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ORIGINAL ARTICLE

Hydrogen peroxide production and resistance to nonoxinol-9 in *Lactobacillus* spp. isolated from the vagina of reproductive age women

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ABSTRACT. Lactic acid production is considered to be the major protection mechanism of lactobacilli against vaginal infections due to genital pathogens. However, some species of *Lactobacillus* are also hydrogen peroxide-producers. Women, who usually use intrauterine dispositive (IUD) and spermicides such as nonoxinol-9 (N-9) as contraceptive methods, increase the risk of acquiring an urinary tract infection and a bacterial vaginosis; some studies have demonstrated that these compounds alter the normal vaginal biota. It is known that they inhibit lactobacilli *in vitro* at concentrations of 0.1% to 1% and that they do not have an effect on the growth of *Escherichia coli*. It is probable that the presence of nonoxinol-9 affects the ecological balance of the vagina by inhibiting the protector lactobacilli.
In this study, we identified *Lactobacillus acidophilus, L. brevis, L. crispatus, L. fermentii* and *L. jensenii* as the species most frequently isolated from women. Seventy-one hydrogen peroxide-producer strains and 48 strains resistant to the inhibitory effect of nonoxinol-9 were detected. *L. brevis* showed the highest number of resistant strains.

Key words: *Lactobacillus,* vagina, H_2O_2, nonoxinol-9.

RESUMEN. La producción de ácido láctico por los lactobacilos se ha considerado la base de su papel protector contra las infecciones vaginales, sin embargo, algunas especies de lactobacilos, también producen peróxido de hidrógeno. Las mujeres que usan dispositivo intrauterino (DIU) y espermicidas, tales como el nonoxinol-9 (N-9) como un método anticonceptivo, incrementan el riesgo de adquirir una infección urinaria y una vaginosis bacteriana (VB). Algunos estudios efectuados demuestran que estos compuestos alteran la biota vaginal, inhibe a muchos de los lactobacilos *in vitro* en concentraciones de 0.1% a 1% y no tiene efecto sobre el crecimiento de *Escherichia coli.* Es probable que la presencia de N-9 afecte el balance ecológico de la vagina por inhibición de los lactobacilos protectores, en especial los productores de peróxido de hidrógeno. En este estudio se identificaron a *L. acidophilus, L. brevis, L. crispatus, L. fermentii* y *L. jensennii* como las especies más frecuentemente aisladas. Se detectaron 71 cepas productoras de peróxido de hidrógeno y 48 resistentes al efecto inhibitorio del nonoxinol-9. *L. brevis* fue la especie con el mayor número de cepas resistentes.

Palabras clave: *Lactobacillus,* vagina, H_2O_2, nonoxinol-9.

INTRODUCTION

The vagina represents a dynamic ecosystem which stays balanced thanks to a fine interaction of factors such as the native bacterial biota.

In healthy adult women the normal vaginal pH is ≤ 4.5. The predominant species of *Lactobacillus* maintain a low pH through their fermenting activity which protects the area against the invasion of undesirable microorganisms.[14] Lactic acid production is considered to be the major protection mechanism of lactobacilli against vaginal infections. Nevertheless, some species of lactobacilli also produce hydrogen peroxide. The inhibition of bacterial growth through the action of substances generated by other species of bacteria is a well characterized antagonistic bacterial mechanism. Like other lactic bacteria, lactobacilli lack the heme group and do not utilize the cytochrome system for terminal oxidation. They possess flavoproteins, which transform oxygen into H_2O_2. This mechanism, together with the absence of the catalase hemoprotein, generates H_2O_2 in amounts that exceed the capacity of the organism to degrade it. It has been proposed that the production of H_2O_2 explains the success of lactobacilli as vaginal colonizers and that it is an inhibitory mechanism that can inhibit or eliminate other members of the microbiota, particularly those that lack or have low levels of catalase or peroxidase enzymes.[2,3,6,10,17]

In vitro, H_2O_2 producing lactobacilli (LB^+) have a bactericidal effect on several bacteria including *Gardnerella vaginalis, Prevotella bivia* and *Neisseria gonorrhoeae*. Several studies have demonstrated that pregnant women who are colonized by a normal biota, present a higher percentage of colonization by LB^+ than women with bacterial vaginosis (BV). Also, women colonized by LB^+ have a lower probability of acquiring a BV, vulvovaginal candidosis (VVC), vaginal tricomoniasis or colonization by any other microorganism than pregnant women colonized by non-H_2O_2 producing lactobacilli (LB^-). The prevalence of LB^+ in the vagina decreases colonization by other microorganisms, including *G. vaginalis, Mobiluncus* spp, *Bacteroides* spp and *Mycoplasma hominis*.[4,5]

