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RESEARCH ARTICLE

Bacteriocins and other bioactive substances of probiotic lactobacilli as biological weapons against *Neisseria gonorrhoeae*

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One sentence summary: Inhibitory effect produced by the cell-free supernatants of *Lactobacillus rhamnosus* L60 on the growth of different gonococcal strains. 1: P-CFS pure cell-free supernatant of *L. rhamnosus* (combined action of organic acid, hydrogen peroxide and, BLIS L60), 2: N-CFS cell-free supernatant neutralized of *L. rhamnosus* (action of H_2O_2 and BLIS L60), 3: BLIS L60: bacteriocin-like inhibitory substance L60, A, B: two different gonococcal strains.

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ABSTRACT

In the search of new antimicrobial agents against *Neisseria gonorrhoeae*, the bacteriocins-producing probiotic lactobacilli deserve special attention. The inhibitory effects of biosubstances such as organic acids, hydrogen peroxide and each bacteriocin-like inhibitory substance (BLIS) L23 and L60 on the growth of different gonococcal strains were investigated. Different non-treated and treated cell-free supernatants of two probiotic lactobacilli containing these metabolites were used. The aims of this work were (i) to evaluate the antimicrobial activity of the biosubstances produced by two probiotic lactobacilli, estimating the proportion in which each of them is responsible for the inhibitory effect, (ii) to define their minimum inhibitory concentrations (MICs) and, (iii) to determine the potential interactions between these biosubstances against *N. gonorrhoeae*. The main antimicrobial metabolites were the BLIS-es L23 and L60 in comparison with other biosubstances. Proportionally, their contributions to the inhibition on the gonococcal growth were 87.28% and 80.66%, respectively. The MIC values of bacteriocins were promising since these substances, when diluted, showed considerable inhibitory activity for all gonococci. In the interaction between bacteriocins, 100% of synergism was found on the gonococcal growth. In summary, this study indicates that both L23 and L60 could potentially serve to design new bioproducts against *N. gonorrhoeae*.

Key words: Gram-negative diplococci; biostrategy; secondary metabolites; therapeutic option; antagonistic activity; synergistic activity; beneficial microorganisms

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INTRODUCTION

The bacterium *Neisseria gonorrhoeae* is the causative agent of gonorrhea. This pathogen was discovered in 1879 by Albert Nisser, although accounts of the disease as such have existed since the fifth-century BC (Barry and Klausner 2009). It is one of the most prevalent sexually transmitted diseases (STD) of bacterial origin worldwide (Tazi *et al*. 2010; Skerlev and Čulav-Košćak 2014). This strictly human pathogen most commonly infects the lower urogenital tract, namely, the urethra of males and endocervix and/or urethra of females of reproductive age. Gonococcal infections in males always cause symptomatic urethritis associated with mucopurulent urethral discharge and can lead to complications such as epididymitis, proctitis, prostatitis and infertility. In women, the cervicitis is generally asymptomatic and can result in serious complications such as pelvic inflammatory disease, ectopic pregnancies and infertility (De Seta *et al*. 2012; Ndowa and Lusti-Narasimhan 2012). Other body sites such as rectum, pharynx and conjunctiva may also be colonized (Biance-Valero *et al*. 2013; Jenkins, Nessa and Clark 2013). The rise to the upper reproductive tract occurs in both sexes and gonococcal dissemination by hematogenous spread to the joints and skin, although rarely, can also occur (Won *et al*. 2011; Guillot *et al*. 2012; de Vries 2014). Further, *N. gonorrhoeae* may also cause neonatal conjunctivitis leading to blindness (CDC 2013). As with other sexually transmitted infections, gonococcal infections have been shown to substantially increase not only the risk of acquiring and transmitting human immunodeficiency virus but also the susceptibility to *Chlamydia*, syphilis, trichomoniasis and/or HSV-2 infections (Sonnenberg *et al*. 2013; Kenyon, Buyze and Colebunders 2014).

In the most recent epidemiological data reported by the World Health Organization (WHO) on the curable STDs, a global index of 448 million new cases per year was estimated, with between 106 and 200 million of them due to gonococcal infections, affecting individuals of 15–50 years of age (WHO 2012a,b). Thus, the infections produced by this Gram-negative diplococcus represent an important public health problem in many developed and developing countries, due to its magnitude and potential sequels. Prevalence rates of this disease vary greatly among countries in the developed and developing world, being the highest in the Western Pacific (China, Japan, the Philippines, Malaysia, Vietnam, Australia), Southeast Asia (India, Korea, Thailand, Bangladesh), Africa, followed by Latin American and the Caribbean countries (LAC; Barbee and Dombrowski 2013; Nesa *et al*. 2013). In LAC, gonorrhea infection is a serious problem but up-to-date information is largely lacking (Starnino *et al*. 2012).

