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# Prevention Strategy of Urogenital Infections by Using Lactobacilli with Probiotic Properties

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## 1. Introduction

Lactic acid bacteria (LAB) constitute a group of Gram-positive nonsporing nonrespiring bacteria, cocci or rods, that produce lactic acid as the major end product during the fermentation of carbohydrates. The term LAB is associated with bacteria involved in food fermentation and bacteria normally associated with the mucosal surfaces of human and animals. The classification of lactic acid bacteria into different genera is based on morphology, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentrations, and acid or alkaline tolerance (Pascual, 2004).

Bacteria belonging to the genus *Lactobacillus* are considered to be the main LAB and the predominant microorganisms in the gastrointestinal and urogenital tracts of humans as well as homeothermic animals. They are also used for elaborating different fermented foods categorized as GRAS (generally considered as safe).

Although there are data on simultaneous colonization of the human vagina by two different species of *Lactobacillus*, which can be homofermentative, heterofermentative or a combination of both (Kaewsrirachan et al., 2006; Pascual et al., 2006), only one species has been isolated from the vaginal tract. Also, there are evidences of their effectivity in the prevention of urogenital infections (Pascual, 2004; Axelsson, 2004).

The urogenital microbiota of a healthy woman comprises approximately 50 species of organisms, which differ in composition according to reproductive stages and exposure to several factors, including antibiotics and spermicides (Pascual, 2004).

In the complex vaginal environment, bacteria of the lactobacilli group ( $10^7$ – $10^8$  CFU g<sup>-1</sup> of vaginal fluid) are the dominant microorganisms in healthy pre-menopausal women, and play an important protective role by limiting growth of pathogenic microorganisms. When lactobacilli are reduced, eliminated, or replaced by pathogenic species, the host has an increased susceptibility to urinary tract infections (UTIs), genital tract infections (GTIs), bacterial vaginosis (BV), vulvovaginal candidiasis (VVC), and infection by *Neisseria gonorrhoeae* or *Trichomonas vaginalis*. Worldwide studies of UTIs or GTIs have revealed increasing antibiotic resistance among pathogenic microorganisms. Our research group has isolated human vaginal lactobacilli, selecting those with beneficial or probiotic properties (Czaja et al., 2007).

Urogenital infections are a major reason for women to visit their family's physician and are generally derived to gastroenterology, gynecology, urology, and infectious diseases specialists. The association between abnormal vaginal microbiota and increased risk for sexually transmitted infections, bladder and vaginal infections, and a higher rate of preterm labor indicate the need to better understand and manage urogenital health (Reid et al., 2004). Urogenital infections, defined here to include those that affect the bladder, kidneys, vagina, urethra, periurethra, and cervix, constitute a worldwide problem (Roos et al., 2006). The majority of UTI's occur in sexually active women (Howes et al., 2008). Risk increases by 3-5 times when diaphragms are used for contraception. Risk also slightly increases with not voiding after sexual intercourse and use of spermicides such as nonoxynol-9, which have been shown to be toxic to lactobacilli. Depletion of disturbances of vaginal lactobacilli biota has been associated with establishment of opportunistic infections like BV (bacterial vaginosis) and an increased risk of acquiring type 1 HIV. Nonoxynol-9 is the active compound in many spermicidal formulas. It is a nonionic detergent that reduces the superficial tension of the human spermatozoon membrane, causing loss of motility, decrease of its glucolytic power and alteration in permeability. It also affects the lipidic content of the human spermatozoon membrane. Nonoxynol-9 is generally used at concentrations of 5% in creams. It is possible that the presence of N-9 affects the ecological balance of the vagina through the inhibition of protective lactobacilli, especially those that produce  $H_2O_2$ . Nonoxynol-9 is a spermicide that has antimicrobial activity. Some studies have shown that lactobacilli present resistance or sensitivity to this compound (Pascual et al., 2006).

Increased risk has not been demonstrated with oral contraceptives, not voiding before intercourse, non-cotton underwear, and use of condoms. The prevalence and incidence of urinary tract infection is higher in women than in men, which is likely the result of several clinical factors including anatomic differences, hormonal effects, and behavior patterns (Standiford et al., 2005).

Historical data indicate that the vast majority of urinary tract infections (UTI) in a suburban, nonhospitalized community is caused by *Escherichia coli*, followed by other *Enterobacteriaceae* and *Staphylococcus saprophyticus*. The most frequent bacterial cause of UTI in adult women is *Escherichia coli*, which is part of the normal gut microbiota. This organism accounts for approximately 85% of community-acquired UTIs and 50% of hospital acquired UTIs (Talan et al., 2008).

However, a recent study reported that by infections *E. coli* were less common and that *Enterococcus faecalis* was the second most prevalent uropathogen. The latter result was also found in hospitalized patients. UTI affect millions of women each year, with an annual societal cost of billions of dollars. More than one quarter of women with a UTI will have a recurrent infection within six months. There are few established options for prevention of UTI other than the use of prophylactic antibiotics. Most uncomplicated UTI cases are resolved within 1 to 7 d of antibiotic therapy (Talan et al., 2008). However, drug resistance to commonly used antibiotics (eg, trimethoprim/sulfamethoxazole) is increasing among uropathogens and patients are experimenting more and more with alternative natural medicines, which appear to contain antiadhesive compounds that are active against uropathogens and can help prevent UTI (Pascual, 2004). The phenological characteristics of the lactobacilli strains including adhesive ability and production of acids, bacteriocins, hydrogen peroxide, and biosurfactants appear to be important in conferring protection to the host. Therefore, effective nonantibiotic methods of prevention are needed. One potential alternative may be probiotic lactobacilli (Reid et al., 2005; Pascual et al., 2008a). The

rationale for the use of probiotics is based on the genitourinary regulatory role played by the commensal microbiota and the need for restoration of this microbial ecosystem after insult. Health care providers who are interested in the therapeutic potential of probiotics require evidence of efficacy from randomized controlled assays, including data on successful local colonization and strain-specific outcomes, and information on product integrity and stability. This article reviews available information on the efficacy and tolerability of probiotics in the treatment and prophylaxis of bacterial vaginosis (BV) and the prophylaxis of UTI (Barrons and Tassone, 2008).

The administration of lactobacilli does not produce adverse effects in the urogenital tract; thus, it effectively prevents urinary tract infections. Several clinical assays have demonstrated that certain *Lactobacillus* species can be given orally or vaginally with resulting colonization of the vagina, reduction in vaginal coliform counts, and even reduction in UTI recurrence (Reid et al., 2004).

