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Synergistic Effect Between Two Bacteriocin-like Inhibitory Substances Produced by Lactobacilli Strains with Inhibitory Activity for *Streptococcus agalactiae*

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Abstract Group B streptococci (GBS) are bacterial species that colonize the vagina in pregnant women and as such may cause serious infections in neonates that passed through the birth channel. The objective of this work was to study the inhibitory activities produced by each bacteriocin-like inhibitory substance (BLIS) of *Lactobacillus rhamnosus* L60 and *Lactobacillus fermentum* L23, and the effects of the combined BLIS-es of these lactobacilli on GBS. The interactions between the BLIS-es were assessed by qualitative and quantitative methods on agar plates. The minimum inhibitory concentrations (MICs) and fractional inhibitory concentrations (FICs) were determined by a modification of the broth microdilution and checkerboard methods, respectively. Antibiotic susceptibilities of all *S. agalactiae* strains were assayed and the results of these tests were evaluated for statistical significance. A 7.5% of GBS isolates were recovered from 760 pregnant women and 91% of those strains were susceptible to each BLIS produced by *L. fermentum, L. rhamnosus*, and also to a mixture of them. The comparisons among the BLIS-es showed statistically significant differences, with a combination of the BLIS-es from the two *Lactobacillus* species being better than the BLIS of each one alone ($P < 0.05$) as GBS growth inhibitors. Synergistic activities between the BLIS-es were found on 100% of susceptible GBS strains, MICs ranges of BLIS of L23 and L60 were 80–160 and 160–320 UA ml^{-1}, respectively. By the checkerboard method, the BLIS-es combination showed synergistic effect on all sensitive strains tested, with values of FICs ranging from 0.131 to 0.218. The BLIS-es produced by these lactobacilli of vaginal origin were able to inhibit *S. agalactiae* isolates. The results indicate that these strains may have probiotic potential for the control of GBS in women and may consequently prevent GBS infections in newborns.

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Introduction

Streptococcus agalactiae belonging to Lancefield's Group B streptococci (GBS) is a significant cause of morbidity and mortality among newborns [11, 16]. *S. agalactiae* is a commensal bacterium of the human gastrointestinal and genital tracts and recent studies have reported asymptomatic colonization rates of up to 36% in healthy women [14, 16, 20, 39]. Many international organizations recommend that all pregnant women must be screened for vaginal colonization at 34–37 weeks of gestation. In Argentina, this prevalence varies widely between geographic areas and even between different populations. For instance, there is a report of a maternal colonization rate of 1.4 and 18.1% in the genitourinary or lower gastrointestinal tract [6]. Moreover, it represents the major cause of bacterial infections in newborns [11].

This bacterium is part of the vaginal microbiota of many women and is therefore strategically located to cause serious infections in neonates, whose immune response is less developed than that of older children and adults. Newborns acquire the microorganisms at delivery from their mothers, who are colonized with GBS in the genital tract [14].

The therapeutic strategies that are currently recommended by the Centers for Disease Control and Prevention based on the risk and/or screening and have contributed to a significant decline in the prevalence of neonatal GBS infection [7].

When lactobacilli are reduced, eliminated, or replaced by pathogenic species, the host has an increased susceptibility to urinary tract infections, and/or genital tract infections [35]. An alternative strategy of biological control for GBS infections could be the use of lactobacilli with strong beneficial properties for human or animal health. Some lactobacilli play a protective role by producing compounds, such as hydrogen peroxide (H_2O_2), lactic acid, and bacteriocins, which inhibit the growth of potential pathogens [25, 26].

Bacteriocins are defined as proteinaceous antimicrobial substances, produced by bacteria that inhibit growth of related or unrelated bacterial species. Bacteriocins are potentially useful for prevention, or as a complement to antibiotic treatment of bacterial infectious diseases [26]. Bacteriocins of lactobacilli have potential applications as inhibitors of pathogens in humans [33, 35].

Two human vaginal lactobacillus strains, *Lactobacillus fermentum* strain L23 and *L. rhamnosus* strain L60, were previously identified and characterized by our group as having probiotic properties and producing bacteriocin-like inhibitory substances [26–28, 35]. The purpose of this work was to study the inhibitory activities of each bacteriocin-like inhibitory substance (BLIS) of *L. fermentum* L23, *L. rhamnosus* L60, and the interaction between the BLIS-es from these lactobacilli, on SGB.

