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Effect of bovine lactoferrin on recurrent urinary tract infections: in vitro and in vivo evidences

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Abstract Uropathogenic *Escherichia coli* (UPEC) strains are the primary cause of urinary tract infections (UTIs). UPEC strains are able to invade, multiply and persisting in host cells. Therefore, UPEC strains are associated to recurrent UTIs requiring long-term antibiotic therapy. However, this therapy is suboptimal due to the increase of multidrug-resistant UPEC. The use of non-antibiotic treatments for managing UTIs is required. Among these, bovine lactoferrin (bLf), a multifunctional cationic glycoprotein, could be a promising tool because inhibits the entry into the host cells of several intracellular bacteria. Here, we demonstrate that 100 µg/ml bLf hinders the invasion of $2.0 \pm 0.5 \times 10^4$ CFU/ml *E. coli* CFT073, prototype of UPEC, infecting $2.0 \pm 0.5 \times 10^5$ cells/ml urinary bladder T24 epithelial cells. The highest protection (100%) is due to the bLf binding with host surface components even if an additional binding to bacterial surface components cannot be excluded. Of note, in the absence of bLf, UPEC survives and multiplies, while bLf significantly decreases bacterial intracellular survival. After these encouraging results, an observational survey on thirty-three patients affected by recurrent cystitis was performed. The treatment consisted in the oral administration of bLf alone or in combination with antibiotics and/or probiotics. After the observation period, a marked reduction of cystitis

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episodes was observed ($p < 0.001$) in all patients compared to the episodes occurred during the 6 months preceding the bLf-treatment. Twenty-nine patients did not report cystitis episodes (87.9%) whereas the remaining four (12.1%) experienced only one episode, indicating that bLf could be a worthwhile and safe treatment in counteracting recurrent cystitis.

Keywords Bovine lactoferrin · *Escherichia coli* CFT073 · UPEC strains · UTIs · Recurrent cystitis

Introduction

Urinary tract infection (UTI) is a general term used to describe any infection of the urinary tract including asymptomatic bacteriuria, cystitis, and pyelonephritis (Kolman 2019).

Asymptomatic bacteriuria arises in patients without urinary symptoms with a bacterial concentration of 10^5 colony forming unit (CFU)/ml or more in two consecutive urine specimens in women or in a single sample in men (Nicolle et al. 2005; Bonkat et al. 2022).

Cystitis and pyelonephritis are symptomatic UTIs involving the bladder and kidneys, respectively. Cystitis is defined as the inflammation of the urinary bladder and it is characterized by dysuria, urinary frequency, and urgency, with or without suprapubic pain. Cystitis occurs due to infectious pathogens and affects about 150 million people each year worldwide (Harding and Ronald 1994; Foxman 2014; O'Brien et al. 2016). Approximately 25% of women presenting with the first episode of bacterial cystitis go on to suffer recurrent cystitis, some having six or more infections in the year following the initial episode (Foxman 2014; O'Brien et al. 2015). Recurrent cystitis is found predominantly in women of all age groups for many years: both sexually active young women and elderly postmenopausal women. A particularly insidious form of recurrent cystitis is the post-coital one, which typically appears 24–48 h after sexual intercourse (Sen 2008).

Pyelonephritis is characterized by inflammation of the renal pelvis and kidney with the concomitant presence of flank pain. An infectious cause of pyelonephritis is evidenced by urinalysis that shows bacteriuria or pyuria, or both (Johnson and Russo 2018). The risk factors for the predisposition to pyelonephritis are sexual activity, new sexual partner, spermicide exposure, and personal history of recurrent UTIs (rUTIs) (Scholes et al. 2005). Only 3% or less of initial events of asymptomatic bacteriuria and cystitis progress to pyelonephritis (Johnson and Russo 2018).

Even if UTIs can be associated with several pathogens with greater clinical impact and potential complications, *Escherichia coli* is the most common uropathogen, leading up to 86% of all UTIs (Flores-Mireles et al. 2015). Although *E. coli* is an environmental commensal colonizer and the predominant non-pathogenic facultative anaerobic bacteria of the intestinal microbiota, some strains are facultative intracellular bacteria able to cause diseases in humans. *E. coli* pathotypes found in humans can be classified into diarrheagenic and non-diarrheagenic extraintestinal pathogenic *E. coli* (ExPEC) (Köhler and Dobrindt 2011; Croxen et al. 2013) including human uropathogenic *E. coli* (UPEC) strains, the primary cause of UTIs when *E. coli* strains ascend via the urethra and colonize the urinary tract. UPEC strains produce several virulence factors, such as adhesins, iron chelators, capsule-forming polysaccharides, flagella, and toxins, which permit these bacterial strains to colonize the host and manipulate the innate immune response (Johnson 1991; Johnson and Russo 2002). Furthermore, UPEC strains after invasion and intracellular multiplication can also persist within the host cells of the urinary tract improving their virulence (Chen et al. 2012). Consequently, these infections require long-term antibiotic therapy (Foxman 2003; Soto et al. 2007; Blango and Mulvey 2010). Nevertheless, current therapeutics are suboptimal, as the prevalence of multidrug-resistant uropathogens is increasing and antibiotic treatment may fail in impeding recurrences (Gupta et al. 2001; Al-Badr and Al-Shaikh 2013; Foxman 2014). Commonly used antibiotics for first-line empirical treatment of UTIs include fosfomycin trometamol, pivmecillinam, and nitrofurantoin macrocrystal (Bjerrum et al. 2009; Minardi et al. 2011; Guglietta 2017), while quinolones are often used as second-line therapy (Minardi et al. 2011; Guglietta 2017). Every year the European Centre for Disease Prevention and Control publishes a report on the state of antibiotic resistance in Europe. The data relating to Italy are alarming: in 2020 the resistance of *E. coli* strains reached 64.5% for aminopenicillins, 37.6% for fluoroquinolones, and 26.4%

for third-generation cephalosporins (WHO Regional Office for Europe 2022). The infections caused by multidrug-resistant *E. coli*, in particular the recurrent infections caused by multidrug-resistant UPEC, can create a significant health problem and diminish the quality of life of the patients. For this purpose, a renewed interest in the use of non-antibiotic treatments for managing UTIs has been observed.

Recommended preventive measures such as adequate water intake, rhythm in urinary emptying, and non-antibiotic prophylaxis such as cranberry, urine acidifiers, probiotics are recommended even if they are not yet completely supported by evidence (Jepson et al. 2012; Juthani-Mehta et al. 2016). On the contrary, D-mannose, studied for a long time, has been shown to be effective and safe in preventing and treating UTIs (Phé et al. 2017) through its binding to *E. coli* FimH adhesins (Han et al. 2012), thus inhibiting adhesion and invasion of the bladder (Hickling and Nitti 2013).

Even if D-mannose shows to be effective and safe in treating and preventing UTIs, the novel non-antibiotic substances against UTIs, beyond to counteracting the bacterial adhesion and invasion, should decrease the inflammatory host response induced by bacteria.

