Effect of Lactobacilli and Antibiotics on *E. coli* Urinary Infections in Mice

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Urinary antibiotic treatments usually affect the normal urethral flora. This work was developed in order to evaluate whether *Lactobacillus fermentum* CRL 1058 could reduce urinary tract infections (UTI) produced by uropathogenic *Escherichia coli* in mice treated with antimicrobial agents.

Animals were inoculated intra-urethrically with agarose beads containing lactobacilli, and were challenged with *E. coli*. Ampicillin (13 mg/kg/dose) was administered orally. The number of microorganisms present at different days was evaluated in the urogenital tract. Serum inflammatory and systemic immune response were also registered.

The use of 5 doses of ampicillin after 3 doses of lactobacilli in agarose beads significantly affected the viability of lactic acid bacteria, while the amount of *E. coli* was not altered. Lactate dehidrogenase (LDH) activity and anti-*E. coli* antibody levels showed no statistically significant difference between the challenged and non-challenged mice.

Lactobacilli reinoculation and 3 doses of ampicillin proved to be a moderately effective treatment since a smaller amount of *E. coli* was recovered from the organs of treated mice than from the controls. The reinforcement of lactobacilli, administered on the 9th day, produced a faster elimination of the pathogen. The ampicillin dose used allowed lactobacilli permanence in the urinary tract, and caused the elimination of the pathogen. Serum LDH values seemed to show an inflammatory immune response. No successful preventive results could be achieved.

We can conclude that lactobacilli and adequately low doses of ampicillin have a positive effect on the treatment of *E. coli* in this UTI model.

Key words ampicillin; lactobacilli; urinary infection; mice *E. coli*

Recurrent urinary tract infection (UTI) is a significant problem for a high percentage of women (2—10%). Most of these infections are attributed to reinfection due to the presence of the antibiotics, having modified the epithelial tissue to an extent which favors a new infection.[1] Sometimes reinfection follows colonization of the periurethral area with intestinal flora and the subsequent ascension and persistence of organisms in the bladder.

Moreover, disturbance of the microbial ecosystem induced by antimicrobial agents leads to the establishment of bacterial species which can be responsible for infection. This colonization may increase the possibility of the development of adverse drug effects.[1]

Lactobacilli and the lactic acid they produce have been considered to constitute the primary ecological barrier against infections caused by genital and intestinal pathogens. Facultative anaerobic lactobacilli constitute 50—90% of the microaerophilic vaginal microflora in women and are usually present at concentrations of 10^7—10^8 CFU/ml of vaginal fluid.[2] These organisms continuously exert forces to maintain an inter-species equilibrium. The practical effect is the exclusion of non-indigenous organisms; however, adverse effects on the colonization by lactobacilli may be produced by antimicrobial agents.

In the last few years there has been a growing tendency to use certain products that have proved to be beneficial for both human and animal health. These products, termed probiotics by Havenaar *et al.*,[3] are viable cultures of one or several microorganisms that, when administered to human beings or animals, produce a beneficial effect on the host by fulfilling the role of the normal endogenous microflora. Preparations containing lactobacilli have been administered to human beings in order to prevent or cure bacterial and micotic vaginitis.[2,4,5] The role of these microorganisms in UTI has been studied in rats.[6]

In a previous study we isolated 40 lactobacillus strains[7] from the vagina of 2-month-old BALB/c mice; we selected one particular *Lactobacillus fermentum* strain (CRL 1058) because of its high hydrogen peroxide production[8] and its *in vitro* ability to adhere to epithelial cells[9] or to polystyrene plates.[10] This strain was used for the preparation of agarose beads, which were inoculated in the urethra of the same type of mice mentioned above. We also determined that three doses of lactobacilli, each of them higher than 10^5 CFU/ml, was the optimal concentration required for these microorganisms to remain in the urinary tract of the animals until the 7th post-inoculation day.[11]

For the present work we selected *Lactobacillus fermentum* CRL 1058 and the uropathogenic strain *Escherichia coli* (UPEC) in order to elucidate antibiotic and lactic acid bacteria influences on the therapeutic treatment and prevention of mice UTI.

MATERIALS AND METHODS

Microorganisms *Lactobacillus fermentum* CRL 1058 was tested in the present work. Isolation, identification, maintenance and characterization procedures have been described previously.[7,11]

The *Escherichia coli* strain was isolated from the infected urinary tract of adult women and identified by biochemical tests according to Orskov.[12] It was also demonstrated to be infectious to 2-month-old BALB/c mice.

