare system.

Nonpharmacological oral supplements, including cranberry proanthocyanidins [14–16] and probiotics [17], have been evaluated for the prevention of UTIs. Although it is recognized that more research is needed, the use of nonpharmacological products to prevent UTIs should be considered a useful and safe alternative to antibiotics in this era of increasing antibiotic resistance [17].

Utipro® (Novintethical Pharma SA, Pambio-Noranco, Lugano, Switzerland) is a nonpharmacological oral medical device which was approved recently for the prevention of UTIs. It contains gelatin-xyloglucan (a natural hemicellulose) as the main ingredient, along with other plant extracts. Xyloglucan belongs to a new class of products, defined as ‘mucosal protectors’, which form a bio-protective film, restoring the physiological functions of the intestinal walls. Results of recent clinical studies have shown that the administration of xyloglucan is a fast, efficacious and safe option for the treatment of acute diarrhea [18].

The rationale for the potential preventive action of Utipro in UTIs is based on the protective properties of xyloglucan in the intestine to avoid the adhesivity of *E. coli* in the ‘intestinal reservoir’ [19], the first step of uropathogenic *E. coli* proliferation which is followed by bacterial migration from the intestinal tract to the perineal region and, therefore, to the urinary tract [20,21]. The fecal–perineal–urethral mechanism indicates that *E. coli* strains residing in the rectal flora serve as a reservoir for UTIs, such as cystitis [20,21]. This mechanism is more frequent in women due to the shorter distance of the perineal region [10,20].

A reduction in the amount of *E. coli* settling in the intestinal mucosa reservoirs may prevent colonization of the perianal region and the urinary tract and reinfection by this micro-organism.

In this study, we investigated whether Utipro, containing the film-forming agent xyloglucan and gelatin, could protect intestinal epithelial cells from *E. coli* adherence and intracellular invasion in an *in vitro* model.

Materials & methods

• Compound

Utipro powder contains a combination of gelatin and xyloglucan, extracted from the seeds of the tamarind tree (*Tamarindus indica*), *Hibiscus*

sabdariffa, propolis, silicon dioxide, magnesium stearate and corn. The product was kindly provided by Novintethical Pharma SA and diluted in bicarbonate solution.

• Cells & reagents

Caco-2 cells (ATCC HTB37) and CacoGoblet™ (Avancell, Spain) were used for the intestinal mucosa model. Caco-2 cells were seeded at a density of 1.5×10^5 cells/well on 0.4 μ M PET transwell inserts (Millipore) in 12-well plates and maintained for 21 days. Caco-2 cells became confluent at day 6 and reached steady state at day 10. Cellular differentiation was completed at day 21. Microvilli and tight junctions were visible by microscopy during cellular differentiation. CacoGoblet is a ready-to-use model for evaluating *in vitro* intestinal absorption. The kit provides a 21-day cell barrier formed by differentiated coculture Caco-2 and human goblet mucus-screening cells (HT29H and HT29-MTX) plated on HTS transwell permeable supports.

In both cases, cells were maintained in DMEM medium with high glucose (Dulbecco’s modified Eagle medium, Lonza, Belgium) supplemented with 10% fetal bovine serum (FBS, Lonza, Belgium), 1% Non-Essential Amino Acid (NEAA, Lonza, Belgium), 4 mM glutamine (Lonza, Belgium), 10 mM hepes (Lonza, Belgium) and 1% penicillin-streptomycin (Lonza, Belgium), at 37°C, 95% humidity and 5% CO₂.

Other reagents used were phosphate buffer solution (PBS; Sigma), Trypsin EDTA (Lonza), HBSS (Sigma), Lucifer Yellow (Sigma), MES (Sigma), Calcium Chloride Dihydrate (Sigma), Magnesium Chloride Hexahydrate (Sigma), Triton X-100 (Sigma) and Thiazolyl Blue Tetrazolium Blue (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide [MTT]; Sigma).