Currently, there are no vaccines against gonococcal infection. Moreover, gonorrhea treatment is complicated by the ability of *N. gonorrhoeae* to quickly develop resistance to antimicrobial therapies. Therefore, the search for effective new antimicrobial agents of potential therapeutic use has been a major challenge for the development of public health control strategies against gonococcal infections (Workowski, Berman and CDC 2010a; Dillon, Trecker and Thakur 2013). Among them, biological tools for the control of infectious diseases are being studied in many countries. For example, in the search of new drugs or products against *N. gonorrhoeae* strains, studies on essential oils, aqueous extracts and/or specific compounds of different medicinal plants from Southern Africa, Canada and Colombia have been recently evaluated (van Vuuren and Naidoo 2010; Cybulska *et al*. 2011; Ruddock *et al*. 2011). Other researchers are turning their attention to the possibility of using non-pathogenic microorganisms that are naturally associated with a healthy status in different human tracts (Spurbeck and Arvidson 2011). Consequently, some probiotic strains that belong mainly to the *Lactobacillus* genus, with strong ability to produce antimicrobial substances such as bacteriocins, organic acids, hydrogen peroxide and/or biosurfactant substances, deserve special consideration (Ruíz *et al*. 2009; Daniele *et al*. 2011; Reid 2012). Special interest in probiotic lactobacilli is based on the fact that until now no genes associated with pathogenicity have been identified in such organisms and therefore they are generally recognized as non-pathogenic safe microorganisms (Sanders *et al*. 2010). In the biomedicine field, the probiotic lactobacilli represent an alternative strategy of biological control for treatment, restoration and maintenance of a balanced microbiota that benefits the host's health (Reid *et al*. 2010; Khani *et al*. 2012).

Bacteriocins are natural antimicrobial substances of protein nature that are produced by a wide variety of bacteria. They are ribosomally synthetized as small peptides, polypeptides or proteins, which may be associated in some cases with lipids or carbohydrates. In general, these biosubstances may have a varied spectrum of antimicrobial activity, inhibiting phylogenetically related or non-related bacteria (Ruíz *et al*. 2005, 2007, 2012; Pascual and Barberis 2011). Although many bacteriocins have correctly been characterized and purified, it is very common to find the BLIS acronym (bacteriocin-like inhibitory substance) to refer to those bacteriocins whose amino-acid sequences have not yet been elucidated (Shokri *et al*. 2013; Zaeim, Soleimanian-Zad and Sheikh-Zeinoddin 2014). Many of these natural substances have a strong biotechnological potential, not only in pharmaceutical industry but also in human and veterinary medicine, basically, due to their wide antimicrobial activity, low eukaryotic cytotoxicity and absence of cross resistance with antibiotics (Montalbán-López *et al*. 2011; Benmechernene *et al*. 2013). On the other hand, although international organisms, such as the Food and Drug Administration (FDA) and WHO have defined probiotics as 'alive microorganisms that when administrated in adequate quantities benefit host health' (FDA 2006), this concept was subcategorized to differentiate the probiotics used as biotherapeutics from those employed in human and animal feeds, and the genetically modified probiotics (design probiotics). According to the FDA, a drug or biotherapeutic agent is defined as any raw material of biological origin which is directly or indirectly used for the preparation of medicines or products that will be employed to diagnose, cure, mitigate, treat or prevent diseases (Hoffman 2008; Ross *et al*. 2008, FDA 2009). Thereby, in the urgent search of new biotherapeutic agents against *N. gonorrhoeae*, the bacteriocins or directly the bacteriocin-producing probiotic lactobacilli could be evaluated as potentially interesting biological options that complement antibiotic therapies. Considering the possibility of developing a bioproduct based on bacteriocin-producing lactobacilli strains, we initiated this *in vitro* study with these following aims: (i) to evaluate the antimicrobial activity of the bioactive substances produced by two probiotic lactobacilli on *N. gonorrhoeae* strains, (ii) to estimate in which proportion each one of them is responsible for inhibitory effect, (iii) to define their minimum inhibitory concentrations (MICs) and (iv) to determine the potential interactions between these biological substances on gonococcal strains.

MATERIALS AND METHODS

Lactobacilli strains

Two vaginal lactobacilli strains, *Lactobacillus fermentum* strain L23 and *L. rhamnosus* strain L60 have been correctly identified by

standard biochemical tests, the API 50 CHL system (BioMèrieux, Inc., France) and 16S r-DNA analysis (Pascual *et al.* 2010). The 16S r-DNA sequencing of both lactobacilli was deposited in GenBank according to the last recommendation of FAO/WHO in 2001 (Pineiro and Staton 2007). GenBank accession no. GQ 455406 and GenBank accession no. EF 495247 have been assigned to *L. fermentum* L23 and *L. rhamnosus* L60, respectively (Ruíz *et al.* 2012). They were selected by our group as potential probiotic bacteria and, for the ability to produce organic acids, bacteriocins and, in the case of L60, also to release hydrogen peroxide in culture supernatant (Pascual *et al.* 2006, 2008a,b, Ruíz *et al.* 2009). Both strains were grown in De Man Rogosa Sharpe (MRS) agar at 37°C, under microaerobic conditions for 24–48 h.

Isolation and identification of pathogenic microorganisms

During a period of 48 months, endocervical samples of *N. gonorrhoeae* isolates were recovered from patients with genital infections, who had been treated in the Gynecology Service at New Río Cuarto Hospital, private assistance centers and Public Hospitals from Córdoba city, in the province of Córdoba, Argentina. These strains were identified by Gram staining followed by standard biochemical tests, and later used as indicator strains (Staley, Brenner and Krieg 2005). They were seeded on Thayer Martin (TM) agar plates (Britania, Argentina), incubated at 37°C with 5% CO_2 for 24 h. Strains were stored at −80°C with sterile Dracron swabs. Each strain was reactivated by re-culturing once in TM plates prior to experiments and then directly seeded on respective agar medium.

