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

SHORT TITLE: Effect of Utipro® against *E. coli* invasion

AUTHORS: Barbara de Servi (1), Francesco Ranzini (1), Nuria Piqué (2)

AFFILIATIONS OF ALL AUTHORS:

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

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

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Summary

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 were evaluated by Trans-Epithelial Electrical Resistance (TEER), Lucifer Yellow assay and microbial counts. **Results**: Utipro® was non-cytotoxic. Utipro® 5 and 10 mg/mL protected cell tight junctions (mean±SD TEER [$\Omega \times cm^2$] 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 of our study indicates 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 for the demonstration of the efficacy of Utipro® to prevent urinary tract infections. Futher research is needed in *in vivo* models and clinical trials.

KEY WORDS: intestinal epithelial cells; urinary tract infection; Utipro®; xyloglucan; gelatin

1. INTRODUCTION

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 (QoL) 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 beta-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 with recurrent UTIs more difficult

58 [3,13]. In this scenario, treatment failure can negatively affect the QoL of
59 patients with recurrent UTIs and can also cause a non-negligible cost for the
60 healthcare system.

61 Non-pharmacological oral supplements, including cranberry
62 proanthocyanidins [14,15,16Howell 2002; Howell et al. 2010; Gupta et al. 2012]
63 and probiotics [17], have been evaluated for the prevention of UTIs. Although it
64 is recognized that more research is needed, the use of non-pharmacological
65 products to prevent UTIs should be considered a useful and safe alternative to
66 antibiotics in this era of increasing antibiotic resistance [17].

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

76 The rationale for the potential preventive action of Utipro® in UTIs is based
77 on the protective properties of xyloglucan in the intestine to avoid the adhesivity
78 of *E. coli* in the “intestinal reservoir” [19], the first step of uropathogenic *E. coli*
79 proliferation which is followed by bacterial migration from the intestinal tract to
80 the perineal region and, therefore, to the urinary tract [20,21]. The fecal-
81 perineal-urethral mechanism indicates that *E. coli* strains residing in the rectal

flora serve as a reservoir for urinary tract infections, 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 microorganism.

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.

2. MATERIALS AND METHODS

2.1. 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.

2.2. Cells and 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 µM PET transwell inserts (Millipore) in 12-well plates

104 and maintained for 21 days. Caco-2 cells became confluent at day 6 and
105 reached steady state at day 10. Cellular differentiation was completed at day
106 21. Microvilli and tight junctions were visible by microscopy during cellular
107 differentiation. CacoGoblet™ is a ready-to-use model for evaluating *in vitro*
108 intestinal absorption. The kit provides a 21-day cell barrier formed by
109 differentiated co-culture Caco-2 and human globet mucus-screening cells
110 (HT29H and HT29-MTX) plated on HTS transwell permeable supports.

111 In both cases, cells were maintained in DMEM medium with high glucose
112 (Dulbecco's modified Eagle medium, Lonza, Belgium) supplemented with 10%
113 fetal bovine serum (FBS, Lonza, Belgium), 1% Non-Essential Amino Acid
114 (NEAA, Lonza, Belgium), 4 mM glutamine (Lonza, Belgium), 10 mM hepes
115 (Lonza, Belgium) and 1% penicillin-streptomycin (Lonza, Belgium), at 37ºC,
116 95% humidity and 5% CO_2.

117 Other reagents used were phosphate buffer solution (PBS; Sigma), Trypsin
118 EDTA (Lonza), HBSS (Sigma), Lucifer Yellow (Sigma), MES (Sigma), Calcium
119 Chloride Dihydrate (Sigma), Magnesium Chloride Hexahydrate (Sigma), Triton
120 X-100 (Sigma), and Thiazolyl Blue Tetrazolium Blue (3-(4, 5-dimethylthiazolyl-
121 2)-2,5-diphenyltetrazolium bromide [MTT]; Sigma).

122 **2.3. Cytotoxicity**

123 Utipro® cytotoxicity was assessed on Caco-2 cells by MTT assay. Firstly,
124 product interference with MTT was tested. A total of 10 mg of Utipro® was
125 incubated in the presence of MTT (0.5 g/mL) for 3 hours at 37°C, 95% humidity,

5% CO_2. Formazan production was qualitatively monitored by direct observation of purple coloring. Non-interference 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 4h at 37ºC, 95% humidity and 5% CO_2. Untreated cells (0 mg/mL) were use 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 3h then MTT solution was replaced with isopropanol (200 µL) and incubated for 10 minutes 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%.