Culture Media and Growth Conditions *L. fermentum*

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was grown as described previously.[7,11] *E. coli* was treated in the same way, except that the culture medium used was Brain Heart Infusion (BHI) (Britania Lab. S.A., Buenos Aires, Argentina) supplemented with 0.5% yeast extract. For both *L. fermentum* and *E. coli*, no more than three-fold subcultures were performed in order to avoid changes in adhesion properties.

***In Vitro* Antibiotic Sensitivity Testing** Antibiotic sensitivity against *L. fermentum* and *E. coli* strains was performed by disk diffusion tests[13] and the Minimum Inhibitory Concentration (MIC) methods[14] described by the National Committee for Clinical Laboratory Standards using Mueller-Hinton agar. The antimicrobial agents evaluated were: ampicillin, mefoxitin, nalidixic acid, neomycin, amikacin, mezlocillin, ampicillin/sulbactam, sulfamethoxazole/trimethoprim, norfloxacin, gentamicin, cephalexin, erythromycin, tetracycline and chloramphenicol. These drugs were provided by Britania Lab. S.A. (Bs. As., Arg.). Antimicrobial agent concentrations ranged from 0.005 to 100 μg/ml. The MIC was defined as the lowest concentration of drug that completely suppressed bacterial growth after 18—24 h of incubation.

Bacterial Inoculation of Animals Adult BALB/c female mice (2 months old) from the breeding stock of our institute, fed *ad libitum*, were used. Just prior to each inoculation, agarose beads with *L. fermentum* were prepared as described in Ref. 7. Three doses of a concentration higher than 10^5 CFU were used, which is the minimum value previously determined for lactobacilli administration.[11] *E. coli* was inoculated in a 0.5% peptone water suspension at an infectious concentration (higher than 10^3 CFU/ml). The inoculation procedure has been described previously.[11] Bacterial doses are indicated in each experiment.

Antibiotic Treatment in Mice Animals were treated orally with ampicillin administered as 1, 3 or 5 doses (13 mg/kg per dose), and with 12 h in between each intake.

Therapeutic Assays A three-fold intra-urethral administration of lactobacilli in agarose beads was given to the mice; afterwards, a suspension of *E. coli* was inoculated as a single dose, and the antibiotic was administered orally (type and doses are indicated in each experiment), with 12 h in between each intake. Control animals were treated without lactobacilli.

Preventive Assays A three-fold intra-urethral administration of lactobacilli in agarose beads was given to the mice; afterwards, the antibiotic was administered orally (type and doses are indicated in each experiment), and a suspension of *E. coli* was inoculated as a single dose; each inoculation was 12 h apart. Control animals were treated without lactobacilli.

Quantification of the Microorganisms in the Organs Animals were sacrificed by cervical dislocation on different days after inoculation. Urethra, bladder, ureters and kidneys were removed and treated as described previously.[11] The homogenized organs were plated out in duplicate onto LBS (Lactobacillus Selective Medium)[15] and onto Mc Conkey (Sigma Chemical Co., St. Louis, MO) agar plates. *L. fermentum*[16] and *E. coli*[12] were identified by biochemical tests.

Serum Analysis Animals were bled from the retro-orbital venous plexus before being sacrificed. Lactate dehydrogenase (LDH), C Reactive Protein (CRP) and antibody analyses were performed. Commercial kits were used to determine LDH (Boehringer Ingelheim, S.A., Bs. As., Arg.) and CRP (Calbiochem. Behring Corp., Bs. As., Arg.). Antibody titres were determined by agglutination reaction using heat-killed lactobacilli and *E. coli* suspensions (2×10^8 CFU/ml). The amount of serum obtained from mice was not enough to determine the antibiotic concentration.

Urine Analysis Urine was collected before animal inoculations and sacrifices. Quantitative counts of polymorphonuclear (PMN) leukocytes, red blood cells and epithelial cells were determined. Urine was also plated out by using standard quantitative-loop methods onto a Cysteine-Lactose-Electrolite-Deficient (CLED) medium (Britania Lab. S.A., Bs. As., Arg.) following the standard technique of Barry *et al.*[17] Biochemical tests were performed in order to identify lactobacilli[16] and *E. coli*[12] strains.