Antibiotic susceptibility testing and detection of β-lactamase enzyme of *N. gonorrhoeae* strains

Antibiotic susceptibility tests were performed on all *N. gonorrhoeae* strains using the method of disk diffusion in accordance with CLSI recommendations for clinical isolates. The antibiotics used for susceptibility testing were penicillin *PEN* (10 IU), ceftriaxone (30 μg), cefixime (5 μg), ciprofloxacin *CPX* (5 mg), tetracycline *TCN* (30 μg), spectinomycin *SPC* (100 μg) and doxycycline *DOX* (30 μg) (CLSI 2009; Unemo *et al.* 2012). Gonococci suspensions from pure cultures were adjusted to a turbidity of 0.5 McFarland scale in sterile distilled water. Then TM agar plates supplemented with 5% defibrinated horse blood and 20 mg L^{-1} of β-nicotinamide adenine dinucleotide were seeded using sterile swabs with each gonococci suspension, and antibiotic disks were placed on these surfaces (Britania, Argentina). Plates were incubated at 37°C with 5% CO_2 for 18–24 h. Inhibition zones were measured and rated as susceptible, intermediate or resistant (Singh *et al.* 2012). All confirmed *N. gonorrhoeae* isolates were tested for β-lactamase production using β-lactamase identification strips (Oxoid Ltd, Hampshire, UK) to detect penicillinase-producing *N. gonorrhoeae* (Llanes *et al.* 2003).

Antimicrobial activity testing of the bioactive substances

Inhibitory effects of each bioactive substances contained in the cell-free supernatant (CFS) of these lactobacilli strains on the *N. gonorrhoeae* strains, such as organic acids, H_2O_2 and of each BLIS elicited from them, were evaluated by well-diffusion test on agar plates. To obtain the different CFSs of each *Lactobacillus* strain, a procedure previously described was carried out (Ruíz *et al.* 2009, 2012). Thus, pure or non-treated CFS (P-CFS) and neutralized CFS (N-CFS) or BLIS L23 of L23 strain were obtained. For the L60 strain, three CFSs were obtained as follows: P-CFS, N-CFS and a neutralized and treated with peroxidase enzyme CFS, called BLIS-L60. Surfaces of TM agar plates were seeded with a sterile swab from a standardized suspension of each clinical isolate of *N. gonorrhoeae* (equivalent to a concentration of 1.5×10^8 CFU ml^{-1}) in TSB broth. Then in three cut wells made on these agar plates, 100 μl of the pure CFS of L60, 100 μl of the CFS neutralized with NaOH 1 N (N-CFS) and 100 μl of the BLIS L60 were dispensed. Similarly for the L23 strain, 100 μl of the pure CFS and 100 μl of the BLIS L23 were dispensed on other cut wells. Plates were incubated for 24 h at 37°C under microaerobic conditions. Antimicrobial activities of each metabolite on bacterial growth of *N. gonorrhoeae* were measured as described previously (Ivanova *et al.* 2000).

Determination of the MIC for BLIS

MICs of each BLIS, L23 and L60 were determined by agar well-diffusion method (Principe *et al.* 2009). Maximum antimicrobial activity of these two BLIS-es elicited from *L. fermentum* L23 and *L. rhamnosus* L60 (data unpublished) were of 640 activity units (AU ml^{-1}), respectively (Pascual *et al.* 2008a,b). A total of 2-fold serial dilutions of each BLIS were made in sterile MRS broth and the following concentrations of the BLIS-es, L23 and L60: 320, 160, 80, 40, 20, 10, 5 AU ml^{-1} were tested. TM agar plates were seeded with gonococci suspensions previously adjusted to a turbidity of 0.5 McFarland standard scale (1.5×10^8 CFU ml^{-1}) in sterile phosphate-buffered saline (PBS). On the surface of those agar plates seven wells were made, each inoculated with 100 μl of the BLIS's serial dilutions, and incubated at 37°C with 5% CO_2 for 18–24 h. As a negative control, a well was inoculated with sterile MRS broth. Inhibition zones of each BLIS-es were measured and the MICs of both BLIS-es were defined as the absolute value and reciprocal of the lowest concentration at which the growths of the *N. gonorrhoeae* strains were inhibited (Ruíz 2013).

Test of interactions between BLIS

A qualitative diffusion method on agar plates was carried out by an adaptation of a technique previously described (Ruíz *et al.* 2007, 2012). Gonococcal suspensions in PBS solution (adjusted to turbidity of 0.5 of the McFarland scale, equivalent to a concentration of 1.5×10^8 CFU ml^{-1}) were seeded with a swab on TM agar plates. Subsequently, two cut wells were made close to each other on the surface of the agar plate. A 100 μl aliquot of each BLIS from L23 and L60 was dispensed on respective cut wells. Plates were incubated for 24 h at 37°C under microaerobic conditions. The inhibition zone produced by two BLIS-es was compared with a control of inhibition of each of the BLIS-es independently. Interactions were interpreted based on the shape of the inhibition zone as follows: (1) a synergistic effect is present when the inhibition zone formed in the area of diffusion of both bacteriocins is larger than the zone for each of the BLIS-es independently, (2) an antagonistic interaction results when the inhibition zone formed in the area of diffusion of both BLIS-es is smaller than the zone for each of the BLIS-es independently and (3) an indifferent effect results if, in the zone of diffusion of both BLIS-es, the inhibition zone of the bacterial growth remains with the same size as compared with the inhibition zone of each BLIS alone (Ruíz *et al.* 2012).

Statistical analysis

All tests were performed in triplicate and mean ± SD were calculated. Differences in inhibitory activities between bacteriocin-producing strains were analyzed by ANOVA ($P < 0.05$), using Sigma Stat Statistical Software, version 3.05, SPSS Inc., Chicago, IL, USA. The Fisher's LSD test was used to determine statistically significant differences between the BLIS-es, BLIS L23 and BLIS L60. A α-value $\leq$0.05 was considered statistically significant, using InfoStat Software, version 2008, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina.