Materials and Methods

Lactobacilli Strains, Cultivation and Identification

Lactobacillus fermentum strain L23 and *L. rhamnosus* strain L60 have been well identified by standard biochemical tests [5], the API 50 CHL system (BioMèrieux, Inc, France) [24], and 16S rRNA analysis [8, 15, 29], and have been the subject of extensive in vitro experimentation. The bacterial sequencing of 16S r-DNA of both lactobacilli was deposited in GenBank according to the last recommendation of FAO/WHO during 2001 [18]. The GenBank accession no. GQ 455406 and GenBank accession no. EF 495247 have been assigned for *L. fermentum* L23 and *L. rhamnosus* L60, respectively. They were selected by our group as a 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 [2, 26–28, 35]. Both strains were grown in De Man Rogosa Sharpe (MRS) agar [10, 34] at 37°C, under microaerobic conditions for 24 h. They were stored at −80°C in MRS broth containing 30% (v/v) glycerol. For re-activation prior to experiments, they were re-cultured twice in MRS broth (BioMèrieux, Inc).

Clinical Isolates

Streptococcus agalactiae isolates was recovered from pregnant women during a period of 24 months; women were patients of the Gynecology Service at New Río Cuarto Hospital, Córdoba, Argentina. These strains were identified by Gram staining followed by standard biochemical tests and, in addition, by Group B-specific latex agglutination (Slidex Strepto-Kit, BioMérieux, Marcy l'Etoile, France) [5], and later used as indicator strains. They were seeded on 5% sheep blood agar plates (BioMèrieux, Inc), incubated at 37°C with 5% CO_2 for 24 or 48 h. Strains were stored at −80°C in tryptic soy broth (TSB) (Merck, Germany) containing 30% (v/v) glycerol. Each strain was reactivated by re-culturing once in TSB broth prior to experiments and then was seeded directly on respective agar medium.

Bacteriocin-like Inhibitory Substances (BLIS-es)

As was described in previous studies by our group, BLIS of each *Lactobacillus* was characterized by proteases, heat sensitivity, and pH stability; and purified through different steps. After these treatments, the antimicrobial activity of the BLIS-es containing supernatants were tested with the plate diffusion assay [25, 26, 35].

L. fermentum L23 and *L. rhamnosus* L60 strains were cultured in MRS broth 37°C with 5% CO_2 for 24 h and the supernatants were removed by ultracentrifugation (4,000×*g* at 4°C for 20 min). To obtain the BLIS, these fractions with biological activity were neutralized by the addition of 1 M NaOH and 0.1 mg ml^{-1} peroxidase (Sigma) to eliminate the inhibitory effects attributed to the organic acids in both strains as well as the hydrogen peroxide produced by the L60 strain [2, 35].

Test of Antimicrobial Activity

Inhibitory effects of the BLIS of L23 and L60 on GBS strains were evaluated by well diffusion test on agar plates [1, 35]. Surfaces of MRS agar plates were seeded with a

standardized suspension in TSB broth of each clinical isolate of *S. agalactiae* (adjusted to turbidity of 0.5 of the McFarland scale, equivalent to a concentration 1.5×10^8 CFU ml^{-1}). A volume of 100 µl of each BLIS-containing supernatants was dispensed on the cut wells of the agar plates and incubated for 24 h at 37°C under aerobic conditions. Antimicrobial effects on bacterial growth of *S. agalactiae* were measured as described previously [19]. A positive control of the inhibitory activity of both BLIS-es was tested using an *E. coli* strain as indicator microorganism.