## 2. Probiotics

### 2.1 The history of probiotics

The first observation of the positive role of some bacteria can be credited to the work of Metchnikoff (1908); who reported on the potential health benefits of probiotics after he observed that Bulgarian peasants that consumed fermented milk products showed long, healthy lives (Sanders, 1999; Senok *et al.*, 2005). At the same time Henry Tissier (1906), a French pediatrician, observed that children with diarrhea had in their stools a low number of bacteria characterized by a peculiar morphology. These bacteria were, on the contrary, abundant in healthy children. He suggested that these bacteria could be administered to patients with diarrhea to help restore a healthy gut biota (WHO/ FAO, 2006).

The application of “health promoting” bacteria for therapeutic purposes has a long tradition in medicine; however, this so-called “bacteriotherapy” has long been considered to be a nonstandard procedure whose efficacy has not yet been proved or is at most based on observation but not on clinical studies (Suvarna and Body; 2005). The successful introduction of probiotics to the market has helped and inspired research in this area to a huge extent. In recent years, there has been a great increase in the number of clinical studies in which the prevention, alleviation, or therapy of diseases has been scientifically investigated not only to find evidence for a health claim for a target group of “healthy consumers” but also to test the medicinal (preventive and therapeutic) application of probiotics (Reid et al., 2006).

The term “Probiotic” derives from the Greek meaning “for life”. It was first introduced in 1965 by Lilly and Stillwell for describing substances secreted by one organism which stimulate the growth of another (Reid *et al.*, 2003). In 1974, Parker referred to “Probiotic” as organisms and substances which contribute to the intestinal microbial balance. However, this term was subsequently redefined by Fuller (1989) as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (Desai, A., 2008). This definition was broadened by Havenaar and Huis in’t Veld (1992) to a mono or mixed culture of live microorganisms which benefits man or animals by improving the properties of the indigenous microbiota (Klaenhammer, 2000; Ranadheera et al., 2010).

At present is defined, by FAO/OMS, as “Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (WHO / FAO, 2001). In a healthy host, a balance exists between members of the microbiota, such that potential pathogenic

and non-pathogenic microorganisms can be found in apparent harmony. During infection, this balance can become disturbed, leading to often dramatic changes in the composition of the microbiota. The most important genus of Gram positive bacteria used extensively as probiotics are *Lactobacillus* and *Bifidobacterium* (Table 1).

<i>Lactobacillus</i> spp.	<i>Bifidobacterium</i> spp.	Others
<i>L. acidophilus</i>		
<i>L. rhamnosus</i>		
<i>L. gasseri</i>		
<i>L. casei</i>		
<i>L. reuteri</i>		<i>Enterococcus faecalis</i>
<i>L. delbrueckii</i> subsp.	<i>B. bifidum</i>	<i>E. faecium</i>
<i>bulgaricus</i>	<i>B. breve</i>	<i>Streptococcus salivarius</i> subsp.
<i>L. crispatus</i>	<i>B. infantis</i>	<i>thermophilus</i>
<i>L. plantarum</i>	<i>B. longum</i>	<i>S. termophilus</i>
<i>L. salivarius</i>	<i>B. lactis</i>	<i>Lactococcus lactis</i> subsp. <i>lactis</i>
<i>L. johnsonii</i>	<i>B. adolescentis</i>	<i>L. lactis</i> subsp. <i>cremoris</i>
<i>L. gallinarum</i>	<i>B.essensis</i>	<i>Propionibacterium freudenreichii</i>
<i>L.plantarum</i>	<i>B.laterosporum</i>	<i>Pediococcus acidilactici</i>
<i>L. fermentum</i>		<i>Leuconostoc mesenteroides</i>
<i>L. helveticus</i>		
<i>L. paracasei</i>		
<i>L. lactis</i>		

Table 1. Microorganisms applied as probiotics. Ranadheera *et al.*, 2010 and Gupta and Garg, 2009.

More than 20 years ago, the production of substances that inhibited pathogen growth on agar plates or the ability to reduce adherence of pathogens *in vitro* defined a probiotic (Chan et al., 1985). Now, the bar has been raised significantly higher, and use of the term ‘probiotic’ needs bacteria to be properly speciated, shown in appropriate formulations to be safe and effective at conferring health benefits on mammalian hosts, and manufactured and sold in a way that accurately reflects what benefits a consumer can obtain (Reid, 2005; Corcionivoschi et al., 2010). Sadly, governments and industry have not yet taken these requirements to heart, and whereas many so-called probiotic products are available, relatively few true probiotic products exist (Table 2).

2.2 Mechanisms of action

Mechanisms by which probiotics exert healthy effects are incompletely understood. Some authors include competitive inhibition with pathogenic bacteria, effects on barrier function, antagonism through the production of antimicrobial substances (acids, hydrogen peroxide and bacteriocins) and modulation of the immune system (Cabana *et al.*, 2006; Almeghaiseeb, 2007). These mechanisms vary according to the specific strain or combination of strains used, the presence of prebiotics [a non-digestible food ingredient which beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon having the potential to improve host health (Gupta and Garg, 2009)] and the condition that is being treated the patient (Devine and Marsh, 2009). Probiotics microorganisms compete with pathogens for nutrients and physical space (Fuller, 1991; Johannsen, 2003).

Appropriateness	
i.	Accurate taxonomic identification
ii.	Normal inhabitant of the species targeted: human origin for human probiotics
iii.	Nontoxic, nonpathogenic, GRAS status
Technological suitability	
iv.	Amenable to mass production and storage: adequate growth, recovery, concentration, freezing, dehydration, storage, and distribution
v.	Viability at high populations (preferred at 10 <sup>6</sup> –10 <sup>8</sup> )
vi.	Stability of desired characteristics during culture preparation, storage, and delivery
vii.	Provides desirable organoleptic qualities (or no undesirable qualities) when included in foods or fermentation processes
viii.	Genetically stable
ix.	Genetically amenable
Competitiveness	
x.	Capable of survival, proliferation, and metabolic activity at the target site in vivo
xi.	Resistant to bile
xii.	Resistant to acid
xiii.	Able to compete with the normal microflora, including the same or closely related species; potentially resistant to bacteriocins, acid, and other antimicrobials produced by residing microflora
xiv.	Adherence and colonization potential preferred
Performance and functionality	
xv.	Able to exert one or more clinically documented health benefits (e.g. lactose tolerance)
xvi.	Antagonistic toward pathogenic/cariogenic bacteria
xvii.	Production of antimicrobial substances (bacteriocins, hydrogen peroxide, organic acids, or other inhibitory compounds)
xviii.	Immunostimulatory
xix.	Antimutagenic
xx.	Anticarcinogenic
xxi.	Production of bioactive compounds (enzymes, vaccines, peptides)

Table 2. Criteria for selection of probiotic strains. Adapted from Klahenhammer (2007).