RESULTS

1. Gonococcal isolation, identification and β-lactamase enzyme production.
   A total of 31 gonococci strains were isolated from several female patients with whitish purulent vaginal discharge suspected of *N. gonorrhoeae* infection, during a period of 48 months. All of the strains were correctly identified as *N. gonorrhoeae* strains by biochemical tests according to the recent recommendations (Staley, Brenner and Krieg 2005; Workowski, Berman and CDC 2010). A proportion of 38% ($n = 12$) of these pathogens were β-lactamase-producing *N. gonorrhoeae* strains and therefore resistant to penicillin and ampicillin.
2. Inhibitory activity on gonococcal strains of organic acids, hydrogen peroxide and the BLIS-es L23 and L60 present in different CFSs of each *Lactobacillus*.
   The inhibitory activities of the P-CFS of both lactobacilli strains on all *N. gonorrhoeae* strains tested were evaluated by the agar well-diffusion technique. Inhibition zones on the growth of the gonococci strains, expressed as the means in mm ± SD, were as follows: 18.33 mm ± 2.48 and 19.01 mm ± 2.69 for the P-CFS of *L. fermentum* L23 and *L. rhamnosus* L60, respectively. A 100% of the gonococci studied were susceptible to the metabolites contained in the CFSs of each *Lactobacillus*. The inhibition produced by P-CFS of *L. fermentum* L23 is due to the combined action of the organic acids and the BLIS L23. In the case of P-CFS of *L. rhamnosus* L60, the inhibitory activity is attributed to the joint action of three synthesized bioactive substances, namely organic acids, H_2O_2 and its BLIS L60. Antimicrobial effects findings with each P-CFS on the growth of gonococcal strains did not show statistically significant differences ($P < 0.05$). Similarly, the inhibitory effects of N-CFSs of both lactobacilli, as well as, CFS neutralized and treated with peroxidase (NP-CSF) of *L. rhamnosus* L60, were also evaluated. In the first case, the N-CFS of both lactobacilli inhibited 100% of gonococci tested. For the L23 strain, this inhibitory activity was directly produced by the BLIS L23, while in the case of L60 strain, the antimicrobial effect was attributed to the combined action of H_2O_2 and the BLIS L60. The average size of the inhibition zones of bacterial growth for the N-CFS of L60 strain was 16.93 mm ± 2.44 for all gonococci tested. Secondly, when the inhibition produced by supernatants containing the BLIS-es of each lactobacilli (N-CFS of L23 strain and NP-CFS of L60 strain) were evaluated, 100% of all gonococci strain isolated were susceptible, with average sizes of inhibition zones of 15.81 mm ± 2.21 and 15.01 mm ± 2.13 for BLIS L23 and BLIS L60, respectively (Table 1; Figs 1 and 2). This inhibitory effect produced by the BLIS-es L23 and L60 on the growth of all gonococcal strains tested did show statistically significant differences ($P < 0.05$).
3. Proportion of inhibitory effect produced by each bioactive substance on gonococcal strains.
   Based on the size of the inhibitory effect found with each bioactive metabolite contained in the different CFSs of lactobacilli, it was possible to estimate in what proportion the organic acids, each of the BLIS-es and H_2O_2 were responsible of the antimicrobial activity observed on the growth of gonococci tested. Consequently, 87.28% of the inhibitory effect produced by N-CFS of *L. fermentum* L23 on all gonococci studied corresponded to the action of the BLIS L23, and the remaining 12.72% was by the organic acid released by this strain of *Lactobacillus*. For the case of *L. rhamnosus* L60, 80.66% of the inhibition produced on the growth of all gonococci strains was due to the BLIS L60, followed by percentage values of 10.20% and 9.14% corresponding to the antimicrobial action of organic acids and H_2O_2 released, respectively.
4. MICs of the BLIS on the growth of *N. gonorrhoeae*.
   MIC of both BLIS-es showed wide ranges of values of inhibitory activity on all gonococci strains. The MIC range of BLIS L23 of *L. fermentum* L23 was 10 AU ml^{-1} for 5 susceptible gonococci tested (16.13%), 20 AU ml^{-1} for 7 susceptible strains (22.58%), 40 AU ml^{-1} for 7 susceptible strains (22.58%), 80 AU ml^{-1} for 10 susceptible strains (32.26%) and for the other 2 gonococci strains (645%) was 160 AU ml^{-1}. These results proved that the BLIS L23, even when very diluted, had a strong antimicrobial activity inhibiting to 93.55% gonococci strains (29 susceptible strains) with an MIC range between 10 and 80 AU ml^{-1}. On the other hand, the MIC range of BLIS L60 was 10 AU ml^{-1} for 5 susceptible gonococci tested (16.13%), 20 AU ml^{-1} for 3 susceptible strains (9.67%), 40 AU ml^{-1} for 4 susceptible strains (12.90%), 80 AU ml^{-1} for 12 susceptible strains (38.71%) and for the remaining 7 gonococci strains (22.59%) was 160 AU ml^{-1}. Thus, the BLIS L60 inhibited 77.41% of gonococci tested (24 susceptible strains) with an MIC range between 10 and 80 AU ml^{-1}, while a lower number of gonococci studied were inhibited by an MIC value of 160 AU ml^{-1} (Table 2, Fig. 3).
5. Interaction between the BLIS-es L23 and L60 on the growth of gonococci.

By a qualitative test performed to study the types of interaction between both BLIS-es, it was determined that these substances showed 100% of synergism in inhibiting the growth of all gonococci tested (Fig. 4). Other types of interactions between the BLIS-es L23 and L60 such as antagonism or indifference were not found.