Women using IUD and spermicides as Nonoxinol-9 (N-9) as contraceptive method, increase their risk of acquiring

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an urinary infection or a BV, some studies have demonstrated that these compounds alter the vaginal biota.[3,11,18,21] N-9 is the active compound in many spermicidal formulas. It forms part of the nonyphenoxypoly (ethylenoxy) ethanols. It is a non-ionic detergent, which reduces the superficial tension of the membrane of human spermatozoon, causing loss of mobility, diminishment of its glucolitic power and alteration of permeability. It also affects the lipidic content of the membrane of human spermatozoon. All of these actions are permanent and irreversible.[12] In addition, it has a toxic effect against *N. gonorrhoeae, Treponema pallidum, Trichomonas vaginalis, Candida albicans, Chlamydia trachomatis, Herpes simplex* virus and HIV type 1. Nevertheless, paradoxically, women using spermicides have a greater vaginal colonization by *Escherichia coli*.[7,11] N-9 is generally used in concentrations of 5% in creams and 12.5% in foams, and it can be present in concentrations of 1.6% to 2.7% after sexual intercourse. It has been demonstrated to inhibit many lactobacilli *in vitro* in concentrations of 0.1% to 1%, but it has no effect on the growth of *E. coli*. It is possible that the presence of N-9 could affect the ecological balance of the vagina through the inhibition of the protective lactobacilli, especially those which produce hydrogen peroxide.[11,13,18,21]

The aim of this work was to identify the species of *Lactobacillus* isolated from vaginal exudates of reproductive age women and to detect the H_2O_2-producing and resistant to N-9 strains.

MATERIAL AND METHODS

Sample sources. Vaginal samples for lactobacilli culture (156 vaginal exudates) were collected as follow: 120 were obtained from Clínica Churubusco de Especialidades y Cirugía Simplificada ISSSTE, Mexico City. From these samples, were isolated 62 strains and were conservated in MRS glycerol at 25% to -70ºC. We isolated 29 strains from 25 samples that were obtained from Laboratorio de Análisis clínicos, Clínica No. 61, Tlanepantla, Estado de Mexico. Seven samples were obtained from Laboratorio de Análisis Clínico, Clínica No. 21, Familiar del IMSS, Mexico City, and four samples more were obtained from Servicio Externo, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional. Samples were taken with a swab from the posterior zone of the fornix of vagina. The pH of the vaginal fluid was taken only from 135 samples. The swab was placed in a tube with thioglycolate broth (Bioxon).

Strain isolation. The swabs were inoculated in MRS agar plates (Difco), incubated at 37ºC in CO_2 partial tension for 24 h. The presumptive colonies were examined through Gram staining technique, catalase and oxidase tests. These colonies were preserved in MRS glycerol at 25% and were also grown in MRS slant agar tubes for further studies. The 62 strains preserved at –70ºC were unfrozen and inoculated in agar plates and MRS broth tubes. They were incubated at 37ºC in CO_2 partial tension for 24 h. Strain purity and correspondence with non-spore forming Gram positive bacilli were verified with the Gram staining technique, catalase and oxidase tests.

Phenotype identification. Identification at the species level of 67 strains was performed with API 50 CH and API 50 CHL systems (bioMérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. The biochemical profile obtained was captured into the APILAB V5.0 identification program for the final identification. Another 19 strains were identified through biochemical tests that had been miniaturized according to the method proposed by Jayne-Williams.[8,9] We began from stocks solutions at 5% of the relevant carbohydrates. These were diluted at 1% in MRS modified broth, added with bromocresol purple indicator. We placed 156 µl in each well of a 96 well microplate, then were added 10 µl of a bacterial suspension to each well. This suspension had been adjusted to tube 2 of a MacFarland nefelometer (6×10^8 UFC/ml) out of a MRS broth tube incubated overnight at 37ºC.

Gas production from glucose. MRS broth with a Durham tubes was used. These were inoculated with the problem strain and incubated at 37ºC, for 24 hours to 7 days. The test was considered positive when the presence of gas in the tube was observed.