2.4. 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 4h at 37ºC and 5% CO_2. Both untreated cell-monolayers and transwells with the filter insert without cells (0 mg/mL of Utipro®) were used as controls.

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

155 **2.5. Evaluation of the properties of Utipro® to preserve the paracellular**
156 **flux**

157 The effects of Utipro® in preserving the paracellular flux within the mucosal
158 barrier model were evaluated in CacoGoblet™ cells by Lucifer Yellow (LY)
159 assay. Cell monolayers were treated with 1.5, 2.5, or 5 mg/mL of Utipro®
160 powder dissolved in bicarbonate solution, in triplicate, and incubated for 4h at
161 37ºC and 5% CO_2. Untreated cells were used as controls.

162 LY assay was performed before and after treatment to measure the degree
163 of porosity of intercellular tight junctions of epithelial cells. Briefly, 0.3 mL/well of
164 LY (100 µM dissolved in HBSS-1% MES buffer) was applied in the AP
165 compartment of the cell monolayer, and 0.75 mL of HBSS-Ca^{2+}/Mg^{2+} was
166 applied in the BL compartment. Cells were then incubated for 2h at 37°C, 95%
167 humidity and 5% CO_2. After incubation, the paracellular flux of LY from the AP
168 to the BL compartment was measured by fluorescence (RFU) using
169 spectrofluorimeter (Tecan Infinite M200) at 428 nm excitation and 535 nm
170 emission. LY flux was calculated with the following formula:

171 *LY Flux = (RFUBL/RFUAP) × 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/sec) was calculated with the following formula:

PAPP = (BL concentration/AP concentration) × (BL volume/Area × time).

To estimate LY concentration in the AP and BL compartments, a standard curve was prepared using 2 fold increasing concentrations of LY (0.0 µM to 200.0 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/sec (internal controls).

2.6. 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 15h. Subsequently, 1h of adsorption time was chosen (data not shown).

CacoGoblet™ cells were pre-incubated for 4 hours with Utipro® (0, 5, 10 mg/mL). After Utipro® treatment, cells were infected with *E. coli* (1×10^{7} cfu/mL) and incubated for 1h. Later, the cell monolayers were washed three times with sterile PBS and treated with 100 mM Penicillin-Streptomycin for 10 minutes. Finally, cell monolayers were washed 3 times with sterile PBS and exposed to 1% Triton X-100 for 10 minutes 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).

2.7 Anti-adherence effects of xyloglucan and 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 triplicates. Cells were incubated for different period of time (1h, 4h and 24h) at 37ºC and 5% of CO_2. In this experiment, duplicate wells of untreated plus bicarbonate solution cell-monolayers were used as negative controls. Bacterial count was analysed by Tali™ Image Cytometer. Changes of those parameters were analysed by comparing the values before and after *E. coli* inoculation and after the addition of xyloglucan and gelatin (1h, 4h and 24 h of treatment).

2.7. 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.

3. RESULTS

3.1. Cytotoxicity

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

3.2. Protective properties of Utipro® on cell monolayers

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

3.3. 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±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±0.65%).

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

Utipro® treatment (4h) 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 (Log_{10}) of 0.9±0.06 (from 2.1×10^4 to 2.4×10^3 average bacteria total count/well); Utipro® 10 mg/mL reduced the intracellular invasion of *E. coli* by a mean±SD (Log_{10} of bacteria total count/well) of 2.1±0.56 (from 2.1×10^4 to 1.2×10^2 average bacteria total count/well) (Figure 4).

3.5 Anti-adherence effects of xyloglucan and gelatin

E. coli was retained in the apical supernatant and in the homogenate mucus (> 6 Log_{10}). 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 gelatine produced a decrease in the number of *E. coli* cells adhered, particularly in the homogenate mucus compartment (from 6.64 $x10^6$ to 3.64 $x10^5$).

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, beingwell 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 aim of this study was to demonstrate the basis of the mechanism of action of a product intended to prevent urinary infections. We consider that the observed protective effects (anti-adhesive and anti-invasive properties) of Utipro® on intestinal epithelial cells is 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 intestinal level is of great importance for the mechanism of action of Utipro®.