Some bacteria can inhibit and prevent the colonization by pathogenic microorganisms by means of a mechanism of steric obstruction or blocking of specific receptors (Amores *et al.*, 2004). LGG and *L. plantarum* have been shown to competitively inhibit the attachment of enteropathogenic *E. coli* 0157H7 to HT-29 human colonic cancer cells (Michail *et al.*, 1997; Alvarez-Olmos and Oberhelman, 2001).

The effect of probiotics may be classified in three modes of action: (i) Probiotics have a direct effect on other microorganisms, commensal and/or pathogenic ones. This principle is in many cases of importance for the prevention and therapy of infections and restoration of the microbial equilibrium. (ii) Probiotics also might be able to modulate the host’s defenses including the innate as well as the acquired immune system. This mode of action is most likely important for the prevention and therapy of infectious diseases but also for the treatment of chronic inflammation. (iii) Probiotic effects may be based on actions affecting microbial products like toxins, host products e.g.bile salts and food ingredients (Oeslschaeger, 2010).

3. Probiotics: effects on other microorganisms

3.1 Production of antimicrobial compounds

Lactobacilli produce a variety of compounds that are inhibitory to both Gram-positive and Gram-negative bacteria. These inhibitory substances include organic acids, bacteriocins, hydrogen peroxide and biosurfactants (Rolfe, 2000).

### 3.1.1 Organic acids

Lactic and acetic acids are the main products of carbohydrates fermentation by LAB. These acids diffuse through the membrane of the target organisms, in their hydrophobic undissociated form. Inside of the bacterial cytoplasm they are exposed to a pH value near to neutrality; subsequently, they dissociate (anion and H<sup>+</sup>), reduce cytoplasmic pH and reduce metabolic activities (Kotikalapudi, 2009; Dalié *et al.*, 2010). This lower cytoplasmic pH inhibits glycolysis, prevents active transport and interferes with signal transduction. Furthermore, the anionic part of the acid cannot diffuse freely through the cell wall and accumulates inside the bacterial cell. Accumulation of anions leads to internal osmotic disorders for the bacteria (Kotikalapudi, 2009).

Among the antimicrobial compounds synthesized by the two human lactobacilli strains used in our work, *L. fermentum* L23 and *L. rhamnosus* L60, we have only researched the antimicrobial activity attributed to the bacteriocins and not necessarily to H<sub>2</sub>O<sub>2</sub> and lactic acid production. In previous reports, our group has shown the probiotic properties and the production of metabolites with biological activity against a wide spectrum of other microorganisms of these lactobacilli strains (Pascual *et al.*, 2008a; Ruiz *et al.*, 2009).

Juarez Tomás *et al.* (2003) tested the antimicrobial activity of lactobacilli strains *in vitro* (*Lactobacillus brevis* CRL 1335 and *L. acidophilus* strains CRL 1259, CRL 1307, CRL 1320 and CRL 1324). They found that these strains were able to inhibit the growth of *E. coli*, *S. aureus*, *S. agalactiae*, *E. faecalis*, *Klebsiella* sp., *N. gonorrhoeae* and *G. vaginalis*. Inhibition was shown to be produced by the low pH of the lactobacilli supernatants, as it disappeared when the supernatants were neutralized.

### 3.2 Bacteriocins

Bacteriocins are antimicrobial substances of protein nature, some of which may contain an associated lipid or carbohydrate, that inhibit growth of related or unrelated bacterial species and are potentially useful for prevention or treatment of bacterial infectious diseases (Riley and Chavan, 2007; Pascual *et al.*, 2008b).

Bacteriocins produced by lactic acid bacteria are divided into five classes based on primary structure, molecular mass, heat stability, and molecular organization : class I, lantibiotics; class II, nonlantibiotic peptides (subclass IIa, pediocin-like bacteriocins with strong antilisterial activity; subclass IIb, bacteriocins whose activity depends on complementary action of two peptides; subclass IIc, secdependent secreted bacteriocins); class III, large, heat labile protein bacteriocins; class IV, bacteriocins consisting of an undefined mixture of protein(s), lipid(s), and carbohydrate(s); and class V, bacteriocins with circular, unmodified posttransductional structure (including AS-48, gaseiricine A, enterocin) (Kemperman *et al.*, 2003; Gutiérrez Merino J, 2005).

Lactobacilli bacteriocins are of interest because of their potential application for inhibition of pathogenic bacteria that affect humans. Two *Lactobacillus* strains from human vagina, *L. fermentum* L23 and *L. rhamnosus* L60, were previously identified and characterized as probiotics and producers of bacteriocins.

In a study carried out by our research group (Pascual *et al.* 2008a,b), we described the isolation, purification, and partial characterization of bacteriocins from *L. fermentum* L23 and *L. rhamnosus* L60. The inhibitory spectrum of these strains was quite broad, including Gram-negative and Gram-positive pathogenic strains and *Candida* species. To evaluate the proteinaceous nature of the antibacterial substances, the effect of proteolytic enzymes (trypsin, protease VI) was tested. Incubation of samples for 1 h at 37 °C with these

enzymes completely inhibited the antibacterial activity. The bacteriocin produced by *L. fermentum* strain L23 was sensitive to several proteases, indicating that the inhibitory material was proteinaceous. Catalase and urease had no effect on its activity. Bacteriocin activity was most stable at acid or neutral pH. At alkaline pH, the bacteriocin became progressively inactivated. The partially purified bacteriocin was further purified by chromatography gel filtration (Sephadex G25). The sample was concentrated by evaporation and diluted in a small volume of phosphate buffer at pH 6.5. Fractions of 2.0 ml were collected, and their activity towards the indicator strain was tested. Fractions F15, F16, F17, and F18 displayed inhibitory activity against *E. coli*. They were pooled and concentrated.