Test of Interactions of Bacteriocin-like Inhibitory Substances

Two qualitative and semi-quantitative methods on agar plates were carried out. For the first one, a suspension in TSB broth of *S. agalactiae* (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 MRS 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 in microaerobic conditions. Interactions were interpreted based on the shape of the inhibition zone as follows: (1) a synergistic effect is described 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 antagonist 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, (3) an indifferent effect results if in the zone of diffusion of both BLIS-es the inhibition zone of the bacterial growth remain with the same size to the inhibition zone of each BLIS. To semiquantify the interactions, a procedure was carried out as follows. On the surface of MRS plates, three wells were cut and 100 µl of BLIS L23 (50 µl of L23 + 50 µl of PBS sterile), 100 µl of BLIS L60 (50 µl of L60 + 50 µl of PBS sterile) and 100 µl of a combination of BLIS-es were dispensed in them, respectively. Plates were incubated as described above and the inhibition zones were measured. Size of the inhibition zones was expressed in millimeter. These experiments were performed in triplicate. The interactions were interpreted as follows: (1) when the diameter of the inhibition zone produced by the mixture of both BLIS-es was >2 mm than those from each independent BLIS it was considered the result of a synergistic effect. (2) If there was a reduction in the inhibitory effect, as a consequence of the interaction produced by the mixture of BLIS-es, in comparison to the inhibitory effects observed for each BLIS alone, it was regarded as an antagonism; (3) an indifferent interaction results when the diameter of the inhibition zone of the mixture of BLIS-es was equal to the size of those inhibitions produced per each BLIS [3, 25].

Determination of BLISs Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentrations were determined by a modification of the broth microdilution method [31]. From an overnight culture of the bacteria to be tested, the optical density (OD) was adjusted to a turbidity of 0.5 in the McFarland standard scale. Twofold serial dilutions of each BLIS were made in MRS broth. In previous studies, our group found an antimicrobial activity of 640 activity units (AU ml^{-1}) for the BLIS from *L. fermentum* L23 [26] and *L. rhamnosus* L60 (data not published). Each microtiter well was inoculated with 100 µl of the BLISs serial dilutions. 25 µl of bacterial suspension was added to each well to a final volume of 125 µl and the plate was incubated at 37°C for 24 h under aerobic conditions. An *E. coli* strain (indicator) was used as a positive control and MRS broth was used as a negative control. The MICs of both BLIS-es were calculated from the lowest concentration at which the growths of the GBS strains were inhibited. The determination of MIC of BLIS-es is based on a colorimetric assay using the reactive 2,3,5 triphenyl tetrazolium chloride (TTC).

Checkerboard Method

Bacteriocin-like inhibitory substances interactions were determined using the checkerboard assay as previously described by Petersen et al. [30]. The BLIS-es initial concentrations used in this experiment were at least double the MIC. Serial dilutions of the BLIS of L23 and L60 along the ordinate and abscissa were made, respectively, in MRS broth. 75 µl of each BLISs dilution was distributed into each well of the microdilution plates and 25 µl of a GBS suspension equal to a 0.5 McFarland turbidity standard was added in a 150 µl final volume. Microplates were incubated at 37°C for 24 h under optimal conditions. After this period, 10 µl of a 5% tetrazolium solution was dispensed into wells and re-incubated for another 2 h under the same conditions. Both positive and negative controls were included as described above. The fractional inhibitory concentration (FIC) index ($\sum$FICs) was calculated as follows: $\sum FIC = FIC\ A + FIC\ B$, 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, $FIC \leq 0.5$; indifference, $0.5 < FIC < 2$; antagonism, > 2.

Antibiotic Susceptibility Testing

Kirby–Bauer disk diffusion tests were performed for each of the clinical isolates according to the methods recommended by CLSI (formerly NCCLS) using Mueller–Hinton agar plates supplemented with 5% sheep blood (Britania, Argentina). Isolates were tested for susceptibility to penicillin (PEN), ampicillin (AM), oxacillin (OXA), gentamicin (GEN), cephalotin (CET), erythromycin (ERY) and clindamycin (CLIN). Classification as "susceptible", "intermediate" or "resistant" was based on the CLSI-recommended breakpoints for inhibition zone diameters [4, 23].

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 ($P < 0.05$). A two-way ANOVA to evaluate the interaction between strains and treatments was used. The mean separation was performed using the Holm–Sidak test ($P < 0.001$) 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 treatments. A α-value ≤ 0.05 was considered statistically significant, using InfoStat Software, version 2008, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina.

Results

A total of 57 *S. agalactiae* strains (7.5%) were isolated from 760 pregnant women at 35–37 weeks of gestation during 36 months. A proportion of 91.23% of *S. agalactiae* strains was susceptible to each BLIS produced by *L. fermentum* L23, and *L. rhamnosus* L60, subsequently referred to as treatments A and B, respectively. Inhibition zones on the growth of the susceptible strains (means in mm ± SD) were as follows: 14.70 ± 1.42, 15.00 ± 1.16, for A and B treatments, respectively. The antimicrobial effects produced by the BLIS-es of both lactobacilli against GBS' strains did not show statistically significant differences ($P < 0.05$). Variations of the inhibition zones measured on the bacterial growth with each type of BLIS were considered. Thus, the 52 strains showed different degree of susceptibility, but all of them were sensitive to each BLIS from *L. fermentum* L23, and *L. rhamnosus* L60.

