

Vaginal Protection by H₂O₂-Producing Lactobacilli

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Background: Peroxide-producing lactobacilli provide protection from infection for the female reproductive tract. However, *in vitro* studies demonstrated that H₂O₂-produced by *Lactobacillus* is not the cause of inhibition of pathogens. It is not exactly known how H₂O₂-producing lactobacilli are involved in the protection of the vaginal environment.

Objectives: This study aimed to evaluate the importance of the interaction between H₂O₂-producing lactobacilli and their host for the resistance of the vaginal biotope.

Materials and Methods: In this study, we used vaginal lactobacilli (11 H₂O₂-producing strains and 11 non-H₂O₂-producing strains). The influence of epithelial cells on the growth and antibacterial activity of lactobacilli were evaluated. The effects of lactobacilli on the antibacterial activity of the epithelial cells, muramidase and lactoferrin were also determined.

Results: Vaginal epithelial cells stimulated the growth and antibacterial activity of H₂O₂-producing lactobacilli in a greater extent than that of the non-H₂O₂-producing lactobacilli. Mainly, the H₂O₂-producing lactobacilli were capable of increasing the activity of the host antimicrobial peptides (muramidase and lactoferrin) as well as the antibacterial activity of the epithelial cells.

Conclusions: The involvement of the peroxide-producing lactobacilli in the protection of vagina was due to their ability to effectively interact with the host. This is expressed on one side to stimulate the growth and antagonistic activity of lactobacilli and on the other side to increase the antibacterial activity of the host defense factors (muramidase, lactoferrin and metabolites of epithelial cells).

Keywords: Epithelial Cells; Host-Pathogen Interactions; Hydrogen Peroxide; Immunity; Innate; Lactoferrin; Muramidase; Vagina; *Lactobacillus*

1. Background

The commensal microflora are important components of the mucosal defense against infections. *Lactobacillus* colonization of the lower female genital tract provides protection from the acquisition of sexually-transmitted diseases or bacterial vaginosis (1-3). The infection and/or proliferation of pathogenic bacteria in vagina are suppressed by lactic acid, H₂O₂ production and others bacteria-generated antimicrobial products (4, 5). The H₂O₂-producing lactobacilli are significant components in this process (6). H₂O₂-producing lactobacilli are isolated from the vaginas of a majority of healthy reproductive-age women (1). The production of H₂O₂ is a predictor for sustained long-term *Lactobacillus* colonization of the vagina (7). With the colonization of H₂O₂-producing lactobacilli, the associated reduced risk of bacterial vaginosis and ascending uterine infection will occur (8).

However, under optimal, anaerobic growth conditions, physiological concentrations of H₂O₂ are not detectable for inactivation of pathogens (9, 10). How can H₂O₂-producing lactobacilli protect the vaginal environment? This question probably has no answer because the study of the properties of lactobacilli was carried out in non-host-associated environments (11). We assumed that the

H₂O₂ produced by lactobacilli provides vaginal resistance, not directly, but by interacting with other protection components of the vaginal environment. This is the primary innate immunity (12) which is represented by cellular elements (epithelial cells and other factors) (13, 14) and humoral factors (15). The present research aimed to verify this hypothesis.

2. Objectives

The purpose of this study was to evaluate the importance of the interaction between H₂O₂-producing lactobacilli and their host for the resistance of the vaginal biotope. We examined how the host (vaginal epithelial cells) can modify the properties of *Lactobacillus* that are vary in their ability to produce H₂O₂ and how these lactobacilli can influence on the properties of the host organism.

3. Materials and Methods

This study was approved by the ethics committee of the institute of cellular and intracellular symbiosis, Orenburg, Russian federation and an informed consent was obtained from all the patients. Specimens were collected exclusively from healthy volunteers.

3.1. Materials

API 50 CH test kit and API CHL medium (bioMerieux, La Balme les Grottes, France), de Man, Rogosa, Sharpe medium and trypticase soy broth (HiMedia, India), Hanks balanced salt solution (HBSS; Gibco, USA), MgSO_4 , MnSO_4 , K_2HPO_4 , glucose, 3,3',5,5'-tetramethylbenzidine, fluorochrome diacetate, hydrogen peroxide, cefuroxime, muramidase (the lysozyme from egg white), lactoferrin from human milk, catalase, and horseradish peroxidase (Fluka, Switzerland) were used.