H_2O_2 production. The qualitative determination of the H_2O_2 produced by the strains was demonstrated using a version of the qualitative method reported by Eschenbach *et al*,[21] as modified by Felten *et al.*[3] It was inoculated 5 µl of a bacterial suspension of *Lactobacillus,* adjusted to the 0.5 tube of the MacFarland nefelometer (1.5×10^8 UFC/ml) on a MRS agar plate with 25 mg/100 ml of 3,3',5,5'-tetramethylbenzidine (TMB, SIGMA) and 1 mg/100 ml of peroxidase (SIGMA) added. Peroxidase generates O_2 out of the H_2O_2 produced by the lactobacilli and the TMB dyes the colonies with a blue color when oxidation occurs in the presence of O_2. After 24 h of incubation at 37ºC in CO_2 tension, the colonies that produced H_2O_2 turned to a blue color. Since peroxidase is unstable, the plates were used within three days following their preparation. The positive control strain for the production of peroxide was *L. acidophilus* ATCC 4356.

Resistance pattern to Nonoxinol-9 (N-9): Since there is no method in the bibliography to determine the resistance pattern to this kind of substances, the conditions conventionally used to make an antibiotic sensibility test were implemented using the method of disc diffusion (Kirby-Bauer), as modified by Hooton *et al.* [7] We tested 72 strains. From the growth of each strain in the MRS slant agar tube an MRS broth tube was inoculated and incubated for 24 hours at 37°C in CO_2 partial tension. After growth, we adjusted a bacterial to the 0.5 of the MacFarland nefelometer and it was massively inoculated onto Müeller-Hinton agar plates enriched with 5% sheep blood. The inoculum was left to dry for 10 minutes. Using a pair of tweezers flamed with 70% alcohol, a paper filter previously impregnated with 10 µl of the contents of a 13% N-9 ovulet (*Lorophyn*, Laboratorios Columbia) was colocated over the plates. Strains that presented growth inhibition disk zones greater or equal to 10 mm in diameter around the disc were considered resistant, strains with growth inhibition disk zones lesser or equal to 9 mm in diameter were considered sensitive.[11,13]

RESULTS

We obtained a total number of 156 samples, only 135 had their pH value determined and only in 87 samples were isolated lactobacilli. The greatest number of lactobacilli strains were isolated from samples with an acidic pH. Table 1 shows the pH of the samples, the number of samples with that pH and the number of samples in which lactobacilli were found.

A total of 93 facultative aerobic strains were isolated out of the 156 samples. Two different species of lactobacilli were found in five of these samples, meaning that those five women were colonized by two species, while in the rest of the samples only one species of *Lactobacillus* was isolated. Eighty six samples were used out of the 93, since seven samples lost viability in the posterior inoculations or did not recover from the preservation process at -70ºC.

We detected 25 heterofermentative strains and 61 homofermentative strains by means of the production of gas from glucose. Identification was done with the API 50 CHL and 50 CH system, but since the total number of tests was not available, only 67 were identified by this method. The remaining 19 strains were identified through miniaturized biochemical tests. Among the heterofermentative species found were: *L. brevis*, *L. fermentum* y *L. paracasei* subsp. *paracasei*, all identified with the API system; among the homofermentative species the following were identified by API: *L. acidophilus*, *L. coprophilus*, *L. crispatus*, *L. delbrueckii* subsp. *delbrueckii*, *L. plantarum*, *Lactococcus lactis* y *Leuconostoc lactis*; while using biochemical tests the following were identified *L. jensenii*, *L. leichmannii* and some strains denominated *L. gasseri/acidophilus*, since these two species are biochemically indistinguishable; other strains were denominated *Lactobacillus* spp., since their species could not be determined through this method. Table 2 shows the identified species, the number of isolations, and the identification method.

Table 1. Sample pH and number of samples in which *Lactobacillus* were isolated.

Vaginal fluid pH	No.	Samples number
4	31	19
5	33	24
6	27	10
7	24	10
8	17	9
9	3	0
not determined	21	15
Total	156	87

Table 2. Species of *Lactobacillus* isolated from vaginal exudates.

Species	No. of isolated strains	Identification method
L. acidophilus	19	API
L. brevis	15	API
L. coprophilus	3	API
L. crispatus	12	API
L. delbrueckii subp. *Delbrueckii*	1	API
L. fermentum	10	API
L. gassei/acidophillus	5	MBT
L. jensenii	6	MBT
L. leichmannii	5	MBT
L. paracasei subp. *paracasei*	1	API
L. plantarum	2	API
Lactobacillus spp	3	MBT
Lactococcus lactis	3	API
Leuconostoc lactis	1	API
Total	86	

API: API 50 CHL and 50 CH identification system; MBT: miniaturized biochemical tests.