By both qualitative and semi-quantitative methods, a synergistic interaction between the substances was shown. On 100% of susceptible *S. agalactiae* strains, synergistic activities between the BLIS-es by a well diffusion method were found. This synergistic effect between the bioactive agents was seen as a clear ovoid zone of inhibition on growth in agar plates (Fig. 1). To quantify this synergistic effect, measurements expressed in millimeter were made (Table 1). A synergistic effect was observed with inhibition zones >2 mm compared with each antimicrobial activity of BLIS-es. There was neither an indifferent nor an antagonistic interaction between the substances evaluated either by qualitative or semi-quantitative method (Fig. 2).

Fig. 1 Synergistic effect between BLIS-es of *Lactobacillus fermentum* and *Lactobacillus rhamnosus* on the growth of *Streptococcus agalactiae* by a qualitative test

The MIC range of BLIS of *L. fermentum* L23 was 80 UA ml^{-1} for 23 strains, which represented 44% of the susceptible GBS tested, and for the other 29 strains (56%) was 160 UA ml^{-1}. On the other hand, the MIC range of BLIS of *L. rhamnosus* L60 was 160–320 UA ml^{-1} for 31 susceptible GBS strains (60%), and for the other 21 strains tested (40%), respectively.

By the checkerboard method, the BLIS-es combination showed synergistic effect on all susceptible strains tested with values of FIC ranged from 0.131 to 0.218.

Patterns of antibiotic resistance of all strains tested by Kirby–Bauer diffusion method showed the following percentages: 54% to *AM* ampicillin (10 μg); 31% to OXA oxacillin (1 μg); 42% to GEN gentamicin (10 μg); 17.31% to CET cephalotin (30 μg) and 35% to ERY erythromycin (15 μg). PEN penicillin (10 U) resistance was not demonstrated and the same happened with CLIN clindamycin (2 μg).

Discussion

The results of prevalence of GBS found in this study are coincident with the carriage rate of GBS presented in

Table 1 Size of the inhibition zone produced by the BLIS-es of *Lactobacillus fermentum* L23, *Lactobacillus rhamnosus* L60, and a mixture of both BLIS-es on the *Streptococcus agalactiae* strains