The fraction collected after C18 reversed-phase HPLC exhibited activity against the indicator strain *E. coli*. The corresponding elution profile from reversed-phase HPLC, recorded at 220 nm, revealed one peak collected in the fraction eluted at 30 min. When an aliquot of the 30-min fraction was subjected to agar well diffusion assay, a zone of inhibition was produced in the agar. The fractions with antibacterial activity (F15, F16, F17, F18) obtained by chromatography assays were analyzed by TLC on silica gel plates.

The bacteriocin L23 produced by *L. fermentum* strain 23 showed a wide inhibitory spectrum, including some lactobacilli. A noteworthy observation was the inhibition of the pathogenic Gram-negative bacteria *E. coli*, *Proteus vulgaris*, *P. mirabilis*, *Klebsiella pneumoniae*, and *Neisseria gonorrhoeae*. In general, bacteriocins from lactic acid bacteria are active only towards Gram-positive bacteria. A wide inhibitory spectrum, as observed here for *L. fermentum* and *L. rhamnosus* L60, seems to be common among bacteriocin-producing isolates from the genus *Lactobacillus* (group III). Bacteriocin L23 did not show inhibitory activity against species of vaginal microbiota, including lactobacilli. Strain L23 secretes an antibacterial substance other than lactic acid, which is heat stable and only moderately sensitive to enzyme treatment. Several characteristics of the component responsible for the antibacterial activity suggest that it contains an unusual acidic amino acid present in a novel peptidic agent (Ruiz et al., 2009).

### 3.1.3 Interactions of bacteriocins

Also, the interactions between pairs of bacteriocins that inhibit the growth of urogenital pathogens were studied. To evaluate types of interaction between L23 and L60 bacteriocins, 207 isolates were considered. Synergistic interaction between these two bacteriocins was found in 68.6% of the cases (Fig. 1). Interactions were interpreted based on the shape of the inhibition zone, as follows: (1) lack of interaction (indifference) is indicated by growth at a right angle; (2) a synergistic effect results in concave growth between the two inhibition zones; (3) an antagonistic effect results in a junction in which growth covers the angle formed by the inhibition zones. A synergistic effect was observed with inhibition zones > 2mm compared with each antimicrobial activity of L60 or L23. Bacteriocin interactions were also determined using the checkerboard assay as previously described by Petersen et al. (2006). The initial concentrations of bacteriocins used in this experiment were at least the double of that of MIC. Serial dilutions of bacteriocins of L23 and L60 along the ordinate and abscissa were made, respectively, in MRS broth. The fractional inhibitory concentration (FIC) indexes ( $\Sigma$ FICs) were calculated as follows:  $\Sigma$ FIC= FIC A + FIC B, where FIC A is the MIC of A in the combination/MIC of A alone, and FIC B is the MIC B in the combination / MIC of B alone. The FIC was interpreted as follows: synergy,  $\text{FIC} \leq 0.5$ ; indifference,

0.5<FIC<2; antagonism >2. There was neither an indifferent nor an antagonistic interaction between the substances evaluated either by qualitative or semi-quantitative method.

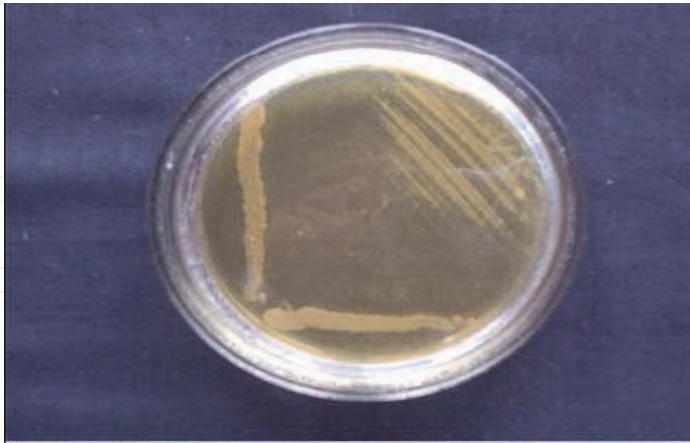


Fig. 1. Synergistic interaction between bacteriocins from L23 and L60 strains against *E. cloacae*

Indicator strains	Number of strains	Indifferent interaction (%)	Antagonistic interaction (%)	Synergistic interaction (%)
<i>Escherichia coli</i>	100	45 (45)	–	55 (55)
<i>Klebsiella pneumoniae</i>	8	2 (25)	–	6 (75)
<i>Klebsiella oxytoca</i>	10	1 (10)	–	9 (90)
<i>Proteus mirabilis</i>	14	9 (64.28)	–	5 (35.71)
<i>Proteus vulgaris</i>	2	–	–	2 (100)
<i>Enterobacter cloacae</i>	14	1 (7.14)	–	13 (92.85)
<i>Citrobacter freundii</i>	5	2 (40)	–	3 (60)
<i>Serratia marcescens</i>	4	1 (25)	–	3 (75)
<i>Acinetobacter baumannii</i>	5	–	–	5 (100)
<i>Pseudomonas aeruginosa</i>	3	–	–	3 (100)
<i>Staphylococcus epidermidis</i>	6	–	–	6 (100)
<i>Staphylococcus saprophyticus</i>	15	3 (20)	–	12 (80)
<i>Staphylococcus aureus</i>	4	–	–	4 (100)
<i>Streptococcus agalactiae</i>	2	–	–	2 (100)
<i>Enterococcus faecalis</i>	7	–	–	7 (100)
<i>Neisseria gonorrhoeae</i>	8	–	–	8 (100)
Total % of interactions observed		31.40	0	68.59

Table 3. Percentage of interactions between bacteriocins produced by *Lactobacillus fermentum* strain L23 and *Lactobacillus rhamnosus* strain L60.

Sensitive species that showed synergistic interaction in 100% of cases were: *A. baumannii*, *P. aeruginosa*, *S. epidermidis*, *S. aureus*, *S. agalactiae*, *E. faecalis*, *P. vulgaris*, and *N. gonorrhoeae*. Smaller percentages of synergistic interactions were found for *E. cloacae*, *K. oxytoca*, *S. saprophyticus*, *K. pneumoniae*, *C. freundii*, and *S. marcescens*. Indifferent interaction between L23 and L60 bacteriocins was found in 31.4% of the total cases. In addition, the highest percentages of indifferent interaction were observed for *P. mirabilis* and *E. coli*. Antagonistic interaction between L23 and L60 bacteriocins was not observed (Table 3).