Statistical Analysis Experimental values (mean and standard deviation) obtained from 3 to 4 animals were analyzed according to the Student's *t*-test.

RESULTS

***In Vitro* Results. Antimicrobial Sensitivity Testing** *L. fermentum* and *E. coli* sensitivity for different antibiotics is expressed in Table 1. Both microorganisms were resistant to ampicillin, mefoxitin, mezlocillin and ampicillin/sulbactam. Sensitivity to sulfamethoxazole/trimethoprim, norfloxacin, gentamicin, erythromycin, tetracycline and chloramphenicol differed between *L. fermentum* and *E. coli*.

***In Vivo* Results. Therapeutic Assay Using Five Doses of Ampicillin** (*L. fermentum*/*E. coli*/Ampicillin): The mice received a three-fold dose of *L. fermentum* (3.80×10^8 CFU each), one dose of *E. coli* (4.30×10^7 CFU), and five doses of ampicillin (Fig. 1). The number of pathogens recovered on the 6th and 8th day after challenge in the bladders of the treated animals was lower ($p < 0.05$) than in the bladders of the control mice. However, since the lactobacilli were killed by the antibiotic treatment, this

Table 1. Activity of Different Antimicrobial Agents against *L. fermentum* and *E. coli*

Antimicrobial agent	MIC (μg/ml)	
	L. fermentum	*E. coli*
Ampicillin	100.00	100.00
Mefoxitin	100.00	100.00
Nalidixic acid	1.00	0.5
Neomycin	1.00	0.5
Amikacin	1.00	0.5
Mezlocillin	5.00	100.00
Amp/sulbactam	5.00	1.00
Sulf/trimeth	50.00	1.00
Norfloxacin	1.00	0.1
Gentamicin	1.00	0.5
Cephalexin	10.00	0.5
Erythromycin	0.005	0.5
Tetracycline	0.005	100.00
Chloramphenicol	0.005	50.00

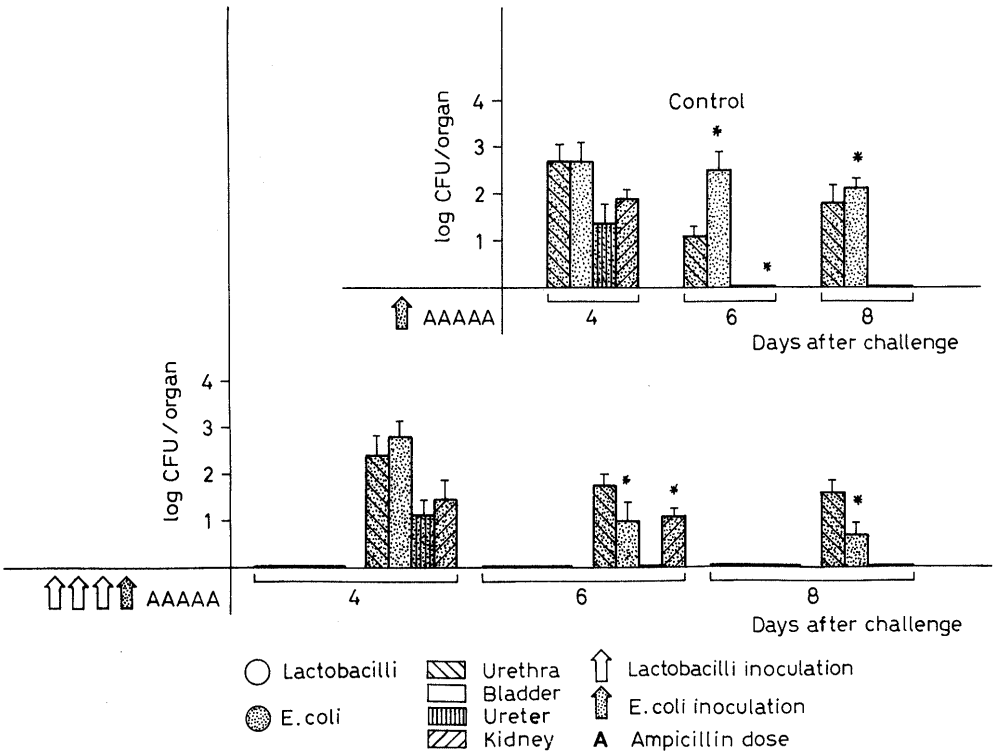

Fig. 1. Intra-urethral Administration of a Three-Fold Dose of *L. fermentum* in Agarose Beads (3.80×10^8 CFU per Dose) (White Arrows) and One Dose of *E. coli* (4.30×10^7 CFU) (Black Arrow), with 12 h in between Each Dose