• Cytotoxicity

Utipro cytotoxicity was assessed on Caco-2 cells by MTT assay. First, product interference with MTT was tested. A total of 10 mg of Utipro was incubated in the presence of MTT (0.5 g/ml) for 3 h at 37°C, 95% humidity, 5% CO₂. Formazan production was qualitatively monitored by direct observation of purple coloring. Noninterference was observed.

Caco-2 cells were then cultured at 120,000 cells/well with either 10 mg/ml Utipro powder or Utipro dissolved in bicarbonate solution, in 96-well culture plates by triplicate and incubated

for 4 h at 37°C, 95% humidity and 5% CO₂. Untreated cells (0 mg/ml) were used as control. After incubation, cell culture medium was removed and replaced with 200 µl of MTT solution (0.5 mg/ml MTT) per well. Plates were incubated again for 3 h then MTT solution was replaced with isopropanol (200 µl) and incubated for 10 min under agitation to dissolve the purple formazan produced by viable cells into a colored solution. Absorbance was read at 570 nm (Microplate Autoreader Infinite® M-200, Tecan, Durham, NC). Absorbance values were normalized to viability percentage relative to the Utipro untreated control cells. The cytotoxic effect of Utipro concentration was considered acceptable when the viability value was higher than 50%.

- **Evaluation of the properties of Utipro to preserve tight junctions of mucosa epithelial cells**

The effects of Utipro in preserving the tight junctions of epithelial cells were evaluated in CacoGoblet cells using Trans-Epithelial Electrical Resistance (TEER). Cell monolayers were treated with 0, 1.5, 2.5, 5 or 10 mg/ml of Utipro powder dissolved in bicarbonate solution, in triplicate, and incubated for 4 h at 37°C and 5% CO₂. Both untreated cell monolayers and transwells with the filter insert without cells (0 mg/ml of Utipro) were used as controls.

TEER was applied to measure the barrier integrity by placing the appropriate electrodes in the apical (AP) and basolateral (BL) positions according to the manual instructions (Millicell® ERS meter, Millipore, MA, USA). TEER measurements were carried out just before the addition of Utipro and after 4 h of treatment. Final TEER values ($\Omega \times \text{cm}^2$) of cell monolayers were obtained after subtracting the TEER value produced by the filter insert without cells.

- **Evaluation of the properties of Utipro to preserve the paracellular flux**

The effects of Utipro in preserving the paracellular flux within the mucosal barrier model were evaluated in CacoGoblet cells by Lucifer Yellow (LY) assay. Cell monolayers were treated with 1.5, 2.5 or 5 mg/ml of Utipro powder dissolved in bicarbonate solution, in triplicate, and incubated for 4 h at 37°C and 5% CO₂. Untreated cells were used as controls.

LY assay was performed before and after treatment to measure the degree of porosity of intercellular tight junctions of epithelial cells. Briefly, 0.3 ml/well of LY (100 µM dissolved in HBSS-1% MES buffer) was applied in the AP compartment of the cell monolayer, and 0.75 ml of HBSS-Ca²⁺/Mg²⁺ was applied in the BL compartment. Cells were then incubated for 2 h at 37°C, 95% humidity and 5% CO₂. After incubation, the paracellular flux of LY from the AP to the BL compartment was measured by fluorescence (RFU) using spectrofluorimeter (Tecan Infinite M200) at 428 nm excitation and 535 nm emission. LY flux was calculated with the following formula:

$$\text{LY Flux} = (\text{RFUBL}/\text{RFUAP}) \times 100$$

where RFUB are fluorescent units detected at the BL compartment and RFUAP are fluorescent units detected at the AP compartment. The apparent permeability (PAPP, cm/s) was calculated with the following formula:

$$\text{PAPP} = (\text{BL concentration}/\text{AP concentration}) \times (\text{BL volume}/\text{Area} \times \text{time}).$$

To estimate LY concentration in the AP and BL compartments, a standard curve was prepared using twofold increasing concentrations of LY (0–200 µM) in a 96-well plate (100 µl, in triplicate). Acceptance criteria were: expected LY flow in untreated cell monolayer lower than 10%, and expected PAPP coefficient less than 2.3×10^6 cm/s (internal controls).