3.2. Bacterial Strains

Vaginal clinical isolates of *Escherichia coli* (7 strains) and *Staphylococcus aureus* (5 strains) were provided by the Institute of Cellular and Intracellular symbiosis (Russian Federation). *Lactobacillus* spp. (47 strains) was isolated from the vagina of 45 healthy women and identified on a set of morphological, cultural and biochemical properties (API 50 CH test kit according to the manufacturer's instructions). Thereafter, the ability of lactobacilli to produce hydrogen peroxide was determined. For this purpose, lactobacilli (~ 109 CFU/mL) were incubated in the medium (0.8 mM MgSO_4 , 0.3 mM MnSO_4 , 11.5 mM K_2HPO_4 , 11.5 mM glucose) at 37°C for three hours with aeration. The concentration of H_2O_2 was determined in supernatants colorimetrically through the detection of the absorbance induced with 3,3', 5,5'-tetramethylbenzidine (16). The lactobacilli were distributed depending on the ability to generate H_2O_2 (Table 1). Most isolates of lactobacilli (76.6%) were capable of producing H_2O_2 in concentrations of 0.8 to 6.4 mM. For further research, we selected 11 strains of lactobacilli with the most frequent level of production of H_2O_2 (1.6 ± 0.5 mM) as well as 11 strains that did not produce H_2O_2 . The *Lactobacillus* strains were grown in de Man, Rogosa, Sharpe medium (MRS) at 37°C without aeration. Culture supernatants from *Lactobacillus* (CSL) were prepared by centrifuging the overnight cultures of the bacteria at 3000 g for 15 minutes at room temperature and then filtering the supernatant to remove the bacteria and particulate matter (0.22 μm syringe filter).

3.3. Vaginal Epithelial Cells

Human vaginal epithelial cells were obtained from the lateral walls of the vaginas of 45 healthy females during the follicular phase of the menstrual cycle. For the elimination of concomitant microflora, the epithelial cells were placed in Hanks balanced salt solution containing 50 mg/mL cefuroxime and 10 mg/mL muramidase for one hour and then washed three times with a tenfold volume of buffered saline (pH 7.0 - 7.2). Live epithelial cells were counted in a hemocytometer after staining with fluorochrome diacetate, using luminescence microscopy. Epithelial cells (106 cells/mL) in HBSS were incubated at 37°C with shaking. Culture supernatants from epithelial cells (CSE) were prepared by centrifuging the six-hour culture

of epithelial cells at 2000 g for 15 minutes at room temperature and filtration (0.22 syringe filter).

3.4. The Influence of Vaginal Epithelial Cells on Growth and Antibacterial Activity of Lactobacilli

For this purpose, the CSE or HBSS were incubated with lactobacilli (~106 CFU/mL in MRS) for one hour at 37°C in a 1:7 ratio. A part of the mixture was further diluted in buffered saline (pH 7.0-7.2) to yield 800 - 1000 CFU on control plates and plated on agar enriched with MRS broth. The colonies were counted after an overnight incubation at 37°C without aeration. The other part of the mixture was incubated overnight and used to determine the influence of epithelial cells on the antibacterial activity of lactobacilli. For this, CSL or the growth medium (MRS) was incubated with *E. coli* and *S. aureus* (~ 106 CFU/mL) for one hour at 37°C in a 1:1 ratio. The mixtures were then diluted in buffered saline (pH 7.0 - 7.2) to yield 800 - 1000 CFU on control plates and plated on agar enriched with trypticase soy broth. The colonies were counted after an overnight incubation at 37°C. All the samples were tested in triplicate and the percentage of inhibition was determined relative to the colonies formed on the control plates.

3.5. The Influence of Lactobacilli on Antibacterial Activity of Epithelial Cells, Muramidase and Lactoferrin

For studying the modifications of the antibacterial activity of epithelial cells, CSL or growth medium (MRS) were incubated with epithelial cells (~ 106 CFU/mL) for 6 hours at 37°C in a 1:7 ratio and the supernatant was separated. Thereafter, CSE or HBSS were incubated with *E. coli* and *S. aureus* (~ 106 CFU/mL) for one hour at 37°C in a 1:7 ratio. The mixtures were further diluted in buffered saline (pH 7.0-7.2) to yield 800 - 1000 CFU on control plates and plated on agar enriched with trypticase soy broth. The colonies were counted after an overnight incubation at 37°C. All the samples were tested in triplicate and the percentage of inhibition was determined relative to the colonies formed on the control plates.

