Curr Microbiol (2011) 63:360–365
DOI 10.1007/s00284-011-9989-y

Ureaplasma urealyticum and *Mycoplasma hominis* Sensitivity to Bacteriocins Produced by Two Lactobacilli Strains

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Received: 12 May 2011 / Accepted: 20 July 2011 / Published online: 30 July 2011

Abstract The purpose of the present study was to determine the inhibitory activities of two bacteriocins, produced by lactobacilli, against genital mycoplasmas. In this study, infections produced by genital mycoplasmas were studied; of these, 1.3% were caused by *Mycoplasma hominis*, 10.7% by *Ureaplasma urealyticum* and 5.6% by *U. urealyticum* + *M. hominis*. *U. urealyticum* was isolated from 75 out of 123 patients with genital mycoplasmas, while *M. hominis* was isolated from 9 patients (7.3%) and both *U. urealyticum* and *M. hominis* from 39 patients (31.7%). Bacteriocins, L23 and L60, produced by *Lactobacillus fermentum* and *L. rhamnosus*, respectively, appear to be two novel inhibitors of bacterial infection with potential antibacterial activity. Both bacteriocins proved to be active against 100% of strains tested; MICs of bacteriocin L23 ranged between 320 and 160 UA ml^{-1} for 78% of the *M. hominis* strains and between 320 and 80 UA ml^{-1} for 95% of the *U. urealyticum* strains. In addition, bacteriocin L60 was still active at 160 UA ml^{-1} for a high percentage (56%) of *M. hominis* strains, and at 80 UA ml^{-1} for 53% of the *U. urealyticum* strains. Interestingly, these antimicrobial substances produced by lactobacilli showed an inhibitory activity against genital mycoplasmas even when diluted. Altogether, our study indicates that the bacteriocins, L23 and L60, are good candidates for the treatment or prevention of genital infections in women.

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Introduction

Mycoplasmas, the smallest, free-living microorganisms, widespread in nature, and several species have been isolated from humans [23]. Genital mycoplasmas represent a complex group of microorganisms that have been associated with a wide array of infectious diseases in adults and children. *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Mycoplasma genitalium* and *Ureaplasma parvum* are thought to be associated with diverse genitourinary infections [12]. Several studies were concentrated on the role of mycoplasmas as sexually transmitted agents, with emphasis on *M. hominis* and *U. urealyticum*, which have been isolated from the genital tract of men and women with high frequency. *M. hominis* and *U. urealyticum* are associated with several diseases of the urogenital tract, but they are not usually detected by routine microbiological analyses. They cause many disorders such as non-gonococcal urethritis (NGU), postdelivery fever, infertility, and pelvic inflammatory disease [10].

The increasing resistance to first-line antimicrobial agents among these bacterial pathogens has led to research for an effective natural alternative to common therapies used for treating genital infections [15].

Lactobacilli are the main microorganisms that inhabit the human vaginal tract. They can maintain the ecological equilibrium of the tract by protecting it against pathogenic microorganisms. In the past few years, there has been an increasing tendency to use probiotic microorganisms to restore the ecological equilibrium and to protect the human vaginal tract against infections [3, 7]. The exogenous application of lactobacilli to the host as probiotic agents appears to offer optimism as an alternative to antimicrobial treatment and prophylaxis [18, 25].

Depleted vaginal lactobacilli and recurrent infection are associated with an increased risk of sexually transmitted diseases, preterm labor, multiple antibiotic resistances, and significant reduction in quality of life [24].

Lactobacilli produce many different bacteriocins with similar activity, which play an important role in maintaining the vaginal health. In addition, they produce lactic acid and hydrogen peroxide (H_2O_2), which can prevent the overgrowth of pathogenic microorganisms [27]. It is, therefore, urgent to identify and characterize probiotics lactobacilli as a natural alternative to the antibiotics usually applied for treatment of these urogenital disorders, as discussed by Zhou et al. [29].

The aim of the present study was to determine the antimicrobial activities of two bacteriocin-producing strains, *L. fermentum* L23 and *L. rhamnosus* L60, which inhibit the growth of *M. hominis* and *U. urealyticum*.