- **Evaluation of the protective properties of Utipro against *E. coli* invasion of intestinal epithelial cells**

The effects of Utipro against *E. coli* invasion of CacoGoblet cells were evaluated by inoculating 1×10^7 cfu/ml of *E. coli* (ATCC 8739) in each well. Previously, the optimal time period for *E. coli* adsorption was assessed at 1, 3, 6 and 15 h. Subsequently, 1 h of adsorption time was chosen (data not shown).

CacoGoblet cells were preincubated for 4 h with Utipro (0, 5, 10 mg/ml). After Utipro treatment, cells were infected with *E. coli* (1×10^7 cfu/ml) and incubated for 1 h. Later, the cell monolayers were washed three-times with sterile PBS and treated with 100 mM penicillin-streptomycin for 10 min. Finally, cell monolayers were washed three-times with sterile PBS and exposed to 1% Triton X-100 for 10 min to produce cell lysates and release the internalized bacteria. Quantitative values of intracellular bacteria were obtained by bacterial counting in cell

lysates and the results were Log_{10} -transformed (Log_{10} total bacteria count/well).

• Antiadherence effects of xyloglucan & gelatin

In a similar manner, we evaluated the protective effect exerted by the film-forming agent xyloglucan and gelatin. After microbial adsorption of *E. coli* (ATCC 8739) and without washing, 5 mg/ml of xyloglucan and gelatin (PL422 and PL423 powder dissolved in bicarbonate solution) were added onto the cell monolayers, in triplicate. Cells were incubated for different time periods (1, 4 and 24 h) at 37°C and 5% CO_2 . In this experiment, duplicate wells of untreated plus bicarbonate solution cell monolayers were used as negative controls. Bacterial count was analyzed by Tali™ Image Cytometer. Changes in those parameters were analyzed by comparing values before and after *E. coli* inoculation and after the addition of xyloglucan and gelatin (1, 4 and 24 h of treatment).

• Statistical analysis

A descriptive analysis of quantitative data was performed. Mean and standard deviation of TEER, LY (%) and bacterial count (Log_{10}) values were calculated from Utipro-treated and untreated cell monolayers.

Results

• Cytotoxicity

Utipro treatment of Caco-2 cells for 4 h showed no cytotoxic effects. Cell viabilities were greater than 88% using Utipro powder (88.6%) or Utipro dissolved in bicarbonate (88.5%) (Figure 1).

• Protective properties of Utipro on cell monolayers

CacoGoblet cell monolayers treated with Utipro for 4 h showed higher TEER values compared with untreated cells. Mean \pm SD ($\Omega \times \text{cm}^2$) TEER values were 66.83 ± 0.288 and 71.33 ± 0.288 with Utipro 5 and 10 mg/ml, respectively, while the mean \pm SD ($\Omega \times \text{cm}^2$) TEER value in untreated cells was 59.17 ± 0.00 (Figure 2).

• Protective properties of Utipro to preserve the paracellular flux

Utipro did not alter cell permeability within the mucosal barrier model. Utipro maintained the paracellular flux between AP and BL compartments of treated cells independently of the concentration assayed. Mean \pm SD (%) LY flux values were 10.64 ± 0.51 (1.5 mg/ml Utipro), 8.70 ± 1.37 (2.5 mg/ml Utipro) and 9.90 ± 0.25 (5 mg/ml Utipro) (Figure 3), similar to LY flux values obtained in untreated cells ($10.08 \pm 0.65\%$).