Number of SGB strains tested	Average of inhibition zone in mm (average ± SD)			
	BLIS of L23	BLIS of L60	Mixture of BLIS-es	Type of interactions
1	7.33 ± 1.15	7.66 ± 0.57	17.66 ± 0.57	Synergism
2	7.00 ± 0	7.33 ± 0.57	18.33 ± 1.15	Synergism
3	7.00 ± 0	7.00 ± 1.00	18.00 ± 0	Synergism
4	6.66 ± 0.57	6.33 ± 0.57	16.66 ± 0.57	Synergism
5	7.33 ± 0.57	7.66 ± 0.57	18.33 ± 0.57	Synergism
6	7.33 ± 0.57	6.33 ± 0.57	17.66 ± 0.57	Synergism
7	7.33 ± 0.57	7.66 ± 0.57	18.66 ± 0.57	Synergism
8	8.00 ± 0	8.33 ± 0.57	18.33 ± 0.57	Synergism
9	7.00 ± 1.00	6.66 ± 0.57	17.33 ± 0.57	Synergism
10	7.00 ± 0	7.66 ± 0.57	17.66 ± 0.57	Synergism
11	8.00 ± 0	7.66 ± 0.57	18.66 ± 0.57	Synergism
12	7.00 ± 0	7.33 ± 0.57	17.66 ± 0.57	Synergism
13	7.00 ± 0	7.33 ± 0.57	18.00 ± 0	Synergism
14	8.00 ± 0	8.66 ± 0.57	20.66 ± 0.57	Synergism
15	6.33 ± 0.57	8.66 ± 0.57	19.33 ± 0.57	Synergism
16	6.66 ± 0.57	7.00 ± 1.00	18.33 ± 0.57	Synergism
17	7.33 ± 0.57	8.33 ± 0.57	20.33 ± 0.57	Synergism
18	7.00 ± 0	7.33 ± 0.57	18.33 ± 0.57	Synergism
19	7.00 ± 0	7.66 ± 0.57	19.33 ± 0.57	Synergism
20	8.00 ± 0	7.33 ± 0.57	21.00 ± 0	Synergism
21	8.00 ± 0	8.33 ± 0.57	20.33 ± 1.15	Synergism
22	8.00 ± 0	6.66 ± 0.57	19.66 ± 0.57	Synergism
23	7.00 ± 0	7.66 ± 0.57	20,00 ± 0	Synergism
24	7.66 ± 0.57	6.33 ± 0.57	18.66 ± 0.57	Synergism
25	7.66 ± 0.57	8.33 ± 0.57	20.33 ± 0.57	Synergism
26	6.33 ± 0.57	7.66 ± 0.57	19.00 ± 0	Synergism
27	8.00 ± 0	8.66 ± 0.57	21.00 ± 0	Synergism
28	7.00 ± 0	7.33 ± 0.57	18.66 ± 0.57	Synergism
29	7.00 ± 1.00	8.00 ± 0	18.66 ± 1.15	Synergism
30	7.66 ± 0.57	6.66 ± 0.57	19.33 ± 2.08	Synergism
31	7.00 ± 0	7.66 ± 0.57	18.33 ± 1.15	Synergism
32	8.00 ± 0	8.33 ± 0.57	20.00 ± 1.00	Synergism
33	8.00 ± 0	7.66 ± 0.57	19.66 ± 1.52	Synergism
34	7.00 ± 0	6.66 ± 0.57	18.33 ± 0.57	Synergism
35	7.66 ± 0.57	7.33 ± 0.57	19.33 ± 0.57	Synergism
36	8.00 ± 0	8.33 ± 0.57	18.33 ± 0.57	Synergism
37	8.33 ± 0.57	7.33 ± 0.57	18.66 ± 0.57	Synergism
38	6.33 ± 0.57	7.00 ± 0	17.33 ± 0.57	Synergism
39	7.00 ± 0	7.66 ± 1.15	19.33 ± 0.57	Synergism
40	7.00 ± 0	7.66 ± 0.57	17.66 ± 0.57	Synergism
41	6.66 ± 0.57	7.00 ± 1.00	18.33 ± 1.52	Synergism
42	7.33 ± 1.15	8.00 ± 1.00	18.00 ± 1.00	Synergism
43	7.33 ± 0.57	7.66 ± 057	19.66 ± 0.57	Synergism
44	7.33 ± 057	8.66 ± 0.57	18.33 ± 0.57	Synergism
45	8.00 ± 1.00	8.33 ± 0.57	19.00 ± 1.00	Synergism
46	7.66 ± 0.57	7.33 ± 0.57	18.00 ± 1.00	Synergism
47	8.00 ± 0	9.00 ± 1.00	20.33 ± 0.57	Synergism

Table 1 continued

Number of SGB strains tested	Average of inhibition zone in mm (average ± SD)			
	BLIS of L23	BLIS of L60	Mixture of BLIS-es	Type of interactions
48	8.00 ± 1.00	9.33 ± 0.57	17.66 ± 0.57	Synergism
49	7.66 ± 0.57	8.00 ± 1.00	18.66 ± 0.57	Synergism
50	8.00 ± 0	8.33 ± 0.57	17.66 ± 0.57	Synergism
51	7.66 ± 0.57	7.33 ± 0.57	18.66 ± 0.57	Synergism
52	8.00 ± 0	8.66 ± 0.57	18.00 ± 1.00	Synergism

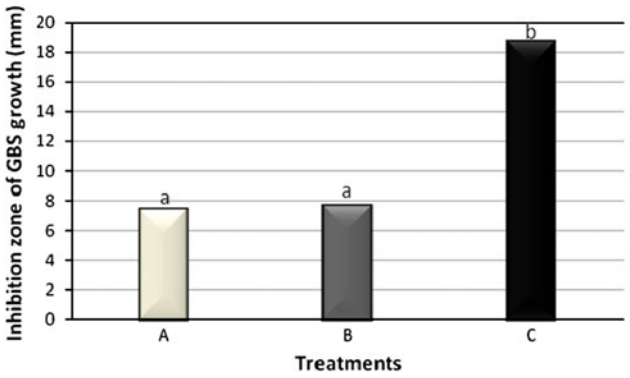

Fig. 2 Comparative inhibitory effect produced for the BLIS-es of *Lactobacillus fermentum*, *Lactobacillus rhamnosus*, and the mixture of both on the growth of *Streptococcus agalactiae* strains. Reference: **a** BLIS of *L. fermentum* L23, **b** BLIS of *L. rhamnosus* L60, **c** mixture of both BLIS-es. By Fisher's LSD test, *different letters* on the *column* indicate statistically significant differences ($P \leq 0.05$)