Mulet-Powell et al. (1998) were the first to describe interactions between bacteriocins from lactic acid bacteria

(LAB). Here, we report rates of inhibitory activity and interactions between two bacteriocins produced by two potential probiotics from lactobacilli of human vagina. Mulet-Powell et al. showed antagonistic interaction between LAB bacteriocins, whereas we found no antagonistic interaction between L23 and L60 from cell-free supernatants (Ruiz et al., 2009).

### 3.1.4 Hydrogen peroxide

Hydrogen peroxide ( $H_2O_2$ ) is produced by most LAB in the presence of oxygen. LAB are unable to produce catalase; therefore, they can not degrade hydrogen peroxide that, after accumulation, develops its oxidative properties with the production of powerful oxidants such as singlet oxygen, superoxide radicals, and the hydroxyl radical. Reactive oxygen species can cause irreversible damage to a number of cell components such as enzymes, membrane constituents and DNA (Schurman, 2001; Dalié et al., 2010).

$H_2O_2$  is produced by many *Lactobacillus* strains in different amounts. There are techniques for measuring these compounds. In a qualitative method that was carried out by Eschenbach et al. (1989), a LAB strain was plated onto MRS agar containing 5 mg 3,3',5,5'-tetramethylbenzidine (TMB) and 0,2 mg horseradish peroxidase (HRPO). Peroxidase generates  $O_2$  from  $H_2O_2$  and TMB dyes the colonies with a blue color when oxidation occurs in the presence of  $O_2$  (Pascual et al., 2008a). Pick and Mizel's phenol red solution method was used for testing  $H_2O_2$  production by macrophages. This qualitative method is modified when working with BAL strains. A 50  $\mu$ l aliquot of centrifuged microorganisms is placed onto a 96-well plate. Then, 50  $\mu$ l of reagent 1 (2 ml of phenol red, 2 ml of HRPO, 46 ml DPBS buffer and 10 ml 1N sodium hydroxide) is added.  $H_2O_2$  was measured indirectly by the 600nm absorbance of phenol red (Strus et al., 2005). As was described in previous studies by our group, a number of cultured lactobacilli generate hydrogen peroxide at inhibitory levels on many pathogenic genitourinary microorganisms. Production of  $H_2O_2$  by the *Lactobacillus* species is considered to represent a nonspecific antimicrobial defense mechanism of the normal vaginal ecosystem. Eschenbach et al. (1989) detected 96%  $H_2O_2$ -producing (LB+) strains. In the present study, we found 62% LB+ and 38% non-  $H_2O_2$ -producing strains (LB-). Species with the largest number of LB+ strains were *L. acidophilus* and *L. fermentum*.

### 3.1.5 Biosurfactants

Biosurfactants are microbial amphiphilic polymers and polyphilic polymers that tend to interact with the boundary between two phases in a heterogeneous system, defined as the interface (Rivardo et al., 2009; Gudín et al., 2010). They comprise a wide range of chemical structures, such as glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids and neutral lipids. Several biosurfactants exhibit antibacterial, antifungal and antiviral activities (Gudín et al., 2010). These molecules alter surface hydrophobicity and therefore inhibit the adhesion of pathogenic microorganisms to infection sites. The release of biosurfactants by probiotic bacteria *in vivo* can be considered as a defence mechanism against other colonizing strains in the urogenital tract (Rodrigues et al., 2006; Gudín et al., 2010). Reid and Bruce (2001c) found that *L. fermentum* RC-14 produces large amounts of biosurfactants. These compounds inhibit the adhesion of a broad spectrum of urogenital pathogens.

### 3.2 Immune modulation

Recent studies have clarified the importance of the immunoregulatory ability of probiotics for exertion of their preventive and therapeutic effects on several diseases. The epithelial barrier consists of a dense mucous layer containing secretory IgA and antimicrobial peptides as well as dynamic functional complexes that regulate permeability between cells (Ohland and MacNaughton, 2010). When barrier function is interrupted due to several factors such as chronic psychological stress, epithelial ion secretion and permeability is enhanced, binding of luminal bacteria to surface epithelia increases, the uptake of luminal antigens through follicle associated epithelium increases and mucosal inflammation initiates (Zareie *et al.*, 2006). There is evidence that consumption of probiotic strains can improve the integrity of the intestinal barrier and the upregulation of mucin production (Devine and Marsh, 2009).

Stimulation and modulation of the mucosal immune system by probiotics reduces production of pro-inflammatory cytokines through activity on NFkB pathways, increase in production of anti-inflammatory cytokines, such as IL-10 and host defence peptides such as b-defensin 2, enhancement in IgA defences and influence on dendritic cell maturation (Devine and Marsh, 2009). It has also been shown that probiotics are able to regulate lymphocyte cell proliferation *in vitro*, as well as the production of specific and nonspecific antibodies (Amores *et al.*, 2004).

The immune system is roughly divided into the acquired immune system, consisting mainly of B lymphocytes and sensitized T lymphocytes, and the innate immune system, consisting mainly of macrophages and NK cells. The ratio of involvement of the systems varies depending on conditions of infection such as species of microorganisms and the amount and site of infection. Mouse studies have clarified that direct activation of macrophages by probiotics increased the bactericidal effect of macrophages on pathogenic bacteria. Probiotics strains have also been reported to promote proliferation of phagocytes such as macrophages and neutrophils in the bone marrow and spleen (hematopoietic tissues). Thus, activation of the innate immune system may be important in the infection preventing effect of probiotics. However, probiotics are also able to protect the integrity of the mucosal barrier against the destructive action of pathogenic microorganisms (Oelschlaeger T, 2010).

### 3.3 Probiotic effects on microbial toxins

One of the most important groups of bacterial virulence factors are toxins. The effectiveness of certain probiotics in suppressing diarrhoea is most likely based on their ability to protect the host against toxins. This protection can result from inhibition of toxin expression in pathogens. Certain probiotics are even able to protect against cyanobacterial and fungal toxins. The basis of the observed protective effect is rather a physicochemical interaction between toxin and a probiotic than a metabolic inactivation (Musa *et al.*, 2009; Oelschlaeger, 2010). This mechanism of action of probiotics is not considered in this chapter.