In this respect, lactoferrin (Lf) with its antimicrobial and anti-inflammatory properties could be a promising tool to counteract UTIs as well as manage rUTIs. Lf is an 80 kDa multifunctional cationic glycoprotein of natural immunity expressed and secreted in humans by glandular epithelial cells and by neutrophils in infection and inflammation sites (Valenti and Antonini 2005; Rosa et al. 2017). Bovine Lf (bLf), possessing high sequence homology (69%) and identical functions with human Lf (hLf), is applied in most of the in vitro and in vivo studies (Rosa et al. 2017; Lepanto et al. 2019a) and it is approved as a Generally Recognized as Safe (GRAS) substance by the United States Food and Drug Administration (U.S. FDA 2014) and as a dietary supplement by the European Food Safety Authority (European Food Safety Authority 2012).

The bLf anti-microbial activity can be both dependent or independent of its iron-binding ability (Valenti and Antonini 2005; Rosa et al. 2017). Through its capability to chelate two ferric ions per molecule until pH value as low as 3.0, bLf exerts a bacteriostatic action by producing an iron-deficient environment, thus reducing bacterial growth in infection sites (Rosa et al. 2017). Independently from its iron-chelation ability, bLf is able to exert a bactericidal activity through the perturbation of bacterial membranes of both Gram-negative and Gram-positive bacteria via the binding to lipopolysaccharides (LPS) or lipoteichoic acid, respectively (Rosa et al. 2017). In Gram-negative bacteria, cellular lysis is performed through the direct interaction of bLf with LPS (Appelmelk et al. 1994; Brandenburg et al. 2001) and bLf ability to sequester Ca^{2+} thus inducing the LPS release (Rossi et al. 2002). In Gram-positive bacteria, the bactericidal activity of bLf is mediated by electrostatic interactions between the negatively charged of bacterial lipid layer and the positively charged of bLf surface, causing changes in the membrane permeability (Rosa et al. 2017). Moreover, independent on iron-chelation, bLf is also able to inhibit: (i) bacterial adhesion and invasion through its competitive binding with surface components of host cells and/or bacteria, thus decreasing bacterial–host cell interaction and bacterial internalization (Longhi et al. 1993; Alugupalli and Kalfas 1997; Kawasaki et al. 2000; Lepanto et al. 2019b), (ii) bacterial intracellular survival through still unknown mechanisms (Lepanto et al. 2019b), (iii) biofilm formation through its iron chelation ability (Singh et al. 2002) and (iv) genotoxicity as cell DNA damage with the mechanism(s) of action not yet completely elucidated (Lepanto et al. 2019b).

Moreover, bLf, besides antimicrobial activity, plays a role in a variety of relevant biological activities, particularly by modulating iron and inflammatory homeostasis (Cutone et al. 2017; Rosa et al. 2017). As matter of fact, bLf enters into the cell nucleus (Ashida et al. 2004; Suzuki et al. 2005, 2008; Paesano et al. 2012) where it binds to specific sequences of DNA, thus regulating gene transcription of anti-inflammatory cytokines (Kim et al. 2012).

Little is known about the role of Lf against UPEC infections. Recently, the proteomic analyses in mice infected by UPEC revealed that Lf is a component of urinary exosomes increasing during infection (Patras et al. 2019). In addition, in the co-culture of human bladder epithelial cells and neutrophils, exogenous hLf exerted a protective effect against UPEC infection (Patras et al. 2019). This protective effect was also demonstrated in a murine UPEC infection model (Patras et al. 2019).

Here, we report the role of bLf in counteracting the invasion and intracellular survival of *E. coli* CFT073, a prototype of UPEC strain, on T24 human bladder cancer cell line. In addition, an observational survey conducted by general practitioners, gynecologists and one hematologist on their patients affected by recurrent cystitis and treated with the oral administration of bLf alone or in combination with antibiotics and/or probiotics was performed.

Materials and methods

Bovine lactoferrin

BLf (Batch N.: LLL09MAR21B7, Saputo Dairy, Australia), with high homology of sequence (69%) and identical function with hLf, was generously supplied by Vivatis Pharma Italia s.r.l. BLf purity was checked through SDS-PAGE and silver nitrate staining. BLf purity was about 99%, and its concentration was confirmed via UV spectroscopy according to an extinction coefficient of 15.1 (280 nm, 1% solution). The iron saturation of bLf used, determined via optical spectroscopy at 468 nm, was about 10% according to an extinction coefficient of a 1% solution of bLf completely iron saturated corresponding to 0.54. LPS contamination of bLf, assessed via Limulus Amebocyte assay (26060PA-05, Pyrochrome kit, PBI International, Italy), was 0.5 ± 0.06 ng/mg of bLf. Before each in vitro assay, the bLf solution was sterilized using a 0.2 μm Millex HV filter at low protein retention (Millipore Corp., USA).

Bacterial strain

E. coli CFT073 (700928), a prototype strain of UPEC, was obtained from the American Type Culture Collection (ATCC) (Manassas, USA) and was isolated from the urine of a woman with acute pyelonephritis. To check purity, the strain was streaked on trypticase soy agar (TSA) plates (Oxoid LTD, UK) before the experiments. The inoculum was made by growing *E. coli* CFT073 strain in brain heart infusion (BHI) broth (Oxoid LTD, UK) overnight at 37 °C.

Cell culture

The T24 human bladder cancer cell line (HTB-4) was obtained from the ATCC (Manassas, USA). The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, USA), and 1% penicillin/streptomycin. Cell cultures were maintained at 37 °C in 5% CO_2 atmosphere.

Invasion and survival assays

The T24 human bladder cells were seeded in 24-well tissue culture plates at a concentration of 5×10^4 cells/well for 48 h at 37 °C in 5% CO_2 atmosphere. After 48 h of incubation, the medium was removed and cell monolayers were washed with phosphate-buffered saline (PBS) (Oxoid LTD, UK), and fresh medium, without FBS and penicillin/streptomycin, was added to each well. Cells were incubated for 2 h at 37 °C in 5% CO_2 atmosphere. *E. coli* CFT073, after the growth overnight in BHI broth, was sub-cultured in a fresh medium for 2 h at 37 °C to obtain log-phase bacterial cultures to be used to infect T24 human bladder cell monolayers.

In both the invasion and survival assays, cells ($2.0 \pm 0.5 \times 10^5$ cells/ml) were infected with bacteria at a multiplicity of infection (MOI) of 1 ($2.0 \pm 0.5 \times 10^5$ CFU/ml), 0.5 ($1.0 \pm 0.5 \times 10^5$ CFU/ml), and 0.1 ($2.0 \pm 0.5 \times 10^4$ CFU/ml) in the absence or presence of 100 μg/ml bLf according to the following experimental scheme: (i) cells infected by *E. coli* CFT073; (ii) cells pre-incubated with bLf for 1 h at 37 °C before *E. coli* CFT073 infection; (iii) bLf pre-incubated with *E. coli* CFT073 for 1 h at 37 °C before cell infection; and (iv) bLf added together with *E. coli* CFT073 at the moment of infection. Of note, bLf was used at non-bactericidal and non-cytotoxic concentration corresponding to 100 μg/ml according to experimental procedures to detect the actual inhibition of the bacterial invasion and intracellular survival (Frioni et al. 2014).