another study from Misiones, Argentina [32]. Thus, it is of significant value to compare with other authors who have found larger values of prevalence on a bigger population according to the last studies done [9, 12]. Probably, these differences are not just due to the population sizes tested, but also to genetic differences between the populations, or to geographic distribution of *S. agalactiae* strains.

On the basis of the increasing antibiotic resistance of *S. agalactiae* strains and considering that the maternal GBS colonization continues to be the most important risk factor for developing disease in the newborns it is urgent to find new prevention strategies to replace or improve on the antibiotic therapy. Among the antimicrobial compounds synthesized by the two human lactobacilli strains, *L. fermentum* L23 and *L. rhamnosus* L60 used in this study, we have only researched the antimicrobial activity attributed to the BLIS-es and not necessarily to the H_20_2 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 using these lactobacilli strains [26–28, 35]. Both BLIS-es produced by these two newly described probiotic strains showed in vitro a consistent antimicrobial activity that inhibited growth of *S. agalactiae* strains. These results are partially similar to those obtained by Melancom et al. [19], who showed inhibitory activity with other BLIS-es on a group of Gram-positive bacteria, among them *S. agalactiae*. While other authors, using bacteriocins such as nisin A (commercially used) against *Streptococcus* species observed a considerable percentage of inhibition [21, 22], we have obtained important antimicrobial results with the BLIS-es produced by our lactobacilli. In fact, the low values of MICs of the BLIS-es of *L. fermentum* and *L. rhamnosus* could be compared with the findings of Spinler et al. [37], who although using another bacteriocin, observed a high percentage of susceptibility to this substance, still at low concentrations, of several bacterial pathogens.

This is the first report where it is quantitatively proven that a positive interaction exists, as a synergism between both BLIS-es, especially against a pathogenic microorganism causative of several infections. We have found that a combination of these two BLIS-es results in a bioactive agent that more effectively inhibits growth of these isolates than its separate components alone. When considering the number of sensitive strains tested in each treatment and the differences in inhibition zones found among them we could assume that these are variations due to individual responses of strains that belong to the same species.

There are few studies of *S. agalactiae* surveillance and its patterns of antibiotic resistance among populations of pregnant adults in Argentina and in other Latin American countries, and for this reason as a first investigation, the resistance profiles of our isolates were studied. Although all of the strains tested were susceptible to penicillin, followed by 93% for cephalotin, these profiles were similar to the findings by another report in our country [17]. Our data on the patterns of antibiotic resistance found for the clinical isolates of *S. agalactiae* were significantly higher than results presented by many authors between the years 2000 and 2006, mainly for ampicillin and erythromycin [7, 9, 13, 36, 38]. In the case of the susceptibility to gentamicin among our isolates, we have found a resistance pattern lower than that published in a previous report by Lopardo [17].

We consider that the discovery of antimicrobial agents has created a new era in medicine and in the control of infectious diseases, which is based on the choice and careful use of a large group of low molecular weight inhibitors as was reported by Mota-Meira et al. [21]. Our results are promising, bearing in mind that we only evaluated a metabolite produced by two human lactobacilli with previously proven good probiotic properties. These in vitro findings on the antimicrobial activity and interactions of the BLIS-es of L23, L60 and even a mixture of them could be considered as important new biological agents that when used either alone or together would reduce the growth of *S. agalactiae* and consequently prevent colonization and infections by this microorganism. Preliminary results on FIC could not be compared with those of other authors because in the references consulted, we did not find studies of interaction between BLIS-es. However, the low values of FIC found using this method showed an important and synergistic inhibitory activity of the combined BLIS-es produced by these lactobacilli. Future research on these vaginal lactobacilli and the BLIS-es they produce will be required to evaluate their possible beneficial effect in vivo and to promote and support their recognition as a safe biological product. In addition, the possibility of preventing GBS colonization in women by the direct application of *L. fermentum* L23 and *L. rhamnosus* L60, both BLIS-producing strains, together to their probiotic properties deserve to be considered as biological strategies complementary to the antibiotic treatments commonly used.