DISCUSSION

For many years in Argentina, the population incidence of *N. gonorrhoeae* infection was quite low (<1–4%) (Di Bartolomeo *et al.* 2002; García *et al.* 2008; SNVS 2008). In this study, the number of gonococci strains recovered from the patients was very meaningful, as in some provinces of our country and, particularly in Córdoba, as reported by the last surveillance studies from 2010 to 2012, there was an alarming increase in the rate of infection with this pathogen. In fact, there was an exponential increment in the infection rate ranging from 0.94 (2010) to 1.24 (2011), and reaching 4.74 (2012) per 1 000 000 inhabitants (SNVS 2012, 2013). Some studies on gonococci infections have established various relationships among predisposing factors such as economic and social characteristics, sexual behavior, migratory flows and the augmentation in gonococci infection rates seen worldwide (Trevisan *et al.* 2008; Folch *et al.* 2009).

Table 1. Antimicrobial activity of organic acids, hydrogen peroxide and BLIS produced by *L. fermentum* and *L. rhamnosus* on the growth of *N. gonorrhoeae*.

Strains tested	Average size (mm) ± SD of inhibition zones of growth of gonococci strains				
	L. fermentum L23		*L. rhamnosus* L60		
	P-CFS	BLIS L23	P-CFS	N-CFS	BLIS L60
1	19.7 ± 1.5	17.7 ± 0.5	18.7 ± 0.5	17.0 ± 0.0	14.7 ± 0.5
2	23.3 ± 1.1	21.0 ± 1.0	25.3 ± 0.5	21.0 ± 2.0	18.7 ± 1.5
3	22.3 ± 2.5	18.0 ± 1.7	22.0 ± 2.0	19.7 ± 0.5	17.7 ± 1.1
4	18.7 ± 1.1	17.0 ± 0.0	16.7 ± 1.5	14.0 ± 1.0	13.0 ± 1.0
5	16.0 ± 2.0	14.0 ± 1.0	15.3 ± 0.5	14.0 ± 1.0	13.0 ± 0.0
6	18.3 ± 0.5	15.7 ± 1.1	16.7 ± 1.5	15.0 ± 1.0	14.0 ± 1.0
7	21.0 ± 1.0	18.7 ± 0.5	19.7 ± 1.5	17.7 ± 0.5	16.0 ± 1.0
8	14.0 ± 1.0	13.0 ± 0.0	16.0 ± 1.0	15.0 ± 1.0	14.0 ± 1.0
9	19.0 ± 1.0	15.3 ± 0.5	19.7 ± 0.5	18.3 ± 0.5	16.7 ± 0.5
10	14.3 ± 1.5	13.0 ± 1.0	16.0 ± 2.0	15.0 ± 1.0	14.0 ± 1.0
11	14.3 ± 0.5	13.0 ± 0.0	15.7 ± 1.5	14.0 ± 1.0	13.0 ± 1.0
12	17.0 ± 2.0	16.0 ± 1.0	15.7 ± 2.5	13.0 ± 2.0	12.3 ± 2.0
13	20.7 ± 2.0	17.7 ± 1.5	14.0 ± 0.0	13.0 ± 1.0	12.0 ± 1.0
14	17.7 ± 0.5	15.7 ± 0.5	18.7 ± 1.5	17.0 ± 1.0	15.0 ± 0.0
15	17.0 ± 2.0	16.0 ± 1.0	19.7 ± 2.0	18.0 ± 2.0	16.0 ± 1.0
16	18.7 ± 0.5	16.3 ± 0.5	22.0 ± 1.0	18.7 ± 0.5	17.0 ± 0.0
17	17.7 ± 0.5	16.0 ± 0.0	21.3 ± 0.5	17.7 ± 0.5	15.3 ± 0.5
18	18.3 ± 0.5	16.7 ± 0.5	19.3 ± 1.5	17.0 ± 1.0	14.3 ± 0.5
19	19.7 ± 2.9	17.0 ± 1.0	21.0 ± 1.0	19.0 ± 1.0	16.7 ± 0.5
20	19.3 ± 1.5	16.7 ± 0.5	19.0 ± 1.0	17.7 ± 0.5	15.7 ± 0.5
21	19.0 ± 1.0	16.3 ± 1.1	19.7 ± 0.5	18.3 ± 0.5	16.7 ± 0.5
22	20.7 ± 2.0	17.0 ± 1.0	21.7 ± 1.5	19.7 ± 0.5	17.3 ± 1.1
23	18.0 ± 1.0	15.0 ± 1.0	20.3 ± 1.1	18.7 ± 0.5	16.3 ± 0.5
24	17.3 ± 0.5	15.0 ± 1.0	23.3 ± 1.5	21.3 ± 1.5	19.3 ± 2.3
25	19.7 ± 0.5	16.3 ± 0.5	18.7 ± 0.5	16.7 ± 0.5	14.3 ± 0.5
26	16.7 ± 0.5	13.3 ± 1.1	18.3 ± 1.1	16.7 ± 0.5	14.7 ± 0.5
27	17.3 ± 0.5	14.3 ± 0.5	19.0 ± 0.0	17.3 ± 0.5	15.7 ± 0.5
28	16.3 ± 1.1	13.3 ± 0.5	18.7 ± 0.5	16.3 ± 1.1	14.0 ± 0.0
29	20.3 ± 0.5	19.6 ± 1.5	19.6 ± 1.5	18.0 ± 1.7	15.0 ± 1.0
30	19.6 ± 0.5	18.6 ± 1.5	19.6 ± 1.5	17.3 ± 1.5	15.3 ± 0.5
31	18.0 ± 0.5	17.0 ± 1.0	18.0 ± 1.0	16.0 ± 1.0	15.0 ± 1.0

P-CFS: pure cell-free supernatant; BLIS L23: bacteriocin-like inhibitory substance L23 produced by *L. fermentum* L23; N-CFS: cell-free supernatant neutralized with NaOH 1 N; BLIS L60: bacteriocin-like inhibitory substance L60 produced by *L. rhamnosus* L60.