For further studies only the 82 strains identified as *Lactobacillus* were used, the strains identified as *Lactococcus* and *Leuconostoc* species were omitted.

Regarding the production of hydrogen peroxide by these strains, the method used to determine H_2O_2 detected 71 strains that produced it (LB^+) and 11 strains that did not (LB^-). Table 3 shows the distribution of LB^+ and LB^- strains by species of *Lactobacillus*.

For the sensitivity assay to nonoxinol-9, only 72 out of the 82 strains of *Lactobacillus* were used, since ten strains did not grow in the Müeller-Hinton medium enriched with blood. From 72 strains assayed, were detected 48 resistant (66.67%) and 24 sensitive (33.33%) (Fig. 1). All species presented resistance, but while *L. acidophilus. L. brevis*, *L. crispatus* and *L. jensenii* had the highest number of resistant strains, *L. fermentum* and *L. leichmannii* had the same number of resistant and

Table 3. Distribution by species of *Lactobacillus* spp. strains: H_2O_2- producing (LB+) and non H_2O_2-producing (LB-).

Species	Strains		
	No. total	LB+	LB-
L. acidophilus	19	18	1
L. brevis	15	15	0
L. coprophilus	3	2	1
L. crispatus	12	12	0
L. delbrueckii	1	1	0
L. fermentum	10	4	6
L. gasseri/acidophilus	5	5	0
L. jensenii	6	3	3
L. leichmannii	5	4	1
L. paracasei	1	1	0
L. plantarum	2	2	0
Lactobacillus spp.	3	3	0
Total	82	71 (86.6%)	11 (13.45%)

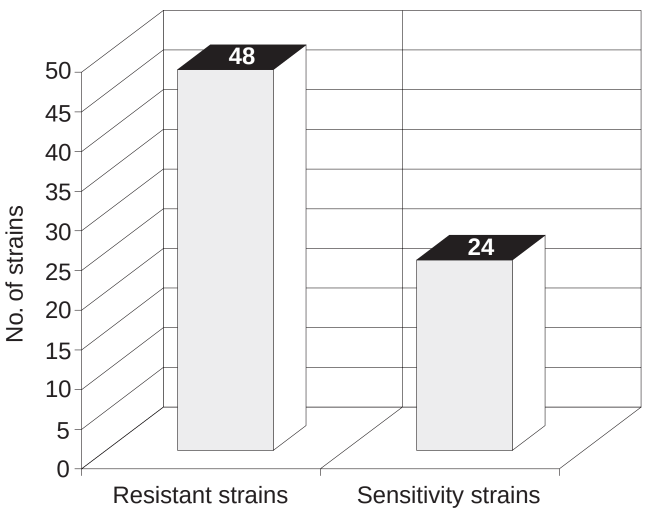

***Figure 1.** Resistance pattern to nonoxinol-9 in* Lactobacillus *spp strains isolated from the vagina of women in reproductive age.*

sensitive strains. *L. gasseri* had two resistant strains and three sensitive. *Lactobacillus* spp had one sensitive strain and two resistant. *L. paracasei* subsp. *paracasei* and *L. plantarum* isolated species were resistant (Table 4).

We detected 37 resistant strains that are H_2O_2-producing (LB^+) and 11 resistant that are non-H_2O_2 producing (LB^-); 19 of the 24 sensitive strains are LB^+ and five are LB^-. Apparently, the majority of the nonoxinol-9 resistant strains are LB^+ (Fig. 2). We did not observe any particular tendency of resistance by species. Nevertheless, *L. brevis* has the greatest number of LB^+ resistant strains, while *L. acidophilus* has nine LB^+ resistant strains and eight LB- sensitive strains (Table 5).

DISCUSSION

The vaginal biota has been widely studied and diverse species of *Lactobacillus* have been identified as the predominant biota.[4,6,12,17,19] In Mexico, Pérez Miravete,[16] identified species of *L. acidophilus, L. delbrueckii, L. plantarum, L. fermentum, L. brevis* , *L. casei* subsp *alolactosus, L. bulgaricus, L. leichmannii* and also a strain of *Leuconostoc mesenteroides* in a group of pregnant women. In other countries, investigators have isolated other species. Rogosa M.[19] also isolated *L. rhamnosus* and *L. cellobiosus.* Onderdonk A.B. and col.[15] isolated strains of *L. plantarum* and *L.*

Table 4. Resistance pattern of *Lactobacillus* spp. to nonoxinol-9 by species.