283

284 In further studies, we will assess the effects of Utipro® in *in vitro* and *in vivo*
285 models of the UTIs using a wide panel of uropathogenic strains and also in
286 randomized clinical studies in subjects susceptible to have UTIs.

287 We used the strain *E. coli* ATCC 8739 since it was used in previous *in vitro* and
288 *in vivo* studies performed by our company with Utipro® and with the film forming
289 agents xyloglucan and gelatin. As already demonstrated in our studies, it has
290 the capacity to adhere and invade intestinal epithelial cells, thus making it
291 suitable for this type of assays. This is in line with its faecal origin
292 (http://www.lgcstandards-atcc.org/Products/All/8739-MINI-PACK.aspx).

293

294 For the first time, we have demonstrated that Utipro® prevents the
295 intracellular invasion of *E. coli* by 2 Log_{10} in an intestinal epithelial cell model,
296 thus reducing the development of *E. coli* reservoirs. We consider that the anti-
297 adhesive and anti-invasive properties of xyloglucan and gelatin allow the
298 expulsion with the faeces of the bacteria embedded in the protective film, thus
299 avoiding bacterial colonization of the perianal region and the urinary tract.

300

301 Further clinical studies assessing the effect of Utipro® in patients with the first
302 symptoms of UTIs willconfirm these results.

303 We consider that the mechanism of action of Utipro® is non-pharmacological,
304 since Utipro® forms a physical barrier on the mucus of intestinal epithelial cells
305 that increases the resistance of cell tight junctions and protects intestinal cells

306 against the adherence of *E. coli*. The xyloglucan-gelatin biopolymer prevents
307 the binding of fimbriae and p-pili to cell oligosaccharides and protects tight
308 junctions from bacterial translocation, indicating a clear effect of resistance to
309 bacterial invasion and the potential development of quiescent reservoirs of *E.*
310 *coli* in the intestinal epithelium model. In previous *in vivo* studies we have also
311 demonstrated the anti-secretory effects of xyloglucan and gelatine after
312 treatment with LPS and cholera toxin, thus demonstrating the protective effects
313 in a model of tight junctions alterations [19]. These results are also in line with
314 those obtained in clinical trials in patients with diarrhea, in which the
315 administration of xyloglucan for 3 days resulted in rapid improvements in
316 diarrheal symptoms (measured as type 6 and 7 Bristol scale stools) and a
317 reduction in the percentage of patients with nausea, vomiting and abdominal
318 pain [18]. The beneficial effects of film forming agents have also been
319 demonstrated in patients with irritable bowel syndrome [28].

320 We consider that the recommended posology assures the required time to exert
321 the preventive action: the device is to be taken orally as 2 capsules per day for
322 5 days in the case of patients who develop the first urinary discomfort
323 symptoms, and as 1 capsule per day for at least 15 consecutive days per
324 month, for the prevention of recurrence (if necessary, the product can be taken
325 for repeated cycles) (Utipro Leaflet, Novintethical Pharma, SA).

326

327 In conclusion, results of our study indicate that Utipro® creates a protective
328 physical barrier on intestinal epithelial cells *in vitro*, which can reduce the
329 settling of *E. coli* reservoirs. These results constitute the first step for the

330 demonstration of the efficacy of Utipro® to prevent UTIs. Futher research is
331 needed in *in vivo* models and in clinical trials.

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334

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339

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341 which could potentially create a conflict of interest with the contents of this
342 paper.

343

EXECUTIVE SUMMARY

- Utipro®, a non-pharmacological oral medical device which was approved recently for the prevention of UTIs, 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.
- This *in vitro* study evaluated whether Utipro® protects intestinal epithelial cells from *Escherichia coli* adherence and intracellular invasion.
- Utipro® was non-cytotoxic.
- Utipro® 5 and 10 mg/mL protected cell tight junctions (mean±SD transepithelial electrical resistance [$\Omega \times cm^2$] 66.83±0.29 and 71.33±0.29, respectively).
- Utipro® 5 and 10 mg/mL protected cells from *E. coli* intracellular invasion (mean±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.