For infection assay, the 24-well tissue culture plates were centrifuged twice at 500 g for 2 min to synchronize adhesion and incubated for 1 h at 37 °C in 5% CO_2 atmosphere.

For invasion assays, the supernatants were removed and T24 bladder cells were washed three times with PBS and incubated in a fresh medium with

100 μg/ml gentamicin (Sigma-Aldrich, Milan, Italy) for 1 h to kill the extracellular bacteria. Then, cell monolayers were lysed in 0.1% *(v/v)* Triton X-100 and plated on TSA plates to count intracellular colony forming units (CFUs). The strain was considered invasive when the invasion efficiency, calculated as the percentage of the ratio between intracellular bacteria and inoculum, was ≥ 0.1%. Data represent the mean of three independent experiments in duplicate.

To evaluate the survival efficiency, after 1 h of incubation with 100 μg/ml of gentamicin, T24 human bladder cells were washed and incubated for a further 24 h in RPMI 1640 medium with a lower concentration of gentamicin (50 μg/ml). After 24 h of incubation, cells were washed, lysed, and plated on TSA plates as described above. The survival efficiency was calculated as the percentage of the ratio between intracellular bacteria recovered at 24 h and those recovered at 2 h. The survival efficiency of *E. coli* CFT073 in the control was set as 100% and the survival efficiency of *E. coli* CFT073 in different bLf treatment conditions was normalized to 100% control. Data represent the mean of three independent experiments in duplicate.

Setting

The objective of this observational study was to evaluate if the treatment with bLf alone or in combination with antibiotics and/or probiotics could reduce the number of cystitis episodes in the patients with recurrent cystitis during the observation period. For this purpose, a small group of subjects with recurrent cystitis treated with oral bLf was evaluated. Each patient was self-control. Data were retrospectively collected by some physicians (general practitioners, gynecologists, and one hematologist) located in the Tuscany, Lazio, and Campania regions of Italy. For many years, bLf has been commercialized in Italy as a nutraceutical product. Ethical approval was not necessary according to National Code on Clinical Trials declaration (Kıraç 2013) because our observation derives from a real-life retrospective study. All patients who arrived at a medical visit affected by recurrent cystitis must signed the informed consent and then they were included in the study. The treated patients did not show allergy to milk proteins. Moreover, safety and tolerability of bLf were also evaluated.

Study population

From June 2021 to January 2022 all patients with recurrent cystitis were included in this retrospective survey. This period included the follow-up time. Patients ranged in age from 22 to 90 years. Data for each patient were collected.

Patients were included in the study as affected from recurrent cystitis if they had suffered from 1 or more episodes of cystitis in the previous 6 months. Recurrent cystitis were characterized by one or more symptoms as stranguria/dysuria, polyuria/pollakiuria, and pelvic pain.

All patients were monitored in the following months to assess their status.

Patients' treatments

The patients suffering from recurrent cystitis were treated with bLf alone or with bLf plus antibiotics (fosfomycin or levofloxacin or ciprofloxacin) and/or probiotics (*Lactobacillus* spp), depending on the seriousness of the patient conditions at the moment of the visit, according to the physician's judgment. All were treated with oral administration of different number of capsules containing 200 mg of bLf (Batch N.: LLL09MAR21B7, Saputo Dairy, Australia, commercialized by Pharmaguida, Italy as Mosiac®). The bLf content in each capsule was confirmed by chemical analysis and corresponded to about 200 mg/capsule. The purity of bLf, checked by SDS-PAGE and silver nitrate staining, was about 99%. The concentration of bLf was assessed by UV spectroscopy on the basis of an extinction coefficient of 15.1 (280 nm, 1% solution). The bLf iron saturation was about 10% as detected by optical spectroscopy at 468 nm on the basis of an extinction coefficient of 0.54 (100% iron saturation, 1% solution). The bLf contained in each capsule derived from the same batch used for the in vitro experiments.

The different dosages ranging from 1 (200 mg/day bLf) to 5 (1000 mg/day bLf) capsules/day were based on the patient's symptoms, considering the presence of one or more symptoms as stranguria/dysuria, polyuria/pollakiuria, and pelvic pain. The administration of 1 capsule/day occurred before meals. When the suggested dose was > 200 mg/day, the capsules were divided in 2 or 3 daily administrations and taken before meals, in order to avoid protein degradation

due to the low pH of gastric juice during digestion (Rosa et al. 2020). Concerning the antibiotics, fosfomycin was administered 3 g/day for two days or in more complicated cases 6 g/day for 2 days; or levofloxacin 250 or 500 mg/day for 7–10 days depending from the severity of infection; or ciprofloxacin 500 mg 2 times a day for 10 days. The administration of 2 capsules/day of probiotics as *Lactobacillus rhamnosus* or *L. reuteri* or *L. acidophilus* or *L. casei* or an association of two or more of them each contained at least 10^{11} total bacteria/day.

Evaluated parameters

The main evaluated parameter is the reduction of cystitis episodes during the treatment with bLf. For this purpose, patients were tested at 1 and 4 months following the bLf treatment start.

A second collected parameter was related to the presence of concomitant intestinal disorders/diseases. Safety and tolerability of bLf were also evaluated.

Statistical analysis

For in vitro experiments, results are expressed as means ± standard deviations (SD). The paired Student's t-test was used to determine the presence of statistically significant differences between bLf treatments and untreated infected cells for invasion and survival efficiency. All statistical analysis were performed by using Prism v7 software (GraphPad Software, USA). In all cases, a p-value < 0.05 was considered statistically significant.

Concerning the observational survey, variables were summarized as median and interquartile range (IQR), or as absolute number and percentage (%), as appropriate. The association between variables was investigated by Pearson product moment correlation coefficient (r) and p-value. The evolution over time of the number of cystitis episodes was investigated by "before-after" plot with connected dots. Within patients, the comparison was investigated by Wilcoxon rank test for paired observations. The incidence rate of cystitis before and after the date of enrolment by treatment groups was expressed as events per 100 persons-month, and 95% confidence interval (95% CI). Given the fact that the number of patients treated with "bLf + probiotics" and that of those treated with "bLf + probiotics + antibiotics" was relatively low (n = 3 and n = 5, respectively) these two groups were merged together (n = 8) for the scope of this analysis. The pre-post study difference in the rate of cystitis episodes was expressed as incidence rate reduction, 95% CI, and p-values. A p-value < 0.05 was considered to be statistically significant. Data analysis was performed by SPSS for Windows, Version 22, IBM, Chicago, Illinois, USA.