With the same interval, mice received 5 oral doses of ampicillin (13 mg/kg per dose). (A). Control animals were treated without lactobacilli (upper graph). Results are expressed as the mean S.D. of the log of CFU/organ from 3 to 4 mice. $*p<0.05$.

therapeutic experiment was not very successful.

LDH and anti *E. coli* antibody levels did not show statistically significant differences between the challenged and control mice ($p>0.05$) (data not shown). Urine samples showed pathogens during the whole treatment (1.10×10^4 CFU/ml) and 2—3 PMN leukocytes/microscopic field ($100\times$) were found on the 8th day.

Preventive Assay Using Five Doses of Ampicillin (*L. fermentum*/Ampicillin/*E. coli*): The mice received a three-fold dose of *L. fermentum* (3.80×10^8 CFU each), five doses of ampicillin and one dose of *E. coli* (4.30×10^7 CFU). This experiment was not successful either, since *E. coli* recovery was similar in the control and treated animals, and lactobacilli did not survive. Again, the response of *L. fermentum* to ampicillin *in vivo* was different from the results obtained *in vitro*. Serum analyses were not significantly different among the treated and control mice ($p>0.05$).

Assay Using Lactobacilli Reinoculation and Three Doses of Ampicillin (*L. fermentum*/Ampicillin/*L. fermentum*; without *E. coli* Challenge): Animals were inoculated with a three-fold dose of 5.20×10^8 CFU of *L. fermentum* in agarose beads. 12 h after the last dose, the mice were orally administered three doses of ampicillin, and 7 d later another three-fold dose inoculation of lactobacilli was administered (6.80×10^8 CFU). Figure 2 shows that lactobacilli remained in the urinary tract of the animals reinoculated with lactobacilli and treated with ampicillin until the 18th day, only after a second three-fold dose. This was demonstrated to be an adequate lactobacilli dose in order to allow *L. fermentum* to survive *in vivo*. No bacteria were recovered on the 25th day.

LDH and anti-lactobacilli antibody levels increased after each inoculation (Table 2; first column). In urine, lactobacilli (1.24×10^3 CFU/ml) were found on the 8th day, and only a few epithelial cells up to the 18th day.

Therapeutic Assay Using Lactobacilli Reinoculation and Three Doses of Ampicillin (*L. fermentum*/*E. coli*/Ampicillin/*L. fermentum*): The animals were inoculated with a three-fold dose of 5.20×10^8 CFU of *L. fermentum* in agarose beads. 12 h later, the mice were challenged with one dose of *E. coli* (6.15×10^7 CFU) and were orally administered three doses of ampicillin; 7 d later, the three-fold dose of lactobacilli inoculation was repeated (6.80×10^8 CFU). Figure 3 shows a high recovery of *L. fermentum* up to the 5th day. However, on the 8th day after the challenge, lactobacilli had dissappeared completely, while at the same time, the amount of *E. coli* started to increase in these mice. Pathogens would not have been eliminated if no lactobacilli reinoculation on the 9th day had been performed. Neither lactobacilli nor pathogens were registered on the 25th day after the challenge.

A largely different pattern was observed in the control mice (upper graph Fig. 3), where three doses of ampicillin were not enough to kill the pathogen, which therefore enabled *E. coli* to infect the urinary tract.

Preventive Assay Using Lactobacilli Reinoculation and Three Doses of Ampicillin Mice were treated as in the previous experiment except that *E. coli* inoculation was performed after the three doses of ampicillin.