Materials and Methods

Lactobacilli and Growth Conditions

L. rhamnosus strain L60 (GenBank accession N° EF495247) and *L. fermentum* strain L23 (GenBank accession N° EF1251481) were identified by standard biochemical tests [6], carbohydrate fermentation patterns, and API 50 CHL system (BioMérieux, Inc, France). Both the strains were grown in De Man Rogosa Sharpe (MRS) agar [9] for 24 h at 37°C, under a 5% CO_2 atmosphere. They were stored at −80°C in MRS broth containing 30% (v/v) glycerol.

The 16S rRNA gene was sequenced using an Applied Biosystems model 3730XL automated DNA sequencing system obtained from Macrogen Laboratories (Korea). The 16S rRNA gene sequence was subjected to a BLAST search www.ncbi.nlm.nih.gov/BLAST/) to reveal identities between sequences. They were selected by our group as potential probiotic bacteria and for their ability to produce organic acids, bacteriocins and, in the case of L60, also to release hydrogen peroxide in culture supernatant [18–20].

Patient Population

A set of 700 vaginal swabs from nonpregnant and sexually active female patients was studied in the Obstetrics and Gynecology Department at the Río Cuarto Hospital. In addition, a survey to record the patients' identity, the presence of clinical manifestations and chronic illnesses was performed. Pregnant women were excluded.

Detection and Identification of Genital Mycoplasmas

MYCOFAST EvolutioN 2 (International Microbio/France) was used for the detection, enumeration, identification, and antibiotic resistance test in a liquid medium. Samples were cultured according to the manufacturers' instructions. Inoculation of the MYCOFAST EvolutioN 2 tray was performed on one row containing ten wells. The tray was incubated at 37°C for 24–48 h, during the growing and antibiotic sensitivities of mycoplasmas/ureaplasmas were evaluated. Growth of genital mycoplasmas caused a colour change in the medium, from orange to fuchsia-red. This colour change is due to the ammonia produced which causes the pH to become alkaline [28]. After incubation, detection, and enumeration of *U. urealyticum* at 10^3, 10^4, and $>10^5$ CCU ml^{-1} (Mycofast CCU means Mycofast Color Changing Unit) on wells 1–3 was carried out. Wells 4–6 were reserved for detection of resistance to doxycycline (DO), roxithromycin (ROX) and ofloxacin (OFX), respectively. Wells 7–9 were reserved for *U. urealyticum* and *M. hominis* identification using resistance profiles to the following antibiotics, respectively: lincomycin (L), trimethoprim-sulfamethoxazole (SXT) and erythromycine (E). Well 10 was reserved for enumeration of *M. hominis*.

Characterization of Bacteriocin

Each bacteriocin used has been characterized in previous articles [1, 5, 18]. Effects of trypsin, type VI protease, and urease on bacteriocin activity were tested. Crude bacteriocin sample was treated with catalase (5 mg ml^{-1}) and dissolved in 50 mM KH_2PO_4 buffer (pH 7.0) at 25°C to completely eliminate a possible inhibitory effect of hydrogen peroxide; finally, heat sensitivity and pH stability were tested [20, 21]. Bacteriocin purification had been described in previous studies by our group, through different steps including a C18 reverse phase HPLC [20].

Study of Antimicrobial Activity of Lactobacilli on Genital Mycoplasmas

Lactobacilli were cultured in MRS broth, and the supernatants with inhibitory activity were separated by 10,500 g^{-1} ultracentrifugation for 20 min at 4°C, neutralized with NaOH 1 N (pH 6,5), and L60 strain was treated with peroxidase (0.1 mg ml^{-1}). Bacteriocin activity was defined as the reciprocal of the highest dilution which produced complete inhibition of the indicator lawn, and is expressed as activity units (AU) per milliliter of culture medium [20].

Antimicrobial activity of lactobacilli was studied from mycoplasmas positive samples.