Results

Bovine lactoferrin inhibits *E. coli* CFT073 invasion and survival in T24 bladder cells

Preliminary experiments were carried out to establish the non-bactericidal and non-cytotoxic concentration of bLf. We found that a 100 µg/ml concentration did not affect the bacterial viability. Moreover, this concentration did not exert any cytotoxic activity. The non-bactericidal and non-cytotoxic concentration of bLf allowed to obtain the actual number of intracellular bacteria inhibited in their entry by this glycoprotein.

Therefore, the ability of 100 µg/ml bLf in the inhibition of invasion and intracellular survival of *E. coli* CFT073 strain into T24 bladder cells was assessed according to different experimental procedures: (i) cells infected by *E. coli* CFT073; (ii) cells pre-incubated with bLf for 1 h at 37 °C before *E. coli* CFT073 infection; (iii) bLf pre-incubated with *E. coli* CFT073 for 1 h at 37 °C before cell infection; and (iv) bLf added together with *E. coli* CFT073 at the moment of infection.

E. coli CFT073 strain shows at MOI 1 a cytotoxicity of about 35% on T24 bladder cells compared to uninfected ones (7.0×10^4 versus 2.0×10^5 cells/well). Therefore, the number of *E. coli* CFT073 invading T24 bladder cells is affected by the percentage of dead cells. In these experiments, even if the number of intracellular *E. coli* CFT073 is very low, bLf shows a significant anti-invasive activity when it was pre-incubated with the cells for 1 h before the infection ($1.0 \pm 0.3 \times 10^2$ CFU/ml corresponding to 0.03 ± 0.007%; $p < 0.01$) and when it was added with *E. coli* CFT073 at the moment of infection ($1.1 \pm 0.2 \times 10^2$ CFU/ml corresponding to 0.03 ± 0.01%; $p < 0.01$) with respect to the

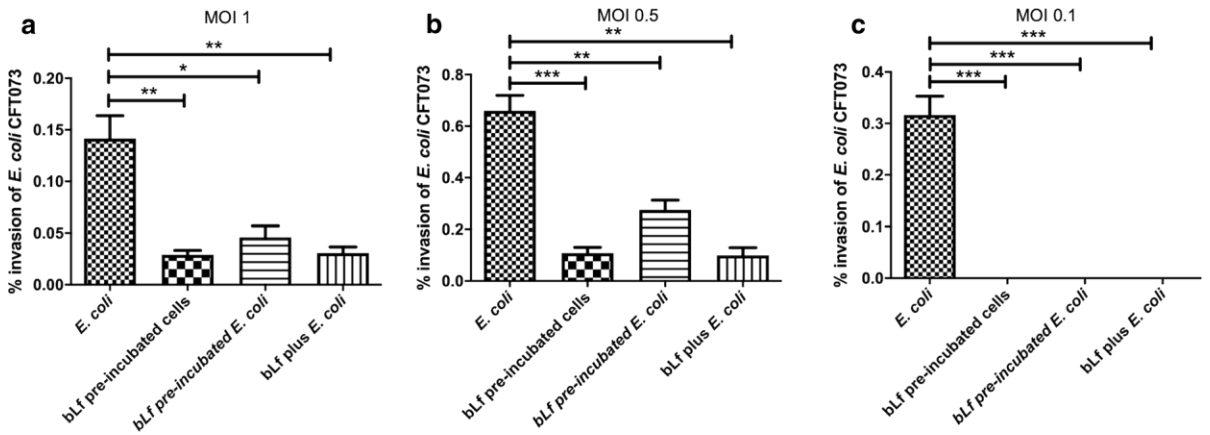

Fig. 1 Percentage of invasion efficiency of the *Escherichia coli* CFT073 strain on T24 bladder cells at multiplicity of infection (MOI) of 1 (**a**), 0.5 (**b**) and 0.1 (**c**) in the absence or presence of 100 μg/ml of bovine lactoferrin (bLf) according to the following experimental scheme: (i) cells infected by *E. coli* CFT073; (ii) cells pre-incubated with bLf for 1 h at 37 °C before *E. coli* CFT073 infection; (iii) bLf pre-incubated with *E. coli* CFT073 for 1 h at 37 °C before cell infection; and (iv) bLf added together with *E. coli* CFT073 at the moment of infection. Data represent the mean ± standard deviation of three independent experiments. Significant values: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

control ($4.3 \pm 0.4 \times 10^2$ CFU/ml corresponding to 0.14 ± 0.04%) (Fig. 1a). A minor significant decrease of invasive bacteria, compared to the control, is observed when bLf is pre-incubated for 1 h with *E. coli* CFT073 before the infection of cell monolayers ($1.7 \pm 0.5 \times 10^2$ CFU/ml corresponding to $0.046 \pm 0.02\%$; $p < 0.05$) (Fig. 1a).

Regarding MOI 0.5, bLf exhibits stronger anti-invasive activity when it was pre-incubated with T24 cells for 1 h before the infection with *E. coli* CFT073 ($2.0 \pm 0.4 \times 10^2$ CFU/ml corresponding to 0.11 ± 0.04%; $p < 0.001$) and when it was added with *E. coli* CFT073 at the moment of infection ($1.9 \pm 0.5 \times 10^2$ CFU/ml corresponding to 0.10 ± 0.05%; $p < 0.01$) with respect to the control ($1.0 \pm 0.6 \times 10^3$ CFU/ml corresponding to 0.66 ± 0.10%) (Fig. 1b). On the other hand, a less evident significant decrease, compared to the control, is observed when bLf is pre-incubated for 1 h with *E. coli* CFT073 before the infection of the T24 bladder cells ($4.0 \pm 0.5 \times 10^2$ CFU/ml corresponding to $0.28 \pm 0.07\%$; $p < 0.01$) (Fig. 1b).

At MOI 0.1, bLf completely inhibits the *E. coli* CFT073 entry in T24 bladder cells in all experimental conditions ($p < 0.001$) compared to the control ($1.0 \pm 0.5 \times 10^2$ CFU/ml corresponding to $0.32 \pm 0.07\%$) (Fig. 1c).

Concerning the survival assay at 24 h post-infection, *E. coli* CFT073 strain used at MOI 1 triggered such a great cytotoxic effect on T24 bladder cells that the cell monolayer was completely detached. No intracellular viable bacteria were found and, consequently, the survival assay couldn't be performed.

At MOI 0.5 and at 24 h post-infection, the number of *E. coli* CFT073 when bLf was pre-incubated with the cells corresponded to $3.1 \pm 0.6 \times 10^2$ CFU/ml (37.41 ± 4.10%; $p < 0.001$); when bLf was pre-incubated with bacteria corresponded to $6.3 \pm 0.5 \times 10^2$ CFU/ml ($52.77 \pm 5.15\%$; $p < 0.001$); and when bLf was added at the moment of infection corresponded to $2.8 \pm 0.7 \times 10^2$ CFU/ml ($33.17 \pm 6.67\%$; $p < 0.001$) compared to $3.6 \pm 0.6 \times 10^3$ CFU/ml (100 ± 6.99%) of the control (Fig. 2a).