Species	No. of strains		
	Total No.	Resistant	Sensitive
L. acidophillus	18	10	8
L. brevis	15	14	1
L. coprophillus	3	2	1
L. crispatus	6	6	0
L. fermentum	10	5	5
L. gasseri/acidophillus	5	2	3
L. jensenii	6	4	2
L. leichmannii	4	2	2
L. paracasei	1	1	0
L. plantarum	1	1	0
Lactobacillus spp.	3	1	2
Total	72	48 (66.67%)	24 (33.33%)

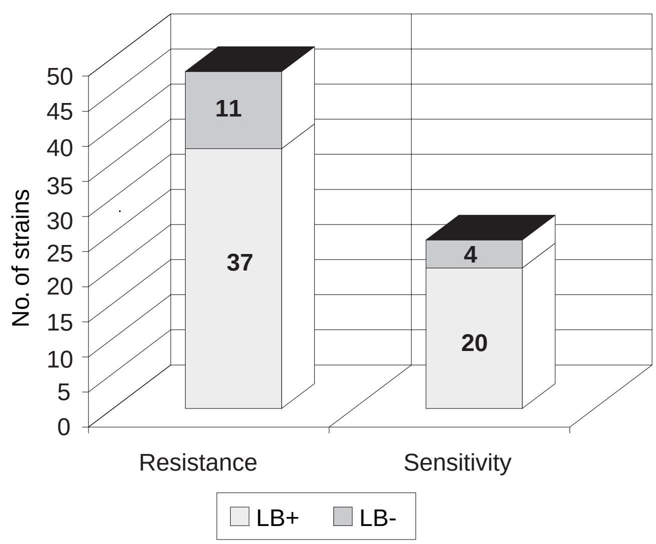

***Figure. 2.** Correlation between peroxide producing strains and resistance to nonoxinol-9.*

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Table 5. Hydrogen peroxide production, resistance to nonoxinol-9 and their correlation.

Species	(No.)	Resistance to N-9		Sensitivity to N-9	
		LB^+	LB^-	LB^+	LB^-
L. acidophilus	18	9	1	8	0
L. brevis	15	13	1	1	0
L. coprophilus	3	2	0	1	0
L. crispatus	6	3	3	0	0
L. fermentum	10	2	3	2	3
L. gasseri/acidophilus	5	2	0	3	0
L. jensenii	6	2	2	1	1
L. leichmannii	4	1	1	2	0
L. paracasei	1	1	0	0	0
L. plantarum	1	1	0	0	0
Lactobacillus spp.	3	1	0	2	0
Total	72	37	11	19	5

LB^+: H_2O_2 producing strains. LB-: non-H_2O_2 producing strains.

leichmannii. Giorgi and col.[4] isolated strains of *L. jensenii, L. crispatus y L. gasseri.* Eschenbach and col.[2] identified species of *L. catenaforme, L. helveticus y L. salivarius.* McGroarty and col.[13] identified several of these species. Antonio and col.[1] reported the identification of strains of *L. ruminis, L. reuteri L. oris* and *L. vaginalis.* Song and col.[20] also report the isolation of several of these species.

In the present study a greater number of species were identified than those informed by Pérez Miravete[16]. Only facultative aerobic lactobacilli were isolated. The homofermentative species identified include: *L. acidophilus, L. crispatus, L. gasseri/acidophilus, L. jensenii* and *L. leichmannii*. The heterofermentative species identified were *L. paracasei* subsp *paracasei, L. plantarum, L. brevis, L. coprophilus y L. fermentum.* Also, three *Lactococcus lactis* subsp *lactis* strains and one *Leuconostoc lactis* strain were identified. In addition, three strains could not be identified through the biochemical methods used. Some strains were named *L. gasseri/acidophilus*, given that these species are indistinguishable in biochemical tests, and the miniaturized biochemical test system did not allow us to differentiate them.

In the majority of the studies done, the most frequently and in greater number isolated species are *L. acidophilus, L. gasseri, L. brevis L. crispatus, L. jensenii and L. fermentum.* With a lower frequency *L. plantarum, L. delbrueckii and L. casei* are isolated, and isolation of *L. helveticus, L. salivarius* and *L. reuteri* is rarely reported. In the present study three strains of *L. coprophilus* were identified but no reports of the isolation of this species from a vaginal sample were found in the bibliography. Thus we do not know if this specie forms part of the normal vaginal biota of Mexican women.