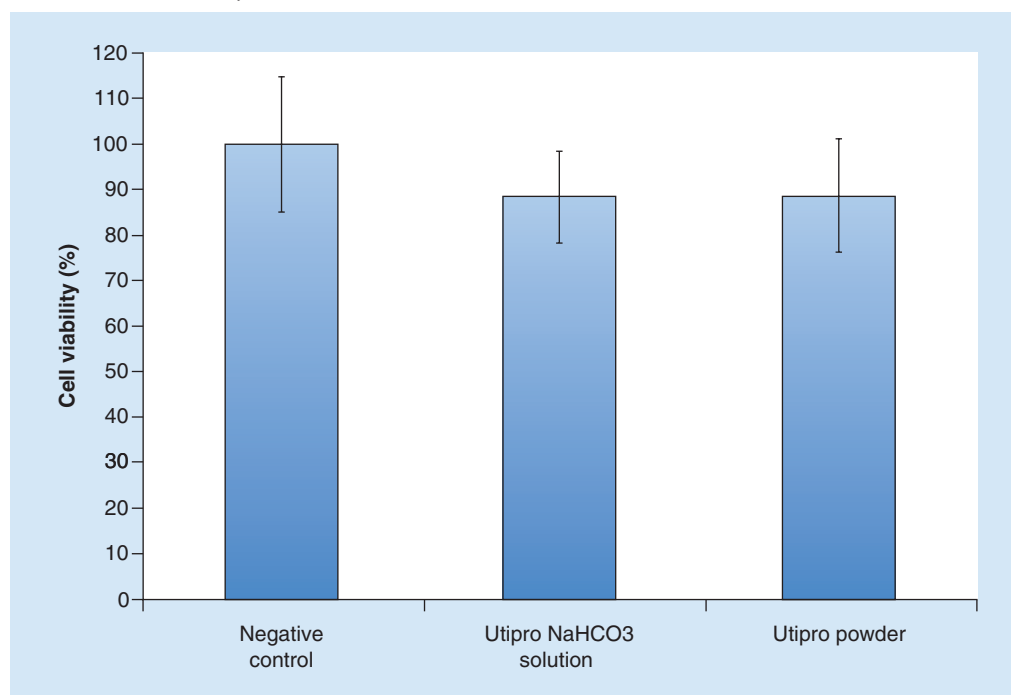


Figure 1. Evaluation of cell viability (%) after 4 h of treatment with Utipro® (10 mg/ml) diluted in bicarbonate solution and with Utipro powder (MTT test).

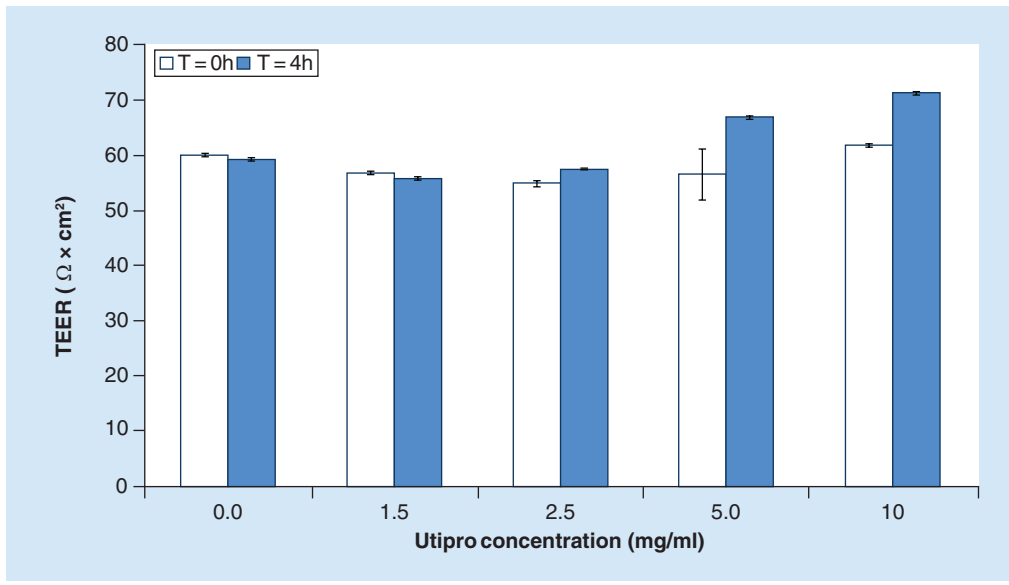


Figure 2. Protective properties of Utipro® to preserve tight junctions among CacoGoblet™ cells. TEER values (mean ± SD, Ω × cm²) increased with Utipro after 4 h of treatment compared to untreated cells.