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References

1. Barberis IL, Albesa I (1994) Assay in vivo with microcin producer *Pseudomonas*. Biomed Lett 49:13–19
2. Barberis L, Pajaro M, Godino S, Pascual M (2002) In vitro inhibition of *Gardnerella vaginalis* growth by bacteriocin produced by *Lactobacillus* strains. Rev UNRC 22:63–70
3. Barcia Hernandez E, Negro lavarez S (2002) Fundamentos de las interacciones farmacodinámicas (I). Anal Real Acad Farm 68(2):126–172
4. Bauer AW, Kirby MM, Sherris JC, Tuurck M (1966) Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 45:493–496
5. Bergey′s Manual of Determinative Bacteriology (1994) Group 4 pp 71–174; Group 5 pp 175–289; Group 17 pp 527–558. In: Holt JG (ed), 9th edn. Williams & Wilkins, Baltimore
6. Bolaños M, Cañas A, Santana O, Perez-Arellano J, de Miguel L, Martin-Sanchez A (2001) Invasive group B streptococcal disease in non-pregnant adults. Eur J Clin Microbiol Infect Dis 20: 837–839
7. Centers for Disease Control and Prevention (CDC) (2002) Prevention of perinatal group B streptococcal disease. Revised guidelines from CDC. MMWR 51:1–18
8. Cepeda C, Santos Y (2000) Rapid and low-level toxic PCR-based method for routine identification of *Flavobacterium psychrophilum*. Int Microbiol 3:235–238
9. Chohan L, Hollier L, Bishop K, Kilpatrick C (2006) Patterns of antibiotic resistance among group B *Streptococcus* isolates: 2001–2004. Infect Dis Obstet Gynecol 57492:1–4
10. De Man JC, Rogosa M, Sharpe ME (1960) A medium for the cultivation of lactobacilli. J Appl Bacteriol 23:130–135
11. Eduards MS, Baker JC (2001) Group B streptococcal infections. In: Remington JS, Klein JO (eds) Infectious diseases of the fetus and the newborn infant. WB Saunders Co., Philadelphia, pp 1091–1156
12. García SD, Eiseth MC, Lazzo MJ, Copolillo E, Barata AD, de Torres R, Vay CA, Famiglietti AM (2003) Group B *Streptococcus* carriers among pregnant women. Rev Argent Microbiol 35(4): 183–187
13. Heelan JS, Hasenbein ME, McAdam AJ (2004) Resistance of group B *Streptococcus* selected antibiotics, including erythromycin and clindamycin. J Clin Microbiol 42(3):1263–1264
14. Hensler M, Liu G, Sobzak S, Benirschke K, Nizet V, Heldt G (2005) Virulence role of Group B *Streptococcus* β hemolysin/cytolysin in a neonatal rabbit model of early-onset pulmonary infection. J Infect Dis 191:1287–1291
15. Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrant E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematic. Wiley, New York, pp 115–175
16. Lindahl G, Stalhammar-Carlemalm M, Areschoug T (2005) Surface proteins of *Streptococcus agalactiae* and related proteins in other bacterial pathogens. Clin Microbiol Rev 18(1):102–127
17. Lopardo H (2007) Antimicrobial resistance in β-hemolytic streptococci in Argentina. In: Mendez-Vilas A (ed) Communicating current research and educational topics and trends in applied microbiology, pp 794–798
18. Pineiro M, Staton C (2007) Probiotic bacteria: legislative framework requirements to evidence basis. J Nutr 137:850S–853S
19. Melancon D, Grenier D (2003) Production and properties of bacteriocin-like inhibitory substances from the swine pathogens *Streptococcus suis* serotype 2. Appl Environ Microbiol 69(8): 4482–4488
20. Moore M, Schrag S, Schuchat A (2003) Effects of intrapartum antimicrobial prophylaxis for prevention of growth B streptococcal disease on the incidence and ecology of early-onset neonatal sepsis. Lancet Infect Dis 3:201–213
21. Mota-Meira M, La Pointe G, Lacroix C, Lavoie M (2000) Minimum inhibitory concentrations of mutacin B-Ny266, nisin A, vancomycin, and oxacillin against bacterial pathogens. Antimicrob A Chemother 44:24–29
22. Mota-Meira M, Morensy H, Lavoie M (2005) In vivo activity of mutacin B-Ny266. J Antimicrob Chemother 56:869–871
23. National Committee for Clinical Laboratory Standards (NCCLS) (2000) Performance standards for antimicrobial disk susceptibility tests, 7th edn. Approved standard M2-A7. National Committee for Clinical Laboratory Standards, Wayne
24. Nigatu A, Ahrne S, Molin G (2000) Temperature-dependent variation in API 50 CH fermentation profiles of *Lactobacillus* species. Curr Microbiol 41:21–26
25. Pascual LM (2004) Bacteriocinogenia en el género *Lactobacillus*: características benéficas de lactobacilos de vagina humana. Doctoral thesis, UNRC
26. Pascual LM, Daniele MB, Giordano W, Pajaro MC, Barberis IL (2008) Purification and partial characterization of novel