On the other hand, generally only one specie is isolated in from women, although there are data about simultaneous colonization of the vagina by two different species of lactobacilli, which can be homofermentative, heterofermentative or combinations of these.[1,4,13] In the present study, two distinct species were identified in five of the samples, meaning that these women were colonized by two different species of lactobacilli. The pairs were *L. jensenii* and *L. plantarum, L. crispatus* and *L. acidophilus, L. acidophilus* and *Leuconostoc lactis, L. acidophilus* and *L. paracasei* subsp *paracasei* and finally *L. acidophilus* and a strain that lost viability in posterior cultures.

Given that lactobacilli are the microorganisms which maintain the acidic vaginal pH and that a pH over 4.5 is considered to be an alteration from the normal vaginal equilibrium, a greater number of isolations would be expected to come form the samples with a pH of 4 or 5. Nevertheless, isolations were done from more alkaline samples with pH of 7 and 8. Distribution of species by pH was varied, and the number of isolations diminished as the pH of the sample got more basic as in the case of *L. acidophilus, L. brevis, L. gasseri/acidophilus* y *L. leichmannii*, though some species were isolated from alkaline and acidic samples: *L. coprophilus, L. crispatus, L. fermentum* y *L. jensenii* (data not shown).

Production of hydrogen peroxide by the *Lactobacillus* species is considered to represent a non-specific anti-microbial defense mechanism of the normal vaginal ecosystem.[2] Eschenbach et al.[2] detected 96% of H_2O_2- producing (LB^+) strains and McGroarty et al.[13] detected 74.6%, while the present study we found 71 LB^+ strains and 11 non H_2O_2-producing (LB^-) strains, corresponding to 86.6% and 13.4% respectively. Species with the largest number of LB^+ strains were *L. acidophilus, L. gasseri/acidophilus, L. brevis, L. crispatus, L. jensenii,* and *L. leichmannii*. These results agree with those published by Antonio et al.[1] In fact, the species reported as LB^- in another study such as *L. delbrueckii* y *L. leichmannii*,[2] were revealed as LB^+ in this study. Also, strains LB^+ and LB^- strains of *L. fermentum* and *L. plantarum* were found. Our results agree with those published by Song et al.[20] and McGroarty et al.[13] They report 50% of these strains as LB^+ and 50% as LB^- of these same species.

Nonoxinol-9 is a spermicide that has anti-microbial activity. Some studies have shown that lactobacilli present resistance or sensibility to this substance. In this study, 48 resistant strains and 24 sensitive strains were found, approximately being 66.67% resistant strains and 33.33% sensitive strains respectively. Thus, we considered the majority of the strains we worked are resistant to this compound. Regarding the pattern of resistance by species, we observed that *L. brevis* and *L. acidophilus* presented the largest num-

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ber of resistant strains to the effect of N-9 and that the rest of the lactobacilli presented more or less the same number of sensitive and resistant strains.

Some authors correlate the susceptibility of lactobacilli to N-9 with their capacity to produce H_2O_2. It has been reported that LB^+ lactobacilli are inhibited *in vitro* by N-9.[7,11,13] This is not the case with LB^- lactobacilli. In contrast, LB^+ lactobacilli are not inhibited by N-9 *in vivo*.[18, 21]

This study was performed *in vitro* and its results seem to indicate that there is a greater number of LB^+ resistant strains. Nevertheless, such disperse results by species do not allow us to tell if this resistance can be related to the production of H_2O_2. This is because many of the species of *Lactobacillus* have about the same number of LB^+ resistant strains and sensitive strains and vice versa, with the exception of *L. brevis*, whose greatest number of resistant strains are LB^+.

Apparently, H_2O_2- producing strains are more resistant to the effect of N-9 at the tested concentration. It appears that the pattern of resistance to N-9 is not common to the genus and only *L. brevis* seems to be resistant. Thus, resistance may be in function of the species of *Lactobacillus* present in the vagina. So, a woman colonized by *L. brevis* would have a lower probability of losing the strain when using nonoxinol-9 as a spermicidal than if she was colonized by any other species. Anyway, this study should be done with a greater number of species isolated from vagina, to obtain a conclusion that is more trustworthy.

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