• Protective properties of Utipro against *E. coli* invasion of the intestinal mucosa

Utipro treatment (4 h) in CacoGoblet cell monolayers reduced the intracellular invasion of *E. coli* compared with untreated cells. Utipro 5 mg/ml reduced the intracellular invasion of *E. coli* by a mean ± SD (Log₁₀) of 0.9 ± 0.06 (from 2.1 × 10⁴ to 2.4 × 10³ average bacteria total count/well); Utipro 10 mg/ml reduced the intracellular invasion of *E. coli* by a mean ± SD (Log₁₀ of bacteria total count/well) of 2.1 ± 0.56 (from 2.1 × 10⁴ to 1.2 × 10² average bacteria total count/well) (Figure 4).

• Anti-adherence effects of xyloglucan & gelatin

E. coli was retained in the apical supernatant and in the homogenate mucus (>6 Log₁₀). After treatment of cell monolayers with xyloglucan and gelatin, bacteria were equally distributed in apical and homogenate mucus compartments at all time points of treatment. Treatment with xyloglucan and gelatin produced a decrease in the number of *E. coli* cells adhered, particularly in the homogenate mucus compartment (from 6.64 × 10⁶ to 3.64 × 10⁵).

Discussion

Utipro has recently been approved as an oral medical device to prevent UTIs. Its components are well known natural products, habitually used in food and drinks, which are well tolerated. The main ingredient of Utipro is gelatin-xyloglucan.

Xyloglucan, from *T. indica* seeds, is a soluble hemicellulose which, combined with gelatin-A, forms an innocuous biopolymer that exerts a physical barrier against intestinal *E. coli* invasion and gut alterations in animals [19].

In the context of UTIs, several studies indicate the fecal tract flora as a potential reservoir of uropathogenic *E. coli* B2 that could increase the risk of urinary tract colonization [21–23]. The persistence of this uropathogenic group in the lower intestinal tract is supported by the activation of several virulence-associated genes that express virulence factors such as adhesins (fimbriae and p-pili), toxins, polysaccharide capsules and siderophores, which can be modulated by environmental conditions, such as changes in pH and osmolarity [21–23]. The expression of a broad variety of virulence-associated genes provides advantages for the colonization of different microhabitats [23].

In this study, we aimed to provide basic evidence that Utipro exerts a protective effect against *E. coli* adhesion and invasion in intestinal epithelial cells. We used established human intestinal epithelial cell models that mimic intestinal mucosa [24,25], and well-known methods, such as TEER and LY, to evaluate the preservation of cellular tight junctions [26,27]. The objective of the study was to demonstrate the basis of the mechanism of action of a product intended to prevent UTIs. We consider that the observed protective effects (anti-adhesive and anti-invasive properties) of Utipro on intestinal epithelial cells are the first step to avoid

urinary colonization, according to the fecal–perineal–urethral hypothesis [20]. Due to the preventive nature of the product, we consider that this step at the intestinal level is of great importance for the mechanism of action of Utipro. In further studies, we will assess the effects of Utipro in *in vitro* and *in vivo* models of UTIs using a wide panel of uropathogenic strains and also in randomized clinical studies in subjects susceptible to UTIs.

We used the strain *E. coli* ATCC 8739 since it was used in previous *in vitro* and *in vivo* studies performed by our company with Utipro and with the film-forming agents xyloglucan and gelatin. As already demonstrated in our studies, it has the capacity to adhere and invade intestinal epithelial cells, thus making it suitable for these types of assays. This is in line with its fecal origin (*E. coli* [Migula], Castellani and Chalmers, ATCC® 8739-MINI-PACK™).

For the first time, we have demonstrated that Utipro prevents the intracellular invasion of *E. coli* by 2 Log₁₀ in an intestinal epithelial cell model, thus reducing the development of *E. coli* reservoirs. We consider that the antiadhesive and anti-invasive properties of xyloglucan and gelatin allow the bacteria embedded in the protective film to be expelled with the feces, thus avoiding bacterial colonization of the perianal region and the urinary

tract. Further clinical studies assessing the effect of Utipro in patients with the first symptoms of UTIs will confirm these results.