The inhibitory effects of L23 and L60 cell-free supernatants were evaluated by means of a modification of the MYCOFAST EvolutioN 2 test. In brief, 100 µl of a mixture formed by the sample containing genital mycoplasmas dissolved in the growth medium and the supernatants of both L23 and L60 (neutralized and treated with peroxidase 0.1 mg ml^{-1}) were dispensed into wells 1, 2, 3, 9, and 10 [19, 21, 27]. Afterwards, two drops of supplement for *M. hominis* were added into wells 9 and 10, followed by two drops of paraffin oil into each well to ensure microaerophily. The tray was incubated at 35–37°C for 24 h. Results were assessed as described above.

Minimal Inhibitory Concentration (MIC) of L23 and L60 Bacteriocin-like Substances on *M. hominis* and U. *urealyticum* Growth

The MIC of L23 and L60 bacteriocin-like substances were evaluated using a modification of the MYCOFAST EvolutioN 2 test. Samples containing genital mycoplasmas dissolved in the growth medium were mixed with serial dilutions (1:2, 1:4, and 1:8) of each of the lactobacilli's bacteriocin-like substances; wells 1, 2, 3, and 7 were inoculated with *U. urealyticum*. MIC of *M. hominis* was performed in wells 9 and 10, using two galleries of the MYCOFAST EvolutioN 2 test. Results were assessed as described above.

Statistical Analyses

All bacterial counts were expressed as means ± SD and were log transformed for each experiment. An analysis of variance (Sigma Stat Statistical Software V 2.0, for Windows NT and 3.1; SPSS) was used for differences in numbers of viable microorganisms from various treatment groups. A P value of <0.05 was considered statistically significant.

Results

In this stduy, vaginal swab samples from 700 women were screened for the presence of genital mycoplasmas. From these samples, 1.3% tested positive for *M. hominis*, 10.7% for *U. urealyticum*, and 5.6% for both *U. urealyticum* and *M. hominis.* Among the 123 positive samples, *U. urealyticum* was isolated from 75 (61%) patients, *M. hominis* from 9 (7.3%) and a mixed infection of *U. urealyticum* and *M. hominis* was detected in 39 patients (31.7%).

U. urealyticum was detected at concentrations of 10^3–$\geq 10^5$ UCC ml^{-1}, while *M. hominis* was identified at concentrations of $\geq 10^4$ UCC ml^{-1} in all of the analysed samples. *U. urealyticum* and *M. hominis* are frequently isolated from the genital tract and should be investigated, especially in patients with leukocytosis and negative bacterial routine.

The antibiotic susceptibility test showed that 1.3% of *U. urealyticum* and 0% of *M. hominis* were resistant to doxycycline. For ofloxacin, the resistance of *U. urealyticum* was of 6.7%. All the *M. hominis* strains were susceptible to doxycycline and ofloxacin; nevertheless, 78% of them showed resistance to roxithromycin. For *U. urealyticum* and *M. hominis* mixed isolates, resistance was of 5.1% for doxycycline, 15.4% for ofloxacin, and 77% of them showed resistance to roxithromycin (Fig. 1). *U. urealyticum* or *M. hominis* isolated from mixed infections exhibited higher levels of antibiotic resistance compared with the same pathogens isolated from single infections.

In previous studies, our group has described the characterization, purification, and probiotic properties of the bacteriocins L23 and L60, produced by *L. fermentum* L23 and *L. rhamnosus* L60, respectively. The synthesis of their metabolites showed a wide spectrum of antimicrobial activity against other pathogenic microorganisms. Both bacteriocins proved to be active on 100% of the *M. hominis* and *U. urealyticum* strains.

In previous studies, our group has found an antimicrobial activity of 640 activity units (UA ml^{-1}) for the bacteriocins produced by L23 and L60 (data not published).

In this study, MICs of bacteriocin L23 ranged between 320 and 160 UA ml^{-1} for 78% of the *M. hominis* strains. In addition, MIC of bacteriocin L23 for 95% of the *U. urealyticum* strains ranged between 320 and 80 UA ml^{-1}. We observed that L60 MIC was 160 UA ml^{-1} for a high percentage (56%) of the *M. hominis* strains, while MIC of bacteriocin L60 was 80 UA ml^{-1} for 53% of the *U. urealyticum* strains.