## 4. Beneficial properties of probiotics

Lactobacilli are able to interfere with genitourinary pathogens by several mechanisms. Other functions of lactobacilli include competitive exclusion of pathogens from the cell surface, co-aggregation with certain pathogenic bacteria, adherence to epithelial cells and biofilm formation based on autoaggregation and surface hydrophobicity (Dunne *et al.*, 2001). Previous studies indicated that autoaggregation of probiotic strains is necessary for

adherence to vaginal epithelial cells, and that co-aggregation leads to formation of a barrier that prevents colonization by pathogens (Boris et al., 1998; Zhou et al., 2004). These are some of the desired characteristics by which specific vaginal lactobacilli strains were selected as potential probiotic agents.

#### 4.1 Autoaggregation assay

The aggregation ability could be described as the clumping of cells of the same strain, known as autoaggregation or self-aggregation (Nikolic et al., 2010).

In a study performed by our research group, autoaggregation was described as the ability to form aggregates within 2 min. (Andreu et al., 1995). Necessary characteristics for *Lactobacillus* strains to serve as effective prophylactic agents include avid adherence to vaginal epithelial cells, interference with the adherence of other bacteria, production of bacteriocins, and production of hydrogen peroxide capable of inhibiting the growth of pathogens (Zhou et al., 2004).

#### 4.2 Surface hydrophobicity

The surface hydrophobicity of lactobacilli was studied by the salt-aggregation test (SAT). The lowest final concentration of ammonium sulfate causing the bacteria to aggregate was defined as the SAT value. Strains were classified into three groups: high surface hydrophobicity (SAT < 0.9 mol/L), intermediate hydrophobicity (SAT 0.9-1.5 mol/L), and hydrophilic (SAT > 1.5 mol/L) (Andreu et al., 1995). Two lactobacilli strains studied by our group showed high hydrophobicity. Thus, we conclude that hydrophobicity is an important mechanism in bacterial adherence.

#### 4.3 Co-aggregation assays

Co-aggregation of probiotic bacterial strains has been suggested to enable them to form a physical-chemical barrier that prevents colonization by pathogenic bacteria. Lactobacilli have been found to co-aggregate with some uropathogenic bacteria and inhibit their growth. A co-aggregation assay is positive when lactobacilli produce aggregates with other strain (Reid et al., 1990). *Lactobacillus fermentum* L23 and *L. rhamnosus* L60 showed co-aggregation with *E. coli*, *G. vaginalis*, and *Candida albicans*, but not with *C. glabrata* (Pascual et al., 2008a). Such co-aggregation could be an important factor in maintaining vaginal health because it produces an area around the pathogen where the concentration of antimicrobial substances produced by these lactobacilli is increased. This would constitute an important host defense mechanism against infection (Kotikalapudi, 2009; Taheri et al., 2009).

#### 4.4 Bacterial adherence

The ability to adhere to epithelial surfaces is considered an indispensable pre-requisite of probiotic strains in order to colonise and then to exert health promoting effects. Bacterial adhesion is initially based on non-specific physical interactions between two surfaces (like hydrophobic interaction), which then enable specific interactions between adhesins (usually proteins) and complementary receptors (Kos et al., 2003; Canzi et al., 2005). To identify bacterial traits related to adhesion ability, potential probiotics strains could be assayed for adherence to cell lines or more frequently, to individual epithelial cells isolated from tissue surfaces by mechanical scraping, brushing or by freezing followed by a rapid thawing (Sillanpää, 2001). Some microorganisms are able to bind to epithelial cells of the

gastrointestinal tract through lectins present in their surface structures. Lectins are carbohydrate-binding proteins or glycoproteins from non-immune origin which agglutinate cells with receptors (Gusilis, *et al.* 2002). Several authors observed a good correlation between adhesion ability and cell surface hydrophobicity (Canzi *et al.*, 2005). Adherence was assessed by counting the number of bacteria adhered to the intact epithelial cells. The number of adhering lactobacilli in the present study was comparable. Such adherence may promote colonization of the vaginal epithelium through formation of a bacterial "film" that tends to exclude pathogens from the mucosa (Reid and Burton, 2002; Pascual *et al.*, 2008a).

#### 4.5 Competitive exclusion

Several studies reported that adhesive probiotic bacteria can prevent the attachment of pathogens and remove them from the urogenital tract. Studies showed that indigenous bacteria isolated from cervical, vaginal, and urethral surfaces of healthy women are able to adhere to human uroepithelial cells *in vitro*. These microorganisms were found to block the adherence of uropathogenic bacteria to uroepithelial cells from women with and without a history of urinary tract infections. Competitive exclusion was most effective with whole viable cells and less effective with cell wall fragments. Analysis of the *Lactobacillus* cell wall preparations suggested that lipoteichoic acid was responsible for the adherence of the *Lactobacillus* cells to uroepithelial cells but that steric hindrance was the major factor in preventing the adherence of uropathogens. Microbiota from the urinary tract may be used as protection against the attachment of uropathogens to the surfaces of uroepithelial cells (Revollo *et al.*, 2006; Kotikalapudi, 2009).

### 5. Applications and beneficial effect of probiotics

There is preliminary evidence that probiotic microorganisms may antagonize the growth of nosocomial pathogens on inanimate surfaces. Among the several health benefits attributed to probiotic bacteria, the modulation of the intestinal microbiota of the host and the capacity to interact with the immune system directly or mediated by the autochthonous microbiota are basic mechanisms. Well-recognized probiotic effects are: 1. Prevention of rotavirus-induced or antibiotic-associated diarrhea as well as alleviation of lactose intolerance symptoms. 2. Reduction of the concentration of cancer-promoting enzymes. 3. Prevention and alleviation of gastrointestinal tract problems in healthy people. 4. Beneficial effects on inflammatory diseases of the gastrointestinal tract (*Helicobacter pylori* infection). 5. Normalization of stool passage in subjects with obstipation or an irritable colon. 6. Prevention of allergies and atopic diseases in infants. 7. Prevention of respiratory tract infections. 8. Prevention as well as treatment of urogenital infections and 9. Hypocholesterolemic effect. Evidence suggests that probiotic microorganisms may have a role in lowering the incidence of vaginal candidiasis, bacterial vaginosis and recurrent lower urinary tract infections (de Vrese and Schrezenmeir, 2008). Certain microorganisms, like *S. aureus* and *E. coli*, are able to adhere to inanimate surfaces by forming biofilms, which consist of an extracellular matrix of polysaccharides. Biofilm formation provides these microorganisms with a survival advantage against their planktonic competitors, and is an optimal environment for proliferation, gene transfer, and quorum sensing within the bacterial population. In this sense, it has been shown that probiotic microorganisms, such as *Lactobacillus* spp., can produce multifunctional molecules, known as biosurfactants, which have antagonistic

antiadhesive properties against microbial pathogens. Biosurfactants, which are amphipathic molecules, have so far found limited application in biomedical sciences; however, indications of their potential clinical applicability are increasing (Falagas and Makris, 2009).