We consider that the mechanism of action of Utipro is nonpharmacological, since Utipro forms a physical barrier on the mucus of intestinal epithelial cells that increases the resistance of cell tight junctions and protects intestinal cells against the adherence of *E. coli*. The xyloglucan-gelatin biopolymer prevents the binding of fimbriae and p-pili to cell oligosaccharides and protects tight junctions from bacterial translocation, indicating a clear effect of resistance to bacterial invasion and the potential development of quiescent reservoirs of *E. coli* in the intestinal epithelium model. In previous *in vivo* studies we have also demonstrated the antisecretory effects of xyloglucan and gelatin after treatment with LPS and cholera toxin, thus demonstrating the protective effects in a model of tight junction alterations [19]. These results are also in line with those obtained in clinical trials in patients with diarrhea, in which the administration of xyloglucan for 3 days resulted in rapid improvements in diarrheal symptoms (measured as type 6 and 7 Bristol scale stools) and a reduction in the percentage of patients with nausea, vomiting and abdominal pain [18]. The beneficial effects of film-forming agents have

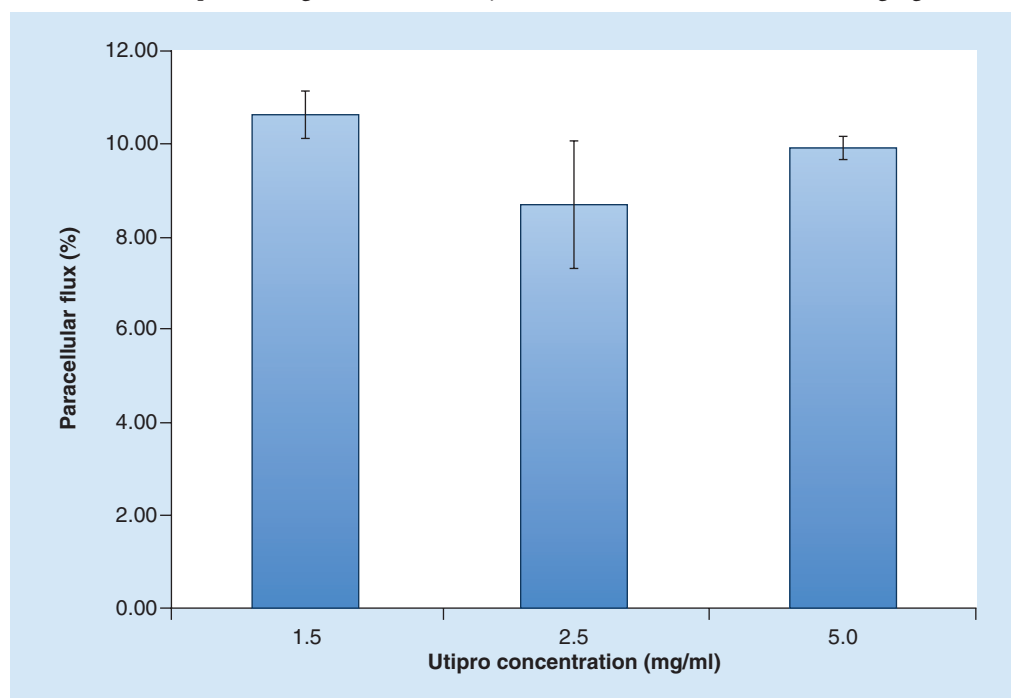


Figure 3. Protective properties of Utipro® to preserve the paracellular flux between the apical and basolateral compartments of CacoGoblet™ cells. Utipro did not alter the cell permeability within the mucosal barrier model. LY flux ± SD (%) values.