The antimicrobial agent killed the lactobacilli (data not shown). Although the lactic acid bacteria were slightly recuperated on the 18th day, this amount was not high enough to eliminate *E. coli* from the mice. LDH and anti

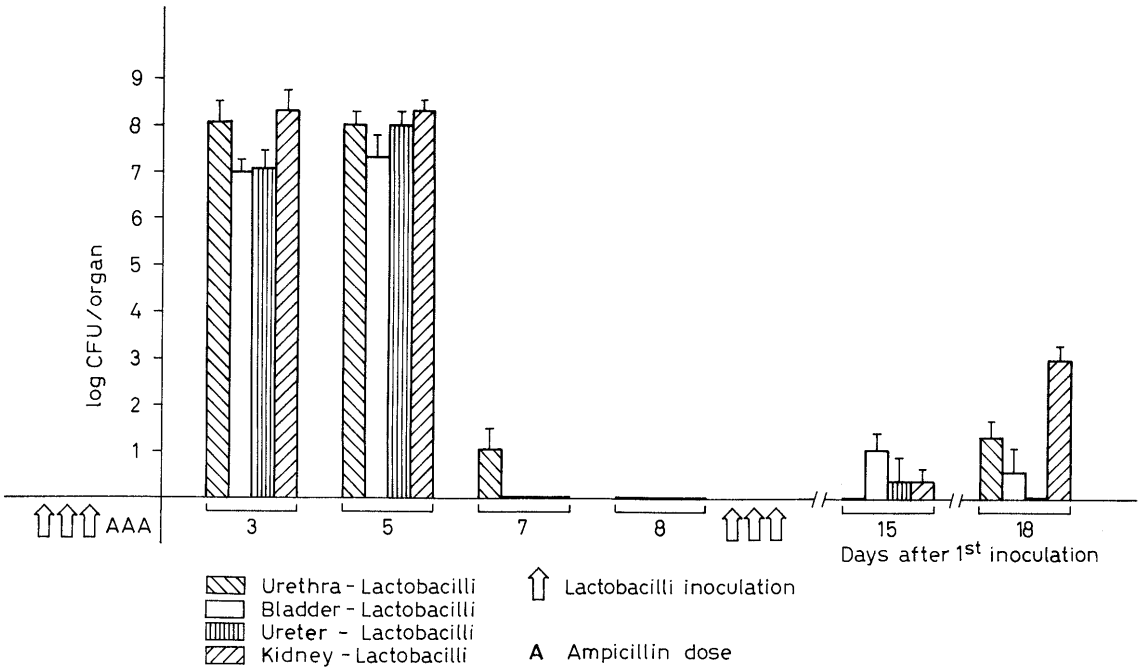

Fig. 2. Intra-urethral Administration of a Three-Fold Dose of *L. fermentum* in Agarose Beads (5.20×10^8 CFU per Dose) (White Arrows) with 12 h in between Each Dose

With the same interval, the mice received 3 oral doses of ampicillin (13 mg/kg per dose) (A). A three-fold dose of lactobacilli reinoculation (6.80×10^8 CFU) (white arrows) was performed on the 9th day. Results are expressed as the mean S.D. of the log of CFU/organ from 3 to 4 mice.

Table 2. Serum Determinations of LDH Enzyme and Antibody Levels in Control (*Lf*-A) (*Ec*-A) and in Challenged Animals (*Lf-Ec*-A) in a Therapeutic Assay Using Lactobacilli Reinoculation and Three Doses of Ampicillin

Days	LDH (IU)			Antibody levels (log inv. dil.)			
	Lf-A	*Ec*-A	*Lf-Ec*-A	Type	*Lf*-A	*Ec*-A	*Lf-Ec*-A
(In.)							
3	642±5.5	770±7.2	811±6.3	*Lf*:	1.80±0.2		2.10±0.2
				Ec:		2.41±0.0	2.25±0.2
5	920±8.2	351±6.3	772±3.5	*Lf*:	1.80±0.3		1.65±0.1
				Ec:		2.11±0.0	2.39±0.3
7	381±5.4	ND	790±6.6	*Lf*:	1.95±0.1		1.50±0.0
				Ec:		2.41±0.0	2.41±0.0
(In.)							
9	595±4.0	200±8.9	855±5.0	*Lf*:	2.11±0.1		1.80±0.1
				Ec:		2.71±0.0	2.41±0.1
15	718±6.1	196±6.8	ND	*Lf*:	1.80±0.2		1.50±0.2
				Ec:		2.41±0.0	2.71±0.0
18	98±4.8*	73±4.8*	169±3.2	*Lf*:	1.80±0.0		1.50±0.1
				Ec:		2.11±0.0	2.55±0.2

Lf: *L. fermentum*, *Ec*: *E. coli*, ND: not determined, A: ampicillin, (In.); *L. fermentum* inoculation. Values determined before urethral inoculations were 85±7.3 IU for LDH enzyme, and 1.20±0.0 for anti-*L. fermentum* and anti-*E. coli* antibody levels. * $p > 0.05$.