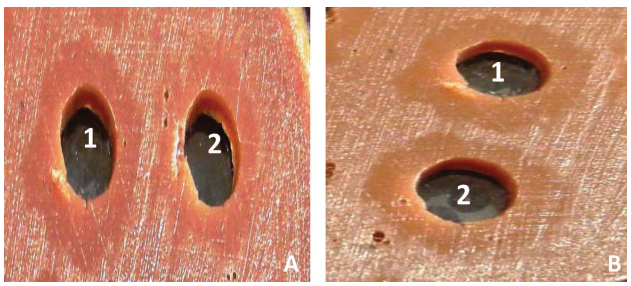

Figure 1. Inhibitory effect produced by the CFSs of *L. fermentum* L23 on the growth of different gonococcal strains. 1: P-CFS pure cell-free supernatant of *L. fermentum* (combined action of organic acid and BLIS L23), 2: BLIS L23 bacteriocin-like inhibitory substance L23 of *L. fermentum*, A, B: examples of two different gonococcal strains.

In relation to the antibiotic profile of gonococcal strains, two conclusions were reached. First, the possible loss of susceptibility to the most common therapeutic agents and second, its correlation with the two last antibiotic options (ceftriaxone or cefixime) recommended by the CDC for the treatment of uncomplicated gonococcal infections, that were later adopted as reference in many other Latin American countries. Presently, there are scant records of antibiotic resistance in gonococci strains available in our country. In fact, those few studies do not necessarily provide a national picture, especially because of the occurrence of cases that are not reported. In this study, the proportion of β-lactamase-producing gonococci strains belonging to this central region was very similar to the percentage values reported in other regions of Argentina and other Latin American countries (Méndez *et al.* 2008; Galarza *et al.* 2012; Starnino *et al.* 2012; Ferreira *et al.* 2013). Moreover, in this study, none of the gonococci strains tested was resistant to the extended-spectrum cephalosporins (ESCs), ceftriaxone and cefixime. These results partially agreed with those obtained by Pagano *et al.* (2012), who evaluated the susceptibility of gonococci strains to these ESCs. Although they found susceptibility to ceftriaxone, an elevated percentage of gonococci showed diminished susceptibility to cefixime. According to this trend of antibiotic resistance or diminished susceptibility of the gonococci strains, the loss of one of these two ESCs currently available in Argentina could happen in a short time. However, in other studies from Brazil, Chile and Venezuela, given the susceptibility to ceftriaxone and cefixime, these antibiotics continue to be a therapeutic option for infections produced by gonococcal strains (Flores Fernández, Márquez Planché and Albarado Ysasis 2012; Costa *et al.* 2013; ISPCH 2013).

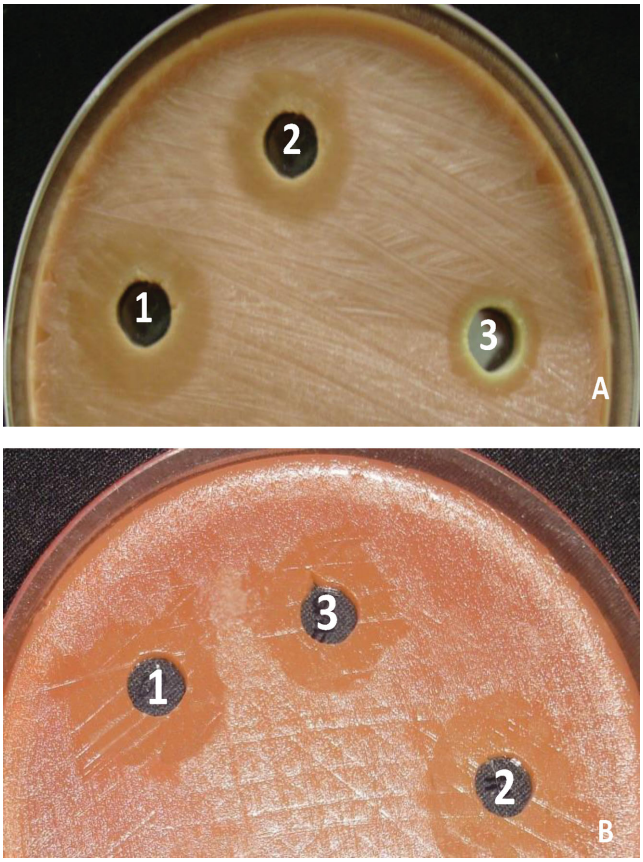

Figure 2. Inhibitory effect produced by the CFSs of *L. rhamnosus* L60 on the growth of different gonococcal strains. 1: P-CFS pure cell-free supernatant of *L. rhamnosus* (combined action of organic acid, hydrogen peroxide and, BLIS L60), 2: N-CFS cell-free supernatant neutralized of *L. rhamnosus* (action of H_2O_2 and BLIS L60), 3: BLIS L60: bacteriocin-like inhibitory substance L60, A, B: two different gonococcal strains.