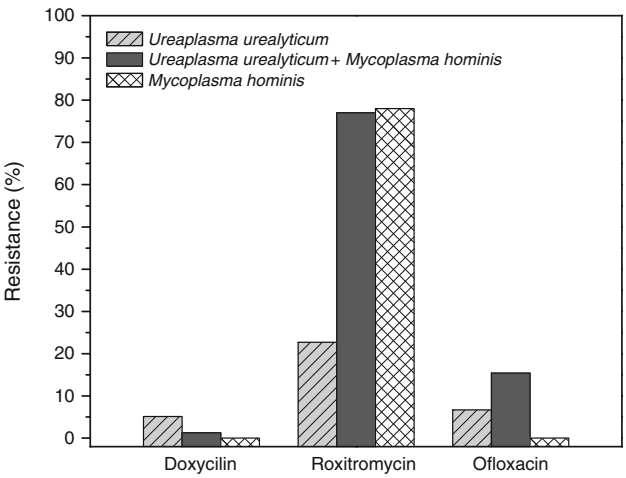

Fig. 1 Distribution of antibiotics resistance percentages of urogenital mycoplasmas

Table 1 MIC values for urogenital mycoplasmas of bacteriocins produced by *Lacbacillus fermentum* and *Lactobacillus rhamnosus*

Microorganisms	MIC (UA ml^{-1})	N° sensitive strains to L23	Percentage (%)		N° sensitive strains to L60
			L23	L60	
M. hominis ($n = 9$)	640	1	11.1	11.1	1
	320	3	33.3	22.2	2
	160	4	44.5	55.6	5
	80	1	11.1	11.1	1
U. urealyticum ($n = 75$)	640	4	5.3	5.3	4
	320	16	21.3	14.6	11
	160	23	30.7	26.7	20
	80	32	42.7	53.4	40

When comparing the results for the majority of the *U. urealyticum* strains, we found that MIC was 80 UA ml^{-1} for both the bacteriocins L23 (32 *U. urealyticum* strains) and L60 (40 strains). On the other hand, MIC of bacteriocins L23 and L60 had the same value of 160 UA ml^{-1} for the majority of *M. hominis* (Table 1).

Discussion

Worldwide studies of Urinary Tract Infections (UTIs) or Genital Tract Infections (GTIs) have revealed increasing antibiotic resistance among pathogenic microorganisms [8]. Our research group has isolated human vaginal lactobacilli, selecting those with beneficial or probiotic properties. These lactobacilli produce different bacteriocins of similar activity, and play an important role in maintaining vaginal health, which can prevent the overgrowth of microorganisms in the vaginal environment. Lactobacilli are able to interfere with genitourinary pathogens by several mechanisms, including competitive exclusion from the cell surface, production of adhesion inhibiting biosurfactant compounds, production of antimicrobial compounds, autoaggregation, surface hydrophobicity, and co-aggregation with other bacterial species. These are some of the desired characteristics by which specific vaginal lactobacilli strains were selected as potential probiotic agents [27].

The genital mycoplasmas represent a group of microorganisms that have been associated with infectious diseases in genitourinary tract. For this reason, it is important to determine the inhibitory activities of these two bacteriocins, produced by lactobacilli, against genital mycoplasmas.

Genital mycoplasmas and ureaplasmas infections are commonly diagnosed by culture. The time-consuming culture requires 2–5 days for *Ureaplasma* spp. and *M. hominis* and up to 8 weeks for *M. genitalium*; however, infectious agents can be detected in less than 8 h by nucleic acid amplification techniques. Recently, a PCR-microtiter plate hybridization assay was developed to detect *M. genitalium*, *M. hominis*, *U. parvum* and *U. urealyticum* in urine samples [12].

In this study, we use the MYCOFAST EvolutioN 2 test as diagnostic method to detect mycoplasmas and ureaplasmas. This method is usually used as an alternative for the detection of genital mycoplasmas in clinical microbiological laboratory. Although the molecular study is confirmatory of the mycoplasma bacteria, the main aspect of this study was to detect the activity of bacteriocins on genital mycoplasmas rather than studying the molecular identification of these microorganisms.