Conversely, at MOI 0.1, because no intracellular bacteria were found in invasion assay (2 h post-infection) in the experimental conditions in which bLf is present, no intracellular bacteria were detected in the survival assay (at 24 h post-infection) compared to $2.0 \pm 0.8 \times 10^2$ CFU/ml (100 ± 9.38%) of the control (Fig. 2b).

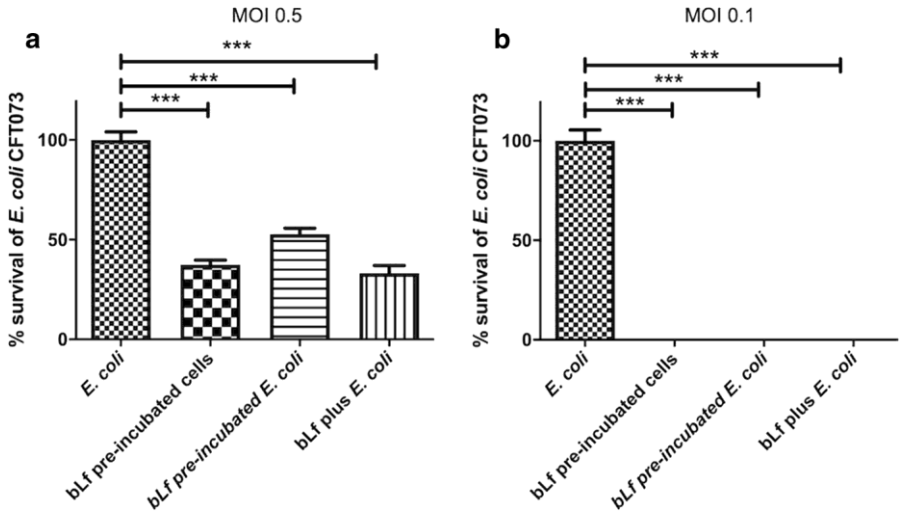

Fig. 2 Percentage of survival efficiency at 24 h post-infection of the *Escherichia coli* CFT073 strain on T24 bladder cells at multiplicity of infection (MOI) of 0.5 (**a**) and 0.1 (**b**) in the absence or presence of 100 µg/ml of bovine lactoferrin (bLf) according to the following experimental scheme: (i) cells infected by *E. coli* CFT073; (ii) cells pre-incubated with bLf for 1 h at 37 °C before *E. coli* CFT073 infection; (iii) bLf pre-incubated with *E. coli* CFT073 for 1 h at 37 °C before cell infection; and (iv) bLf added together with *E. coli* CFT073 at the moment of infection. Data represent the mean ± standard deviation of three independent experiments. Significant value: ***$p < 0.001$

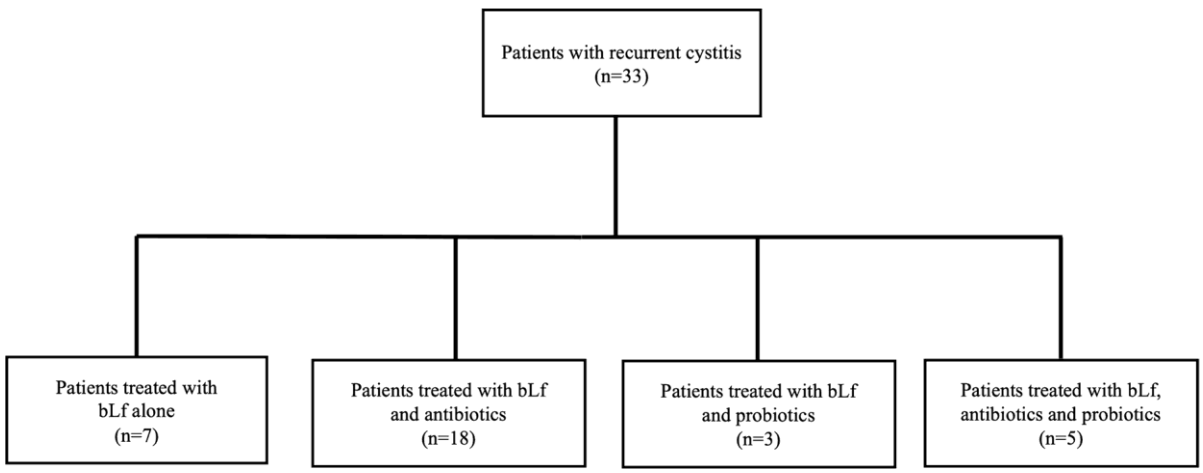

Fig. 3 A total of 33 patients with recurrent cystitis were involved in the study: 7/33 treated with bovine lactoferrin (bLf) alone; 18/33 treated with bLf and antibiotics; 3/33 treated with bLf and probiotics and 5/33 treated with bLf, antibiotics and probiotics

Baseline demographic characteristic for patients with recurrent cystitis

This study involved thirty-three patients as reported in the flowchart (Fig. 3).

The main demographic and clinical characteristics of the whole study group and separately of patients treated with bLf alone are summarized in Table 1.

The study sample includes thirty-one females and two males. Their median age and body weight were 52 years and 60 kg, respectively (Table 1). The median number of recurrent cystitis episodes in the

Table 1 Main clinical characteristics of the whole group of patients and separately of those on treatment with bovine lactoferrin (bLf) alone across the study period

	Whole group (n = 33)	Patients on bLf alone (n = 7)
Age (years)	52 (36–71)	43 (40–69)
Body weight (kg)	60 (55–68)	55 (50–58)
Episodes of cystitis in the past 6 months (n)	4 (3–6)	5 (2–5)
Antibiotic treatment in the past 6 months [n (%)]	33 (100)	7 (100)
Comorbidities [n (%)]		
None	27 (81.8)	6 (85.7)
Anemia	5 (15.2)	1 (14.3)
Diabetes	1 (3.0)	0 (0)
bLf alone [n (%)]	7 (21.2)	7 (100)
bLf and antibiotics [n (%)]	18 (54.5)	0 (0)
bLf and probiotics [n (%)]	3 (9.1)	0 (0)
bLf, antibiotics and probiotics [n (%)]	5 (15.2)	0 (0)

Data are median and interquartile range or absolute numbers and percentage

past 6 months was 4 episodes (IQR: 3–6 episodes) (Table 1). Five patients were affected by anemia, and one was diabetic (Table 1); nine patients (27%) presented gastrointestinal disorders. All patients received bLf across time. BLf alone was administered in seven patients; bLf treatment was associated only with antibiotics in eighteen patients; only with probiotics in three patients, and with both antibiotics and probiotics in the remaining five patients (Table 1). During the study, no patient was on treatment with antibiotics alone.

Seven out of thirty-three patients (21.2%), receiving only bLf, are also separately reported in the last column of Table 1. Among the seven patients treated with bLf alone, the median age and body weight were 43 years and 55 kg, respectively (Table 1-last column). The median number of recurrent cystitis episodes in the past 6 months was 5 episodes (IQR: 2–5 episodes) (Table 1-last column). One patient was affected by anemia (Table 1-last column).