The probiotic concept has focused on two principal areas: Health and human nutrition, and health and animal production. In this chapter we will approach to the study of probiotics in human health.

### 5.1 Probiotics in human health

Several studies have shown the positive use of probiotics in diverse human health problems. They are considered to offer potential therapeutic applications in the prevention and treatment of different diseases (Anuradha et al., 2006; Harish and Varghese, 2006; Rao et al., 2009).

### 5.2 Urogenital tract infections

Among women producing estrogen or receiving estrogen supplementation, the largest part of the vaginal flora consists of lactobacilli, which possesses antimicrobial properties that regulate urogenital microbiota. Genitourinary infections in women are often characterized by an alteration in the local flora from a predominance of lactobacilli to coliform uropathogens as a result of hormone deficiency, sexual activity, contraceptive measures, and other factors (Forsum et al., 2005).

In the complex vaginal environment, bacteria of the lactobacilli group ( $10^7$ – $10^8$  CFU g<sup>-1</sup> of vaginal fluid) are the predominant microorganisms in healthy pre-menopausal women and play an important protective role by limiting growth of pathogenic microorganisms (Reid, 2005; Anukam et al., 2006; Pascual et al., 2008a). When lactobacilli are reduced, eliminated or replaced by pathogenic species, the host has an increased susceptibility to urinary tract infections (UTIs), genital tract infections (GTIs), bacterial vaginosis (BV), vulvovaginal candidiasis (VVC), and infection by *N. gonorrhoeae* or *Trichomonas vaginalis* (Reid et al, 2003; Klebanoff et al., 2004; Ruíz et al., 2009).

The use of probiotics *per se* and mainly lactobacilli has received greater attention as an alternative, inexpensive and natural remedy to restore and maintain the genitourinary health (Reid, 2001a,c).

Reid et al. (2001b) reported the first clinical evidence that probiotic lactobacilli can be delivered to the vagina following oral intake, strain *L. rhamnosus* GR-1 and *L. fermentum* RC-14 were suspended in skim milk and given twice daily for 14 days to 10 women with a history of recurrent yeast vaginitis, bacterial vaginosis and urinary tract infections. Six cases of asymptomatic BV or intermediate BV were resolved within 1 week of treatment. Also, a recent clinical trial showed that oral administration of capsules containing *L. fermentum* RC-14 and *L. rhamnosus* GR-1 was effective as adjuvant in the treatment of patients diagnosed with VVC (Gil et al., 2010).

Pascual et al. (2010) found that *L. fermentum* L23 isolated from vaginal swabs of healthy, non-pregnant, pre-menopausal woman was able to prevent and cure *Escherichia coli* infection in a murine vaginal tract model. The study of vaginal colonization by lactobacilli showed that the human *L. fermentum* L23 strain had the ability to colonize the vaginal tract. A single inoculation was sufficient to establish the probiotic lactobacilli into that niche. Vaginal tract levels of *L. fermentum* L23 remained fairly high for 4 days, with bacterial levels ranging from

4.6 to 3.8 log<sub>10</sub> c.f.u. ml<sup>-1</sup>. On day 5, values decreased to 2.6 log<sub>10</sub> c.f.u.ml<sup>-1</sup>. No growth of L23 strain was observed thereafter (Fig. 2). Infection with the pathogen was maintained in the vaginal tract for more than 7 days (Fig. 3), and this human *E. coli* uropathogenic strain was able to produce a strong infection when inoculated at this concentration, producing significant morphological alterations of the mucosal structure, mainly due to infiltration of polymorphonuclear cells.

The test on the preventive effect produced by strain L23 showed that a single administration of *Lactobacillus* (1x10<sup>8</sup> u.f.c. ml<sup>-1</sup>) inhibited *E. coli* growth and, on the third post-infection day, the *E. coli* growth was not detected, showing that the pathogen was eliminated by the probiotic strain (Fig. 4). The curative effect produced by L23 showed complete inhibition of pathogen's growth after 5 days of treatment (Fig. 5). Thus, *L. Fermentum*, at that concentration, effectively eliminated *E. coli* from the vagina and had no negative effect on the host.

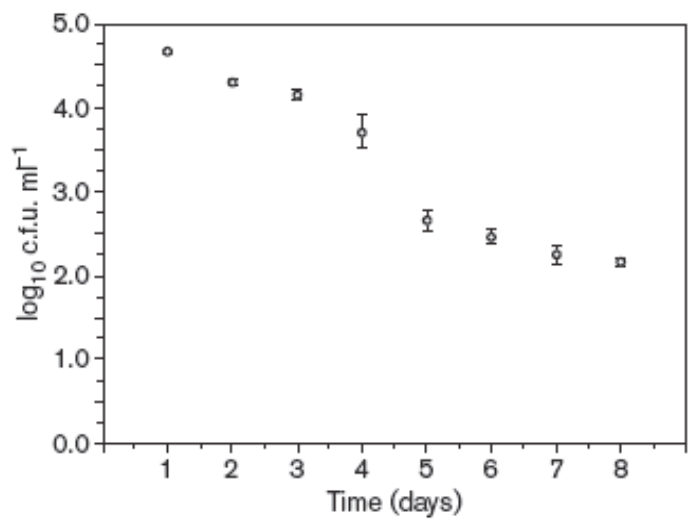


Fig. 2. BALB/c mice vaginal colonization by *L. fermentum* L23. Results are shown as means±SD.