The recovery frequency of *M. hominis* and *U. urealyticum* among patients was similar to that previously reported by Nassar et al. [17]; however, our values were higher than those reported by those authors for *U. urealyticum*. In this study, the isolation frequency for *M. hominis* and *U. urealyticum* in a mixed infection was higher than that obtained by Zhou et al. [29]. These differences could be due to different epidemiological and cultural characteristics, such as distribution of agents in terms of time and geographical region [16].

The high susceptibility that we observed of *U. urealyticum* and *M. hominis* strains to doxycyclin was also observed by Karabay et al. [15] and Bayraktar et al. [4]. On the other hand, resistance of *U. urealyticum* to ofloxacin was higher among our strains than that previously informed by the same authors. We theorize that antibiotic resistance is due to common widespread usage of quinolones in UTIs. Susceptibility to ofloxacin for all of the *M. hominis* strains was similar to that reported by Gokahmetoglu et al. [13], who also found a high sensitivity to quinolones.

The high resistance to roxithromycin among the *M. hominis* strains as well as the *U. urealyticum* and *M. hominis* mixed isolates was similar to that reported by Ardic et al. [2]. Most likely, resistance to macrolides found in our *M. hominis* strains could be associated with an intrinsic mutation in the 23S rRNA; consequently, strains became naturally resistant, as reported by Pereyre et al. [22].

The high resistance observed with mixed pathogens, compared with single infections by *U. urealyticum* or *M. hominis*, represents an interesting finding for all of the assessed antibiotics; in fact, these results concur with those of Fagundo-Sierra et al. [11].

Also, the higher effectiveness of macrolides against infection by *U. urealyticum* and of quinolones against infection by *M. hominis* presented in this study results coincides with those previously reported by Roca [26]. *M. hominis* and *U. urealyticum* were uniformly susceptible to doxycycline, which may be successfully used in the empirical therapy of infected individuals.

Other researchers who used similar commercial detection kits determined that a titter of $\geq 10^4$ CCU ml^{-1} must be considered an evidence of disease [28]. In this study, values greater than 10^4 CCU ml^{-1} indicated that patients carried a sexually transmitted disease.

To our knowledge, there have been no recent studies in the literature concerning the enumeration of *U. urealyticum* and *M. hominis* isolated from the vaginal niche, though a report by Tiodorović [28] examined the urethra of men.

Compounds with antibacterial activity were identified as bacteriocin L23, produced by *L. fermentum* L23, and bacteriocin L60, produced by *L. rhamnosus* L60. Bacteriocins L23 and L60 appear to be two novel inhibitors of bacterial infection with potent antibacterial activity. Here, we report rates of inhibitory activity of two bacteriocins produced by two potential probiotic lactobacilli from human vagina; indeed, the two bacteriocins showed considerable antimicrobial activity against *M. hominis* and *U. urealyticum*. Both bacteriocins proved to be active on 100% of the *M. hominis* and *U. urealyticum* strains, as the ones reported by Hutt et al. [14] who studied other urogenital microorganisms with antibacterial activity. An antimicrobial effect of bacteriocins L23 and L60 was still observed when tested at 1/8 the original testing concentration. This is the first report showing the inhibition of urogenital mycoplasmas by bacteriocins of lactobacilli, and, in particular, evaluating the bacteriocinśs MIC for these types of microorganisms.

These findings are favourable for therapeutic application, especially if one considers that, in recent years; the resistance of these microorganisms to different antibiotics of clinical use has been increasing.

In this way, we could get an idea of the in vitro inhibitory effect of lactobacilli on mycoplasmas and later on, with further studies, we could extrapolate these promising results to a vaginal niche.

As antibiotic treatment may be ineffective in many cases, the application of bacteriocins producing lactobacilli strains could offer an important alternative for the treatment and prevention of genital disorders in women. In this manner, our study showing the in vitro inhibitory effect of lactobacilli on mycoplasmas is encouraging for further studies to determine if these results will extrapolate to the vaginal niche.

Acknowledgments This study was supported by the Secretaría de Ciencia y Técnica de la Universidad Nacional de Río Cuarto.

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