Analysis of bovine lactoferrin doses and concomitant treatments across time

The individual doses of bLf over the follow-up period are reported in Fig. 4.

Three time periods were considered: first 5–8 days (1st period), next 30 (2nd period) and 90 (3rd period) days.

During the 1st period, six patients received 1000 mg/day of bLf, five patients received 800 mg/day, four patients received 600 mg/day, 11 patients received 400 mg/day, one patient received 300 mg/day, and the remaining six patients received 200 mg/day (median bLf dose: 400 mg/day, IQR: 400–800) (Fig. 4). Throughout this 1st period, six patients remained at the prescribed dose for 8 days, twenty-two patients for 7 days, and the remaining five patients for 5 days.

Across the 2nd period, four patients received 600 mg/day of bLf, twenty-two patients received 400 mg/day of bLf, and seven patients received 200 mg/day of bLf (Fig. 4).

In the 3rd period of observation, five out of thirty-three patients, treated with 400 mg/day of bLf in the 2nd period, stopped the treatment due to symptom's resolution. Among the remaining twenty-eight patients, four patients received 600 mg/day of bLf, nine patients received 400 mg/day of bLf, and the remaining fifteen patients received 200 mg/day of bLf (Fig. 4).

Analysis of episodes of cystitis across time

All enrolled patients had recurrent episodes of cystitis during the 6 months preceding the study: 7–8 episodes in two patients, 5–6 episodes in fourteen patients, 3–4 episodes in twelve patients, 1–2 episodes in the remaining five patients (median: 4 episodes, IQR: 3–6 episodes) (Fig. 5a). Among the seven patients treated with bLf alone, the recurrent episodes of cystitis during the 6 months preceding the study were 5 episodes in four patients, 3 episodes in one patient, and 2 episodes in two patients (median: 5

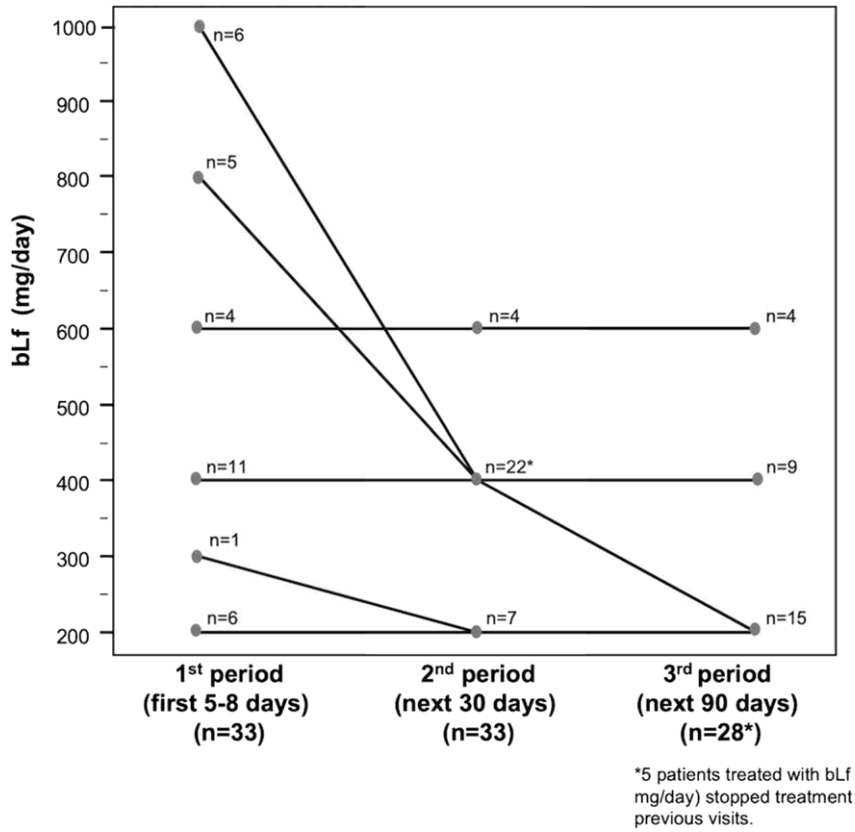

Fig. 4 Analysis of individual bovine lactoferrin (bLf) doses over the follow-up period

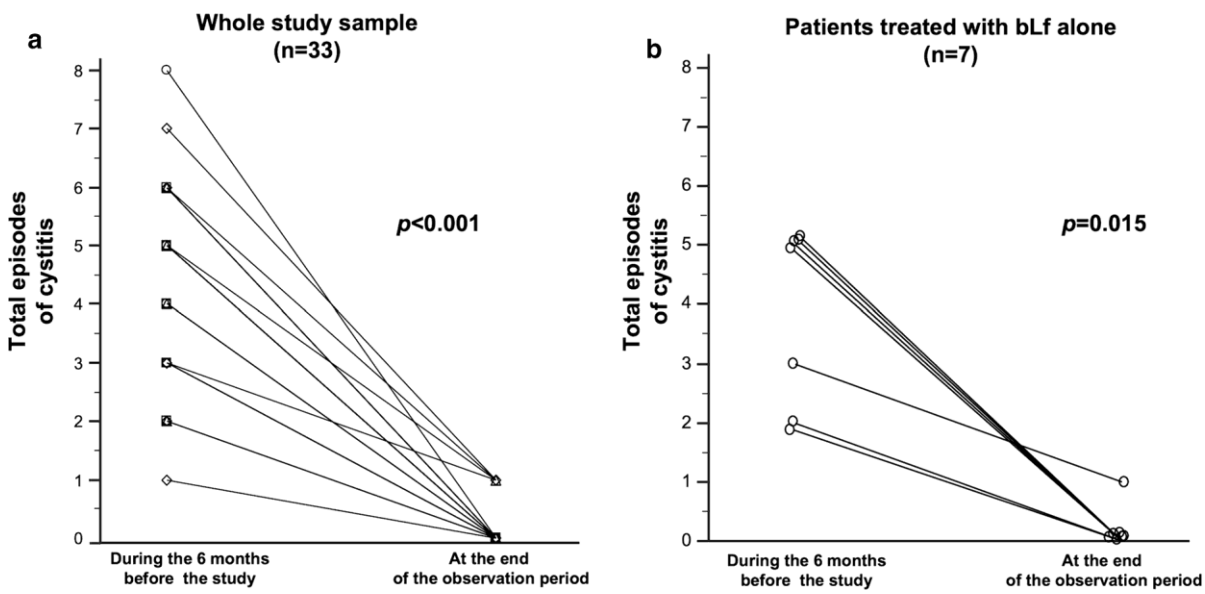

Fig. 5 Before-after plot with connected dots in the whole group of patients (**a**) and separately in patients treated with bovine lactoferrin (bLf) alone (**b**). Data are individual episodes of cystitis occurred during the 6 months before the study and at the end of the observation period

episodes, IQR: 2–5 episodes) (Fig. 5b). On univariate analysis, the number of episodes of cystitis during the 6 months before the enrolment tended to be inversely related to age (r = − 0.32, p = 0.07) indicating that younger patients were more susceptible to cystitis episodes.