In this work, the antimicrobial activities produced by all bioactive substances contained in the supernatants of lactobacilli on the growth of gonococci strains were partially similar to the results of Castro and Rovetto (2006). Although these authors evaluated other antimicrobial substance-producing lactobacilli strains, they found inhibition of growth of several urinary pathogens and a lower number of gonococci strains. The results obtained with each neutralized supernatant of L23 and L60 strains differed from the findings of Juarez Tomas *et al.* (2003). These authors tested the effect of pure and neutralized supernatants of *L. brevis* and *L. acidophilus* on the growth of gonococci, and did not observe any antimicrobial activity in those neutralized fractions, indicating that the inhibitory effect was only due to the organic acids. In contrast, the results obtained in this study with neutralized supernatants partially coincided with those of Amant, Valentin-Bon and Jerse (2002). These investigators employed neutralized supernatants of *L. jensenii* and *L. crispatus,* both H_2O_2-producing lactobacilli strains, and determined that even without the organic acids action, the antimicrobial inhibition observed on the gonococci growth was exclusively produced by the H_2O_2 contained in the supernatants.

The inhibition produced by the BLIS-es L23 and L60 on all the gonococci tested was significantly more representative than the findings previously reported by Ocaña, Pesce de Ruiz Holgado and Nader Macías (1999) and Morency *et al.* (2001). Those studies evaluated the antimicrobial activity of different bacteriocins produced by non-lactobacilli strains, such as nisin (A, Z), mutacins (A, B, I, J, T), epidermin and also of a BLIS produced by *L. salivarius,* and found a lower inhibitory effect on the growth of several microorganisms, including a much reduced number of gonococci strains.

We considered that the high inhibitory activity produced by each of the supernatants, pure CFSs of both lactobacilli, N-CFS of L23 and L60, and the NP-CFS of L60 on the growth of gonococci was of relevance in comparison to a recent study performed by Graver and Wade (2011). In that research, the inhibitory activity of supernatants of three ATCC lactobacilli strains, namely *L. crispatus*, *L. gasseri* y *L. jensenii*, described as strong, weak H_2O_2-producing strains and a non-H_2O_2 producing strain, respectively, was evaluated on gonococci growth. The authors determined that the inhibitory activity against the gonococci strains was only due to the organic acids produced by those lactobacilli and not to H_2O_2 released by *L. crispatus* and *L. gasseri,* respectively, which differs with the results obtained in our study with respect to H_2O_2-producing strain *L. rhamnosus* L60. In addition, the results obtained with our lactobacilli strains showed that antimicrobial activity against the gonococci tested was due to BLIS-L23 and the organic acid of strain L23, while for the L60 strain the inhibition was not only attributed to BLIS-L60 but also to the organic acids and H_2O_2 released by this microorganism. These findings of inhibitory activity produced by each bioactive substance contained in the CFSs of our lactobacilli are of great importance. We believe this to be so because there are only a few reported studies that show that inhibition of gonococci growth, besides it being typically due to effect of organic acids released by lactobacilli, it is also due to other metabolites, such as bacteriocins and H_2O_2. However, although there are many reports on bacteriocins produced by bacteria of different genera, including *Lactobacillus*, there are no reports that specifically focus on biological control of gonococci by bacteriocin-producing lactobacilli strains.

Our results highlight the BLIS-es of both lactobacilli, BLIS-L23 and BLIS-L60, as the two secondary metabolites with higher percentages of inhibition of gonococci growth, compared to other studies that show that the main substances with inhibitory activity on *N. gonorrhoeae* were the organic acids and, in some cases, H_2O_2. Furthermore, to our knowledge, this is the first study where the antimicrobial activities of lactobacilli's bacteriocins against a representative number of clinical isolates of gonococci were evaluated. We believe the results of this study to be very novel and of promising potential utility.

Given that the BLIS-es were the main antimicrobial substances active against the gonococci growth; this led us to determine the MIC of both BLIS-es, BLIS-L23 and BLIS-L60. Our findings on the BLIS's MICs showed a wide range of MIC values, which showed that those substances had a strong inhibitory activity on the gonococci strains, even when they were very diluted. These results partially coincide with those of Mota-Meira *et al.* (2000). These authors, who used bacteriocins synthetized by *Streptococcus* spp., observed inhibition to low values of MIC, but on a much reduced number of gonococci.

In previous works performed by our group, we have already studied by different techniques (qualitative and quantitative tests) the possible interactions, not only between the lactobacilli strains but also with the BLIS-es released by them, to inhibit the growth of several pathogenic microorganisms (Ruíz *et al.* 2009, 2012). In this study, to determine the types of interactions between the BLIS-es, a qualitative test was employed with which the synergism between those antimicrobial substances was the

Table 2. MICs of BLIS-es L23 and L60 produced by *L. fermentum* and *L. rhamnosus* on the growth of gonococcal strains.