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FIGURE LEGENDS

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

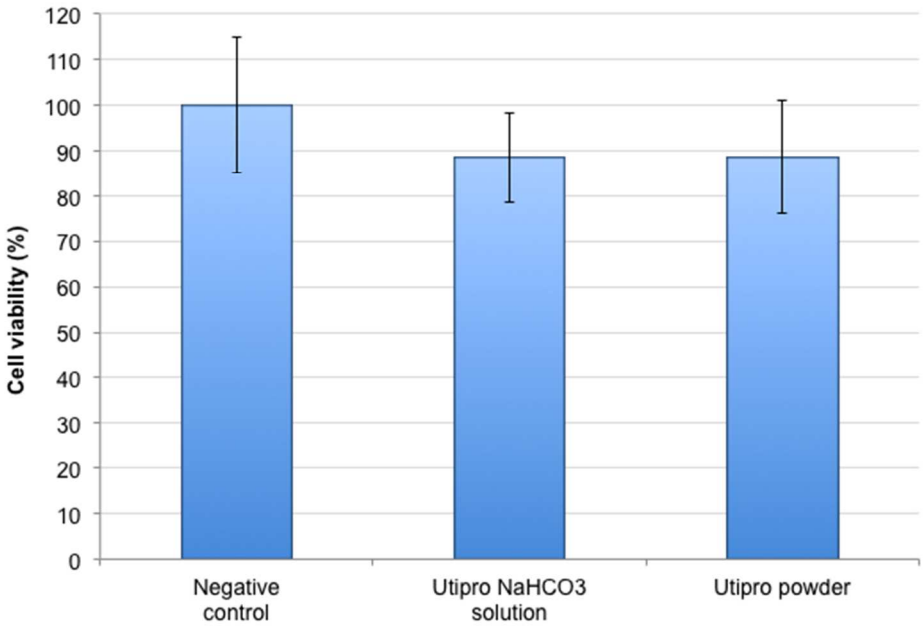
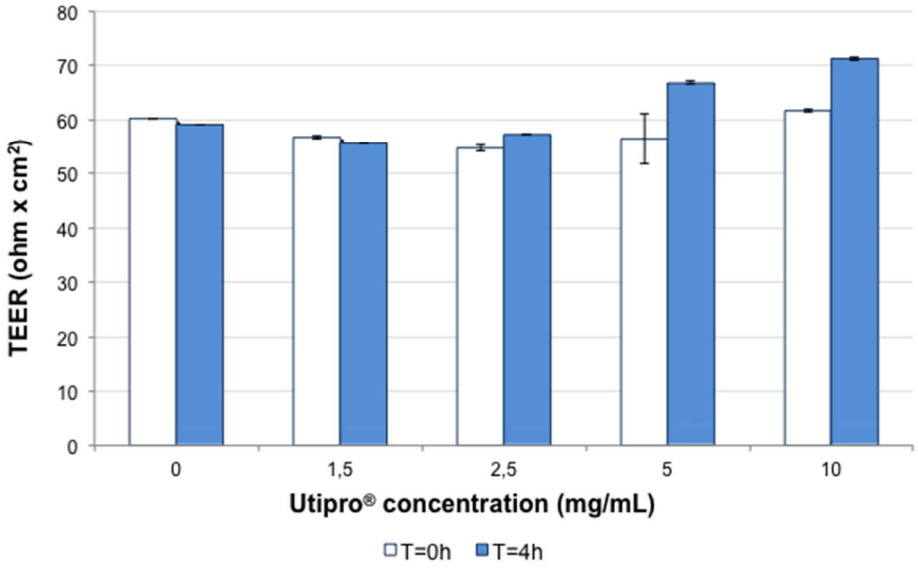


Figure 2. Protective properties of Utipro® to preserve tight junctions among CacoGoblet™ cells. TEER values (mean±SD, Ω × cm^2) increased with Utipro® after 4h of treatment compared to untreated cells.