Following the baseline assessment, nine patients underwent a follow-up visit after 4 months, seven patients after 3 months, nine patients after 2 months, seven patients after 1 month, and the remaining one patient after > 4 months. Of note, the absence of cystitis episodes was evaluated by the lack of clinical symptoms as stranguria/dysuria, polyuria/pollakiuria, and pelvic pain. At the end of the observation period, a marked reduction of cystitis episodes was observed (p < 0.001) as compared to the episodes of the same outcome variable occurred during the 6 months preceding the study (Fig. 5a). In detail, twenty-nine patients did not manifest cystitis episodes (87.9%) whereas the remaining four (12.1%) experienced only one episode of cystitis across time (Fig. 5a). An analysis restricted to patients treated with bLf alone (n = 7) revealed a reduction of cystitis episodes (p = 0.015) which was of similar magnitude to that observed in the whole group of patients (Fig. 5b). In particular, six out of seven patients did not manifest cystitis episodes (85.7%) whereas only one patient (14.3%) experienced a single episode of cystitis over the study period (Fig. 5b).

A further analysis focused on the four patients who were still affected by cystitis at the study end showed that these patients had a higher median number of previous episodes of cystitis as compared to those of the whole group (6 versus 4) (Fig. 6a) and received a low dose of bLf across time that was 200 mg/day in three patients and 400 mg/day in one patient (Fig. 6b).

A further analysis carried out by calculating the incidence rate of cystitis episodes according to treatments before and after the date of enrolment showed that the frequency of cystitis did not differ among the three groups neither before nor after the date of enrolment (Supplementary Fig. S1). Furthermore, the incidence rate reduction (and 95% CI) from pre- to post-study period was statistically significant in each group but of similar magnitude among the three groups (Supplementary Fig. S2). Of note, beyond the only one episode reported by four patients in the observation period, no other cystitis episode has been reported by the patients at the date of April 30th, 2022 (data not shown).

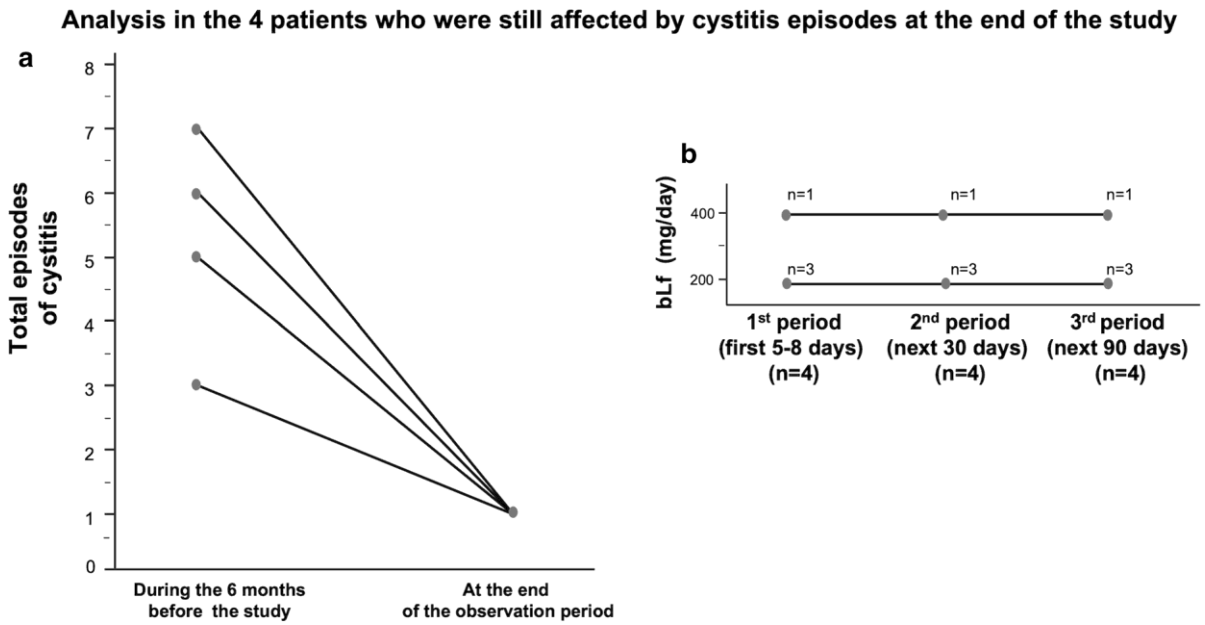

Fig. 6 Before-after plot with connected dots in the subgroup of patients (n = 4) who were still affected by cystitis at the study end (**a**). The individual bovine lactoferrin (bLf) doses across time of these 4 patients are also given (**b**)

Discussion

Lf is an iron-binding glycoprotein with antimicrobial, anti-inflammatory, and immunomodulatory activities (Valenti and Antonini 2005; Rosa et al. 2017).

HLf possesses high sequence homology and identical functions with bLf, which is applied in most of the in vitro and in vivo studies (Rosa et al. 2017; Lepanto et al. 2019a). The native forms of hLf are iron saturated on average of 11%, while bLf of 13% (Vega-Bautista et al. 2019). The antimicrobial properties of Lf were at first fully attributed to the ability of Lf to avidly bind iron, leading to a bacteriostatic effect against iron-requiring pathogens. As matter of fact, there is an intense competition between the host and pathogens for iron acquisition. Lf ability to chelate iron may be a primary mechanism to mitigate bacterial virulence and multiplication including that of uropathogens (Subashchandrabose and Mobley 2015; Bauckman et al. 2019).

Further studies demonstrated that, beyond its iron-binding capabilities, Lf has bactericidal effects because provokes the lysis of several pathogens through its interaction with the LPS of Gram-negative bacteria (Appelmelk et al. 1994; Brandenburg et al. 2001; Elass et al. 2002; Rosa et al. 2017) and the lipoteichoic acid of Gram-positive bacteria (Rosa et al. 2017; Lu et al. 2021). Moreover, bLf is also able to interact with glycosaminoglycans (GAGs) or heparan sulfate proteoglycans (HSPGs), anionic components of host cells (Hu et al. 2021), and with anionic surface structures of facultative intracellular pathogenic bacteria thus hindering bacterial adhesion and entry (Valenti and Antonini 2005 and references therein). The first demonstration of the mucosal protective activity of Lf against injury by adherent and invasive bacteria was shown for *E. coli* HB101 carrying the pRI203 plasmid *Yersinia pseudotuberculosis inv* gene (Longhi et al. 1993). The binding of Lf to anionic components of bacterial surface and to GAGs/HSPGs, can induce a dramatic subversion in bacterial-host cell interaction, thus inhibiting the internalization of Gram-negative bacteria (Di Biase et al. 2004) as well as of Gram-positive bacteria (Antonini et al. 1997; Ajello et al. 2002; Diarra et al. 2003). To demonstrate the importance of the bLf binding to bacterial or cell surface components on the adhesion and invasion process, bLf must be pre-incubated with bacteria or with host cells before cell monolayer infection (Frioni et al. 2014; Sessa et al. 2017; Lepanto et al. 2019b).