Gonococcal strains tested	Inhibition zones (mm) of growth of gonococci strains produced by different concentrations of each BLIS L23 and BLIS L60																MIC AU ml^{-1}	
	640 AU ml^{-1}		320 AU ml^{-1}		160 AU ml^{-1}		80 AU ml^{-1}		40 AU ml^{-1}		20 AU ml^{-1}		10 AU ml^{-1}		5 AU ml^{-1}			
	BLIS L23	BLIS L60	BLIS L23	BLIS L60	BLIS L23	BLIS L60	BLIS L23	BLIS L60	BLIS L23	BLIS L60	BLIS L23	BLIS L60	BLIS L23	BLIS L60	BLIS L23	BLIS L60	BLIS L23	BLIS L60
1	16	14	16	12	14	11	14	10	13	10	0	0	0	0	0	0	40	40
2	21	18	18	18	18	16	16	14	15	13	15	12	0	0	0	0	20	20
3	18	17	17	16	16	16	16	15	15	14	14	11	0	0	0	0	20	20
4	17	13	17	12	16	11	15	0	14	0	14	0	0	0	0	0	20	160
5	14	13	13	12	13	12	11	0	0	0	0	0	0	0	0	0	80	160
6	15	14	14	14	13	13	13	11	0	0	0	0	0	0	0	0	80	80
7	17	15	16	14	15	13	13	11	12	0	0	0	0	0	0	0	40	80
8	13	14	13	14	11	13	0	11	0	0	0	0	0	0	0	0	160	80
9	16	16	14	15	13	15	11	14	0	12	0	0	0	0	0	0	80	40
10	13	14	12	13	12	12	0	11	0	0	0	0	0	0	0	0	160	80
11	13	13	14	12	12	11	12	0	11	0	10	0	0	0	0	0	20	160
12	17	12	16	12	14	11	13	11	11	0	0	0	0	0	0	0	40	80
13	16	12	15	12	14	11	14	0	13	0	0	0	0	0	0	0	40	160
14	15	15	14	14	14	14	11	12	0	0	0	0	0	0	0	0	80	80
15	16	12	15	15	14	13	11	13	0	11	0	0	0	0	0	0	80	40
16	16	17	15	14	14	10	11	0	0	0	0	0	0	0	0	0	80	160
17	16	15	14	12	12	10	9	0	0	0	0	0	0	0	0	0	80	160
18	17	14	15	12	12	10	10	8	0	0	0	0	0	0	0	0	80	80
19	16	17	14	16	13	14	10	9	0	0	0	0	0	0	0	0	80	80
20	17	16	14	14	13	11	12	9	11	0	0	0	0	0	0	0	40	80
21	17	17	15	15	13	11	11	8	8	0	0	0	0	0	0	0	40	80
22	20	18	20	17	18	16	16	14	14	11	12	10	0	0	0	0	20	20
23	20	16	19	16	17	15	16	13	13	11	11	0	0	0	0	0	20	40
24	19	19	19	19	18	18	18	18	16	16	15	13	13	11	0	0	10	10
25	21	14	19	14	18	13	18	13	17	12	15	12	11	11	0	0	10	10
26	17	15	16	13	15	13	15	12	14	12	14	11	10	11	0	0	10	10
27	14	16	13	14	13	13	12	12	12	11	11	10	11	10	0	0	10	10
28	14	14	13	13	13	12	13	12	12	11	12	10	10	10	0	0	10	10
29	19	16	18	15	18	13	16	11	14	0	12	0	0	0	0	0	20	80
30	17	15	18	13	16	11	14	0	11	0	0	0	0	0	0	0	40	160
31	16	15	15	14	13	12	11	11	0	0	0	0	0	0	0	0	80	80

AU: arbitrary unit of BLIS; MIC: minimum inhibitory concentration of BLIS L23 and L60, 0 mm: no inhibition zone observed.

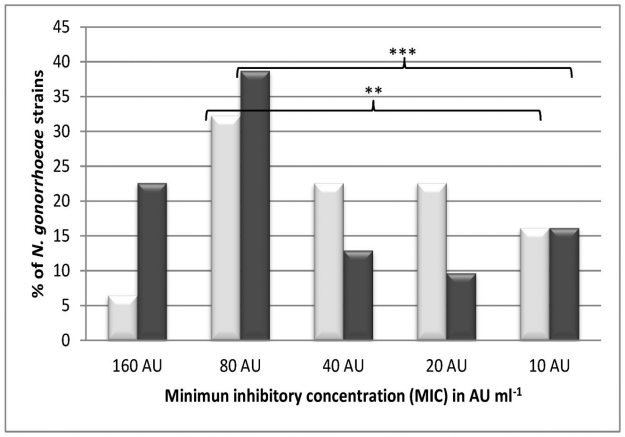

Figure 3. Range of MICs of BLIS-es L23 and L60 on *N. gonorrhoeae* strains. **: more than 93% of the gonococcal strains were inhibited by a MIC range of 10–80 AU ml^{-1} of BLIS L23. ***: 77% of gonococcal strains were inhibited by a MIC range of 10–80 AU ml^{-1} of BLIS L60.

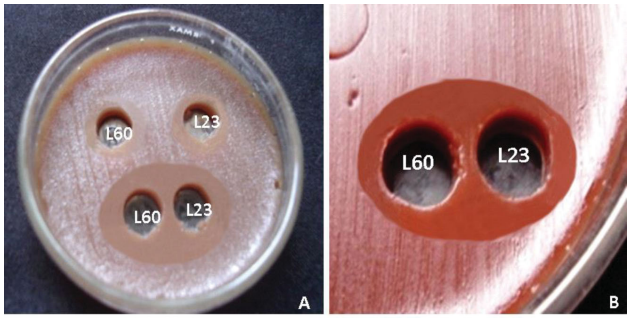

Figure 4. Synergistic interaction between the BLIS-es L23 and L60 on the growth of different *N. gonorrhoeae* strains. L23: bacteriocin-like inhibitory substance L23 produced by *L. fermentum*, L60: bacteriocin-like inhibitory substance L60 produced by *L. rhamnosus*, A, B: different gonococcal strains.

only interaction found against all susceptible gonococci studied. Thus far, despite exhaustive bibliographic searches, we have not found previous reports on the possible interactions of lactobacilli's bacteriocins with specific activity against gonococci growth. Thus, the results obtained in this first work would represent valuable information for the development of biological control strategies for the infections produced by *N. gonorrhoeae*. Our results suggest the potential use of both BLIS-es, L23 and L60 for the design of new antimicrobial substances, and also the combined use of the bacteriocin-producing lactobacilli strain *L. fermentum* and *L. rhamnosus*, which together with other probiotic characteristics previously proven, would positively enhance their antimicrobial effects against *N. gonorrhoeae*.

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Conflict of interest statement. None declared.

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