E. coli antibody levels were not statistically different compared with the control mice ($p > 0.05$).

Serum Analysis LDH level increased whenever a lactobacilli inoculation was performed, that is, in both the *L. fermentum*–ampicillin and *L. fermentum–E. coli*–ampicillin assays (Table 2).

The anti *E. coli* antibody levels (log inverse of dilution) did not vary very much in the challenged animals compared with the control mice (*E. coli*–ampicillin).

Urine Analysis In challenged animals, urine showed 4.50×10^4 and 1.24×10^3 CFU of *E. coli*/ml on the 3rd and 10th days, respectively. In the control mice, urine showed 4.00×10^5 *E. coli* CFU/ml on the 3rd day, 2.00×10^3 CFU/ml on the 10th day and 2.00×10^4 CFU/ml on the 18th day. At the same time there were also 10—15 PMN leukocytes/microscopic field (100×) and a small number of epithelial cells/microscopic field (100×).

Other Serum and Urine Analyses In all experiments, CRP showed negative values. Red blood cells in the urine were also always negative.

DISCUSSION

Although the normal urethral flora is relatively constant during one's lifetime, certain factors can affect this equilibrium. The most common cause of disturbances is the

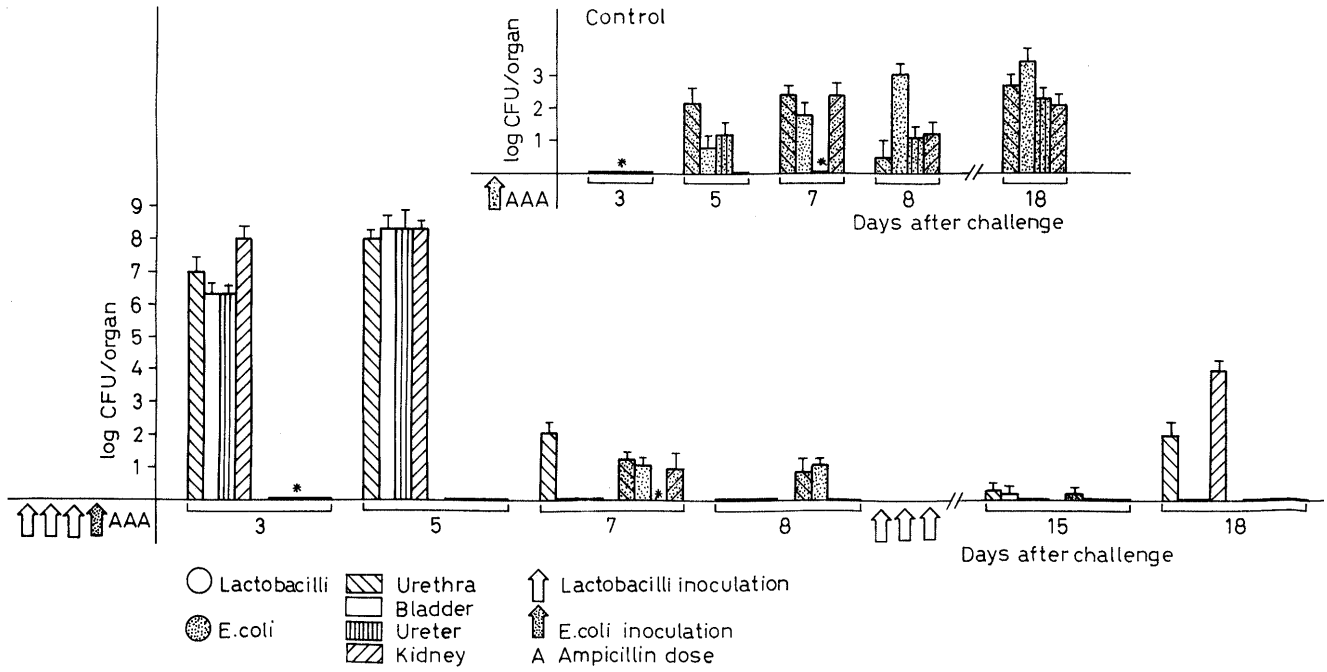

Fig. 3. Intra-urethral Administration of a Three-Fold Dose of *L. fermentum* in Agarose Beads (5.20×10^8 CFU per Dose) (White Arrows) and One Dose of *E. coli* (6.15×10^7 CFU) (Black Arrow) with 12 h in between Each Dose