bacteriocin L23 produced by *Lactobacillus fermentum* L23. Curr Microbiol 56:397–402
27. Pascual LM, Daniele MB, Pajaro C, Barberis L (2006) *Lactobacillus* species isolated from the vagina; identification, hydrogen peroxide production and nonoxynol-9 resistance. Contraception 73:78–81
28. Pascual LM, Daniele MB, Ruiz F, Giordano W, Pajaro C, Barberis L (2008) *Lactobacillus rhamnosus* L60 a potential probiotic isolated from human vagina. J Gen Appl Microbiol 54:141–148
29. Pascual L, Ruiz F, Giordano W, Barberis L (2010) Vaginal colonization and activity of the probiotic bacterium *Lactobacillus fermentum* L23 in a murine model of vaginal tract infection. J Med Microbiol 59:360–364. doi:10.1099/jmm.0.012583-0
30. Petersen P, Labthavikul P, Jones C, Bradford P (2006) In vitro antibacterial activities of tigecycline in combination with other antimicrobial agents determined by chequerboard and time-kill kinetic analysis. J Antimicrob Chemother 57:573–576
31. Principe l, D'Arezzo S, Capone A, Petrosillo N, Visca P (2009) In vitro activity of tigecycline in combination with various antimicrobials against multidrug resistant *Acinetobacter baumanii*. Ann Clin Microbiol Antimicrob. doi:10.1186/1476-0711-8-18
32. Quiroga M, Pegels E, Oviedo P, Pereyra E, Vergara M (2008) Antibiotic susceptibility patterns and prevalence of group B *Streptococcus* isolated from pregnant women in Misiones. Argent Braz J Microbiol 39:245–250
33. Reid G, Jass J, Sebulsky MT, McCormick JK (2003) Potential use of probiotics in clinical practice. Clin Microbial Rev 16:652–658
34. Rogosa M, Sharpe E (1963) Species differentiation of human vaginal lactobacilli. J Gen Microbiol 23:197–201
35. Ruiz F, Gerbaldo G, Asurmendi P, Pascual L, Giordano W, Barberis I (2009) Antimicrobial activity, inhibition of urogenital pathogens, and synergistic interactions between *Lactobacillus* strains. Curr Microbiol 59:497–501
36. Spaetgens R, De Bella K, Ma D, Robertson S, Mucenski M, Davies HD (2002) Perinatal antibiotic usage and changes in colonization and resistance rates of group B *Streptococcus* and other pathogens. Obstet Gynecol 100(3):525–533
37. Spinler JK, Taweechotipatr M, Rognerud CL, Ou CN, Tumwasorn S, Versalovic (2008) Human-derived probiotic *Lactobacillus reuteri* demonstrate antimicrobial activities targeting diverse enteric bacterial pathogens. Anaerobe 14(3):166–171
38. Stiller RJ, Padilla L, Choudhary R, Tinghitella T, Laifer S (2003) Group B streptococcal antibiotic resistance patterns in pregnant women. Conn Med 67(6):323–326
39. Tyrrell G, Senzilet L, Spika J, Kertesz D, Alagaratnam M, Lovgren M, Talbot J (2000) Invasive disease due to group B streptococcal infection in adults: results from a Canadian, population-based, active laboratory surveillance study—1996. J Infect Dis 182:168–173