The most common bacteria found to cause UTIs are *E. coli* strains classified as facultative intracellular pathogens, and, therefore, able to invade host cells. Other bacteria can cause UTIs, but *E. coli* is the culprit of about 80 percent of these infections (Josephs-Spaulding et al. 2021). *E. coli* normally lives harmlessly in the human intestinal tract, but it can cause serious infections if it gets into the urinary tract. The proximity between the anus and the urethra as well as the short length of the urethra are the pivotal causes of the higher risk of infections in women.

In the present study, we report the role of bLf in counteracting the invasion and intracellular survival of *E. coli* CFT073, a prototype of UPEC strain, in T24 human bladder cancer cell line.

To demonstrate the putative inhibition by bLf of invasion of *E. coli* CFT073 strain at different MOI in T24 bladder cells, different experimental procedures were performed utilizing bLf (100 µg/ml) at non-bactericidal and non-cytotoxic concentration. By comparing with the control (0.66 ± 0.10%), the lowest anti-invasive activity was found when bLf was preincubated with MOI 0.5 of *E. coli* CFT073 (0.28 ± 0.07%), while the highest when bLf was pre-incubated with host cells (0.11 ± 0.04%) or added at the moment of infection (0.10 ± 0.05%) (Fig. 1b), suggesting the importance of the bLf binding with host cells. In the survival assay, at MOI 0.5 and at 24 h post-infection, normalizing the control at 100 ± 6.99% the survival percentage of the intracellular bacteria when *E. coli* CFT073 was pre-incubated with bLf before infection was higher (52.77 ± 5.15%) compared to that observed when bLf was preincubated with the cells (37.41 ± 4.10%) and when bLf was added at the moment of infection (33.17 ± 6.67%) (Fig. 2a). Conversely, at MOI 0.1, bLf completely hinders the invasion as well as inhibits intracellular survival independently from experimental conditions performed at time 0 (Figs. 1c and 2b, respectively).

These results indicate the importance of the competitive binding between bLf and anionic components of host cells which probably hides the bacterial entrance sites. The minor efficacy of bLf binding to the bacteria is likely due to bLf non-bactericidal concentration used in in vitro experiments. This concentration of bLf is obliged and chosen to detect the actual number of intracellular bacteria avoiding to

confuse the anti-invasive activity with the bactericidal action of bLf.

Concerning the in vivo role of Lf during bacterial infections, its concentration in the urine increases from 30–75 to 3000 ng/ml (Arao et al. 1999), indicating Lf protective action against UTIs. However, this local protection is only partial due to the incidence of recurrent UTIs.

Therefore, an observational survey on patients affected by recurrent cystitis episodes has been conducted. Thirty-three patients, with the median of 4 recurrent cystitis episodes during the 6 months before the new treatments, were orally treated, according to the severity of cystitis symptoms, with a different amount of bLf as alternative to antibiotics or as co-treatment with antibiotics and/or probiotics. The patients had all been treated with antibiotics in the 6 months before the study.

At the end of the observation period, a marked reduction of cystitis episodes was observed ($p < 0.001$) in all patients as compared to the episodes occurred during the 6 months preceding the study (Fig. 5a). In detail, twenty-nine treated patients did not report cystitis episodes (87.9%) whereas the remaining four (12.1%) experienced only one episode of cystitis across time (Fig. 5a). A further analysis focused on the four patients who were still affected by cystitis at the observation period end showed that these patients had a higher median number of previous episodes of cystitis as compared to those of the whole group (6 versus 4) (Fig. 6a) and received a lower dose of bLf across time consisting in 200 mg/day in three patients and 400 mg/day in one patient (Fig. 6b).

An analysis restricted to the seven patients treated with bLf alone revealed a reduction of cystitis episodes ($p = 0.015$) which was of a similar magnitude to that observed in the whole group of patients (Fig. 5b). Six out of seven patients did not show cystitis episodes (85.7%) whereas only one patient (14.3%) experienced a single episode of cystitis over the study period (Fig. 5b). BLf treatment was safe and well tolerated.

In our study, the concentration of exogenous bLf in the urine of patients was not detected and no data on inflammation markers and on antibiogram were available. Therefore, our observations were based on the subjective symptoms of the patients before and after the treatments.

The results of this survey could lead to hypothesize different mechanisms of action by bLf as demonstrated in other in vitro (Frioni et al. 2014; Cutone et al. 2017; Sessa et al. 2017) and in vivo (Valenti et al. 2017; Lepanto et al. 2018; Cutone et al. 2019) models. These mechanisms are probably related to the bLf local and systemic action. At local level, bLf could counteract the multiplication of uropathogens through iron-withholding as well as bacterial invasiveness through its binding with host cells and bacteria. At systemic level, the activity of bLf seems more complex and could: (i) rebalance iron and inflammatory homeostasis disorders; (ii) decrease the intracellular iron concentration as a consequence of a decrease of pro-inflammatory cytokines; (iii) decrease the intracellular bacterial multiplication related to the decrease of intracellular iron; and (iv) stimulate the growth of intestinal beneficial microorganisms such as *Bifidobacterium* and *Lactobacillus* (Vega-Bautista et al. 2019). Of note, beyond these mechanisms, oral administration of bLf could limit the bacterial transmission from feces to urinary tract and rebalance the gut-bladder axis disorders.

The use of bLf as alternative or supplementary treatment to conventional antibiotics, considering on one hand its local effect against pathogens and on the other hand its systemic effects in balancing especially iron and inflammatory homeostasis disorders, might counteract bacterial infections caused by the drug resistant pathogens.

Conclusions

Recurrent cystitis represent an increasing problem because *E. coli* and other uropathogenic bacteria show a rising resistance to antibiotics. Antimicrobial resistance, fueled by the overuse of antibiotics, is a serious threat to global public health. Therefore, the innovative strategies to counteract antibiotic resistance and bacterial virulence are required.

In our study, in patients with recurrent cystitis a remarkable decrease in the number of such episodes was observed after treatment with bLf alone or adjunctive to antibiotics or probiotics or both.

This study has several limitations, such as the lack of the inflammatory markers data, antibiograms, the sample size not defined according to a formal sample size calculation and its retrospective and observational nature. Therefore, it could be considered a preliminary study for a further wider clinical trial to better explore the efficacy of bLf treatment in patients suffering from recurrent cystitis.

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Author Contributions Conceptualization AMC, RP, PV and LR; Formal analysis, GT; Investigation, ALC, RP, SS, FV, EN, GP, VC, MM, PF, PV, and LR; Writing: original draft, AMC, GT, PV, and LR; Writing-review & editing: CL, MPC, AMC, PV and LR. All authors read and approved the final version.

Declarations

Conflict of interest The authors declare no conflict of interest.

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