With the same interval, the mice received 3 oral doses of ampicillin (13 mg/kg per dose) (A). A three-fold dose of lactobacilli reinoculation (6.80×10^8 CFU) (white arrow) was performed on the 9th day. Control animals were treated without lactobacilli (upper graph). Results are expressed as the mean S.D. of the log of CFU/organ from 3 to 4 mice. $*p > 0.05$.

administration of antimicrobial agents. Antibiotics are frequently used to eliminate symptomatic UTI. However, patients who are immunologically deficient or undergo prolonged antibiotic therapy may develop serious recurrent infections.[1] Since it is now recognized that the indigenous microflora of humans and animals provides protection against infections[18-20] we made an attempt to find out the possible *L. fermentum* CRL 1058 contribution, and to understand its colonization capability in response to antibiotic treatments in the therapy and prevention of *E. coli* UTI in mice.

Agarose beads with *L. fermentum* were prepared in order to increase lactobacilli permanence in the urinary tract and to avoid their clearance by urine.[7,11] We used a lactobacilli strain of mouse origin, based on the host-specificity of the normal flora, and also of lactobacilli.[21] This specificity is not the same in the case of *E. coli*, since it can produce infections in different types of hosts.[22] Furthermore, the *E. coli* strain used has a pyrelonephrytogenic capability since it was able to produce a kidney infection when inoculated intra-urethrically.

Optimal concentrations for both microorganisms have been determined previously.[11] *E. coli* was inoculated as a single suspension dose, since a good colonization was obtained without using agarose beads. It was also demonstrated that agarose did not affect lactobacilli or *E. coli* permanence *in vivo*.

For the present work we selected the antibiotic ampicillin because *L. fermentum* showed resistance to this antimicrobial agent *in vitro* (Table 1). Furthermore, ampicillin is frequently used in Argentina in the treatment of children and pregnant women suffering UTI.

In mice, *L. fermentum* demonstrated a high sensitivity to ampicillin, showing that the results of experiments performed *in vitro* cannot always be interpolated to *in vivo* experiments.

When five doses of the antibiotic were used, lactobacilli did not survive, thus showing it not to be able to exert a preventive effect against *E. coli* in combination with ampicillin. LDH as an indication of the inflammatory response and anti *E. coli* antibody levels showed no difference between animals treated with lactobacilli and the control mice. RCP was also negative, supporting the idea that lactobacilli treatment cannot produce an inflammatory response.

Better results were obtained with *L. fermentum* reinoculations. Administration in this way prolonged the lactic acid bacteria survival in the urethra and therefore produced the beneficial effect of elimination of the pathogen, when adequate antimicrobial and lactobacilli concentrations were used (Fig. 3). LDH was higher in treated animals than in the control (no lactobacilli inoculated) ones. LDH also increased after *L. fermentum* inoculations and decreased during the experiment, as if the lactic acid bacteria first stimulate an inflammatory response and then compete with the pathogen in order to perform its subsequent elimination. Specific immune response did not seem to be important since antibody levels were not high enough in any of the experiments. We suggest, therefore, that the preventive effect observed would not be produced by stimulation of the immune system.[23] The mechanism involved in the decrease of the number of *E. coli* with ampicillin treatment could be a competitive exclusion phenomenon, since lactobacilli did not allow *E. coli* to colonize and to produce infections up to the 18th day. *E. coli* elimination by urine could also be an indication of lactobacilli–pathogen competition, or at least of pathogen difficulty in adhering to the urinary

epithelium. In other experiments where the antibiotic and *L. fermentum* were used together for the prevention of UTI, the lactic acid bacteria could not survive and *E. coli* colonization was not affected. Even so, LDH and antibody levels were not significantly altered. Once again, *in vivo* lactobacilli were directly sensitive to the antimicrobial agent.

The effect observed in this study of lactobacilli therapy against UTI could also be studied and determined in vaginal infections.

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