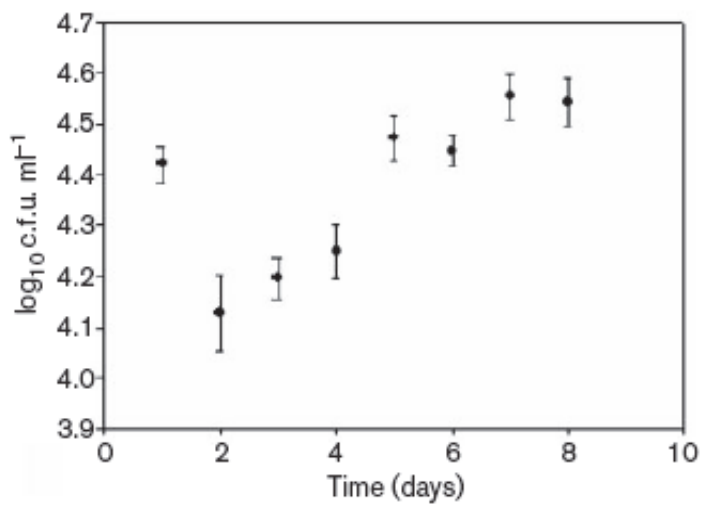


Fig. 3. Vaginal infection of *E. coli* in female mice. Results are shown as means±SD.

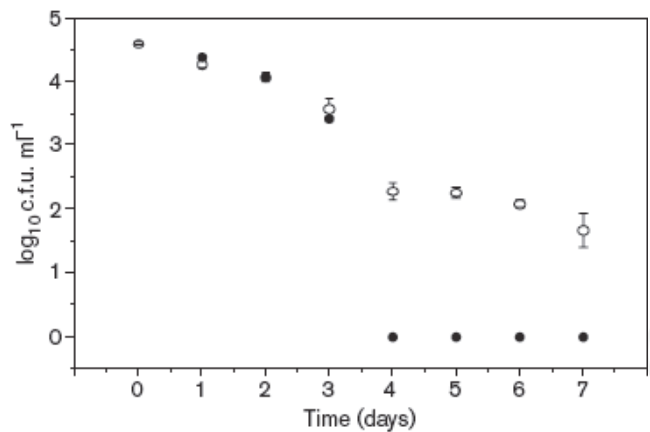


Fig. 4. Preventative effect of *L. fermentum* L23 (o) on *E. coli* (•) in BALB/c mice. Results are shown as means±SD. Significant differences between the untreated control (Fig. 3) and the treated group were found ( $P<0.05$ ).

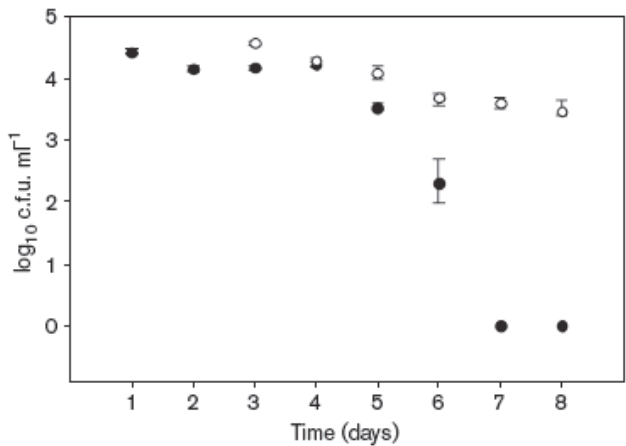


Fig. 5. Curative effect of *L. fermentum* L23(o) on *E. coli*(•) in female BALB/c mice. Results are shown as means±SD.

6. Safety considerations

Probiotics are viable microorganisms, and therefore it is feasible that they could infect the host. First selection criteria mentioned that a probiotic supplement have to be generally regarded as safe microorganisms (Reid *et al.*, 2003; Cabana *et al.*, 2006). Species of *Lactobacillus* or *Bifidobacterium* are normal residents of the gastrointestinal and/or vaginal microbiota and do not display infectivity or toxicity. The risk of infection with these microorganisms is lower (World Gastroenterology Organization Practice Guideline, 2008; Gupta and Garg, 2009). Probiotics are safe for using in healthy people, but should be used with caution in high risk cases such as: People with immune compromise and premature infants. Current WHO/FAO guidelines (2001) recommend that, before using probiotic strains, a number of parameters should be evaluated to prevent health damages, including antibiotic susceptibility patterns, toxin production, metabolic and haemolytic activities, infectivity in immunocompromised animal models, side-effects and adverse incidents in humans (Senok *et al.*, 2005).

## 7. Conclusion

This chapter focuses on a group of lactobacilli, which may protect the vaginal epithelium through a series of barrier mechanisms (adherence), interference mechanisms (co-aggregation with potential pathogens), and production of antimicrobial substances. They appear to be excellent candidates for development as prophylactic agents. The *L. fermentum* L23 and *L. rhamnosus* L60 strains were selected for further studies of possible therapeutic application in the vaginal tract. Further studies are needed to evaluate their immunomodulatory capabilities. The bacteriocins produced by these lactobacilli are strong candidates for treatment or prevention of urogenital disorders in women.

Probiotics do not represent a magic result, but evidence is accumulating that the use of probiotic strains and manipulation of the host's own vaginal/urethral microbiota will provide valuable options to help restore and maintain urogenital health. Once appropriate product formulations with supporting clinical data become available, it will be up to the physician to determine their place in patient management.

## 8. Acknowledgment

Pascual L and Barberis L declare that they own the intellectual property rights associated with *Lactobacillus fermentum* L23 and *Lactobacillus rhamnosus* L60 strains. This work was supported by Secretaría de Ciencia y Técnica, Universidad Nacional de Río Cuarto.

## 9. References

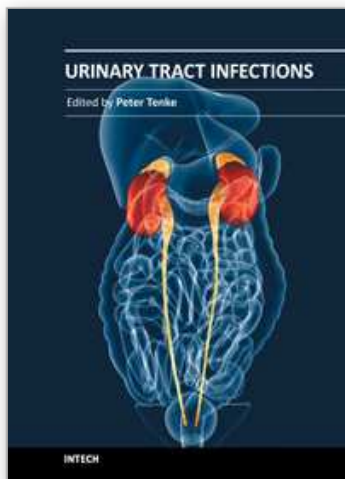
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Urinary tract infections (UTIs) are among the most common bacterial infections worldwide, and they are also the leading cause of hospital-acquired infections. Therefore, the appropriate management of UTIs is a major medical and financial issue. This book covers different clinical manifestations of UTI, with special emphasis on some hard-to-treat diseases, and special conditions in respect of treatment; antibiotic resistance and the available alternative strategies for the prevention and treatment of UTIs and it deals with urinary tract infections in children. The aim of this book is to give a summary about the different aspects of the diagnosis, management and prevention of urinary tract infections for all medical disciplines.

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