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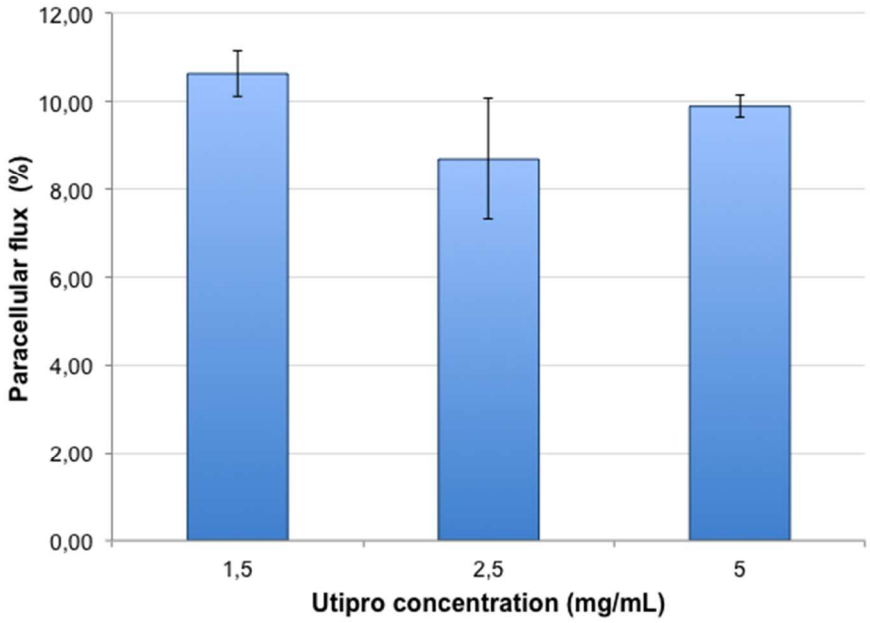
483 **Figure 3**. Protective properties of Utipro® to preserve the paracellular flux
484 between the apical and basolateral compartments of CacoGoblet™ cells.
485 Utipro® did not alter the cell permeability within the mucosal barrier model. LY
486 flux ± SD (%) values.



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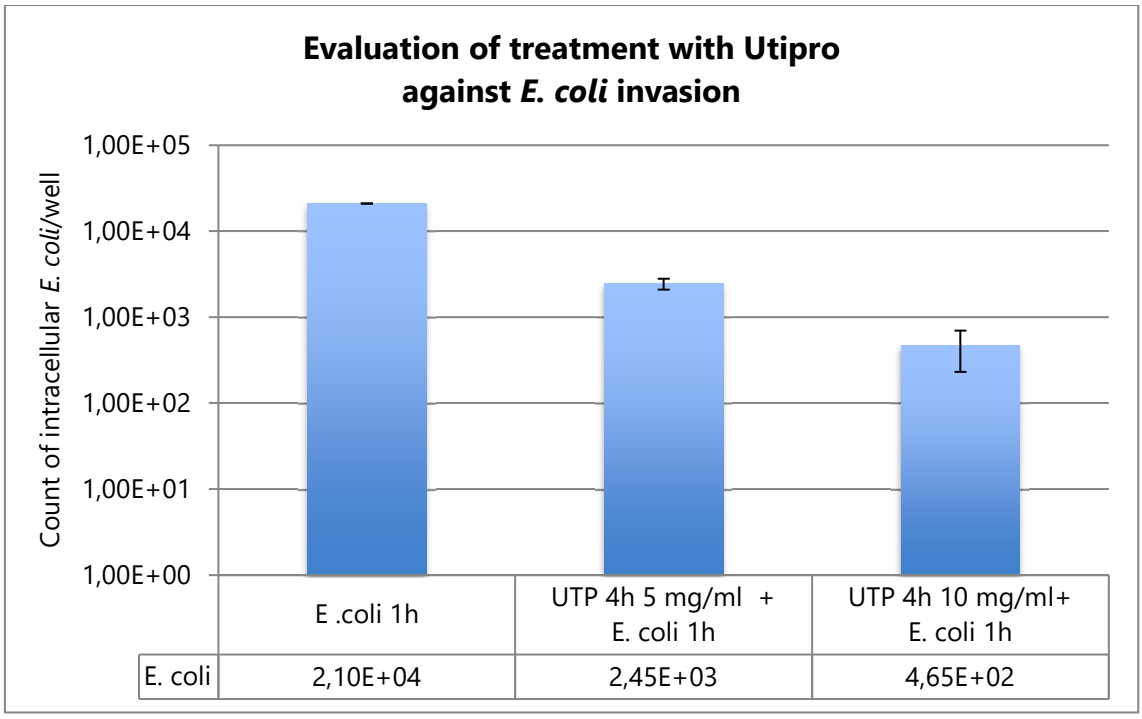
492 **Figure 4**. Evaluation of preventive and anti-absorptive properties of Utipro®
493 against *E. coli* invasion. Four hours of preventive treatment with Utipro® (5 and
494 10 mg/mL) reduced microbial growth (mean bacterial total count/well) >0.9
495 Log_{10} compared to untreated cells (0 mg/mL).



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