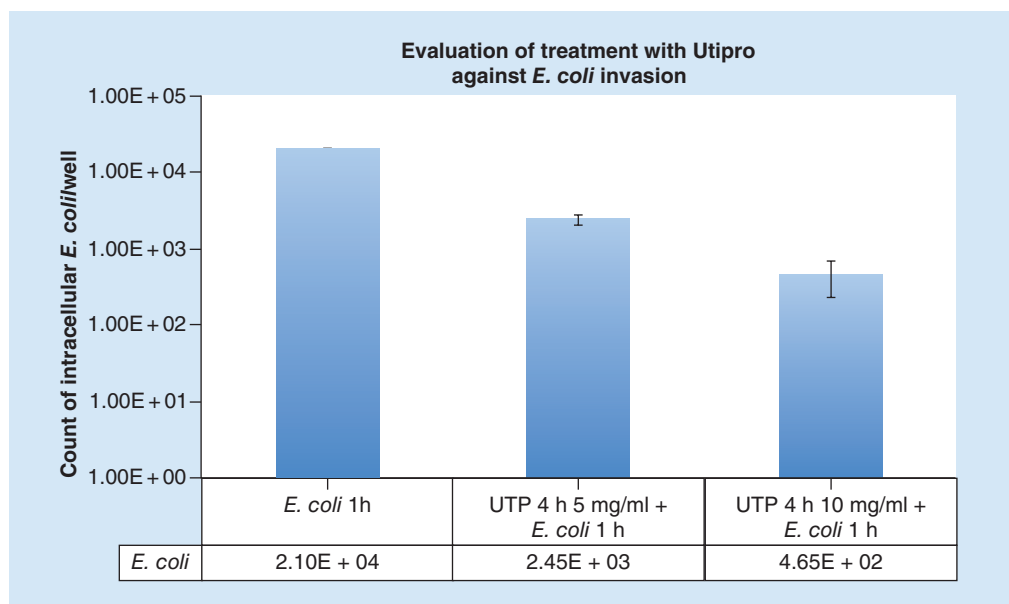


Figure 4. Evaluation of preventive and antiabsorptive properties of Utipro® against *E. coli* invasion. Four hours of preventive treatment with Utipro (5 and 10 mg/ml) reduced microbial growth (mean bacterial total count/well) $>0.9 \text{ Log}_{10}$ compared to untreated cells.

also been demonstrated in patients with irritable bowel syndrome [28].

We consider that the recommended posology assures the required time to exert the preventive action: the device is to be taken orally as 2 capsules per day for 5 days in the case of patients who develop the first symptoms of urinary discomfort, and as 1 capsule per day for at least 15 consecutive days per month, to prevent recurrence (if necessary, the product can be taken for repeated cycles [Utipro Leaflet, Novintethical Pharma, SA]).

In conclusion, the results of our study indicate that Utipro creates a protective physical barrier on intestinal epithelial cells *in vitro*, which can reduce the settling of *E. coli* reservoirs. These results constitute the first step in the demonstration of the efficacy of Utipro to prevent UTIs. Further research is needed in *in vivo* models and in clinical trials.

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EXECUTIVE SUMMARY

- Utipro®, a nonpharmacological oral medical device which was approved recently for the prevention of urinary tract infections, contains gelatin-xyloglucan (a natural hemicellulose) as the main ingredient, along with other plant extracts.
- Xyloglucan belongs to a new class of products, defined as ‘mucosal protectors’, which form a bioprotective film, restoring the physiological functions of the intestinal walls.
- This *in vitro* study evaluated whether Utipro protects intestinal epithelial cells from *Escherichia coli* adherence and intracellular invasion.
- Utipro was noncytotoxic.
- Utipro 5 and 10 mg/ml protected cell tight junctions (mean \pm SD transepithelial electrical resistance [$\Omega \times \text{cm}^2$] 66.83 ± 0.29 and 71.33 ± 0.29 , respectively).
- Utipro 5 and 10 mg/ml protected cells from *E. coli* intracellular invasion (mean \pm SD reductions in total bacteria counts [Log_{10}] 0.9 ± 0.06 and 2.1 ± 0.56 , respectively) and bacterial adherence.
- *In vitro*, Utipro created a protective physical barrier on intestinal epithelial cells, which is able to reduce the settling of *E. coli* reservoirs.

Financial & competing interests disclosure

The study was supported financially by Novintethical Pharma SA. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial

interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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