For studying the modifications of the antibacterial activity of muramidase and lactoferrin, lactobacilli (~109 CFU/mL) were incubated in a medium (0.8 mM MgSO_4 , 0.3 mM MnSO_4 , 11.5 mM K_2HPO_4 , 11.5 mM glucose) at 37°C for three hours with aeration and the supernatant was separated. The supernatants or the previously-described nutrient medium were mixed with the solution of muramidase (400 $\mu\text{g/mL}$) or lactoferrin (1400 $\mu\text{g/mL}$) in 1:1 ratio and incubated for one hour at room temperature. Afterwards, for neutralizing H_2O_2 , the mixture was treated with catalase (8 U/mL). The minimum bactericidal concentrations of muramidase and lactoferrin were determined by plating the test strains on agar plates.

3.6. Statistical Analyses

The effects of the culture supernatants from the epithelial cells were dichotomized as stimulation or inhibition (more than 10%) of growth of lactobacilli relative to the control. Categorical variables were compared between the groups by chi-squared or Fisher's exact tests. Continuous variables were compared by the Student t-test or the Mann-Whitney U test, depending on the distribution of the data. All the tests were two-sided with P value < 0.05 considered as statistically significant (17).

4. Results

4.1. The Influence of Vaginal Epithelial Cells on Growth and Antibacterial Activity of Lactobacilli

Vaginal epithelial cells stimulated the growth of H₂O₂-producing lactobacilli significantly more than non-H₂O₂-producing lactobacilli (Table 2). The inhibition of growth of lactobacilli was observed rarely and was not associated with their ability to produce H₂O₂.

At baseline, H₂O₂-producing lactobacilli strongly inhibited the growth of *E. coli* compared to non-H₂O₂-producing lactobacilli. The antibacterial activity of H₂O₂-producing lactobacilli against *E. coli* after pre-incubation with the supernatant of vaginal epithelial cells increased to a greater degree than that of non-H₂O₂-producing lactobacilli (Table 3).

Initially, we did not find any relationship between the inhibition of the growth of *S. aureus* and the production of H₂O₂ by lactobacilli. The antibacterial activity of H₂O₂-

producing lactobacilli against *S. aureus* after pre-incubation with the supernatant of vaginal epithelial cells increased to a greater degree than that of the non-H₂O₂-producing lactobacilli (Table 3).

4.2. The Influence of Lactobacilli on Antibacterial Activity of Epithelial Cells, Muramidase and Lactoferrin

The antibacterial activity of the metabolites of vaginal epithelial cells was enhanced by lactobacilli. Increased antimicrobial activity of vaginal epithelial cells depended upon the ability of lactobacilli to produce H₂O₂ (Table 4).

The result of the influence of metabolites of lactobacilli on muramidase and lactoferrin showed increase of the antimicrobial activity of these proteins (Table 5). The supernatants of H₂O₂-producing lactobacilli reduced the minimal inhibitory concentration of muramidase against *E. coli* and *S. aureus* 52.3% and 22.0%, respectively. Non-H₂O₂-producing lactobacilli modified the bactericidal activity of muramidase less significantly. The bactericidal activity of lactoferrin under the influence of metabolites from lactobacilli changed identically with the bactericidal activity of muramidase (Table 5). Therefore, treatment by supernatants of peroxide-producing lactobacilli reduced the minimal inhibitory concentration of lactoferrin against *E. coli* and *S. aureus* 40.4% and 23.4%, respectively, whereas non-H₂O₂-producing lactobacilli almost did not modify the activity of lactoferrin. Mainly, peroxide-producing lactobacilli were capable of increasing the activity of the host antimicrobial peptides.

Table 1. The Ability of Lactobacilli to Produce Hydrogen Peroxide

Concentrations of Hydrogen Peroxide, mM	Total No. of Strains	Frequency, %
0 ≤ x ≤ 1.1	13	27.66
1.1 < x ≤ 2.1	16	34.04
2.1 < x ≤ 3.1	7	14.89
3.1 < x ≤ 4.1	6	12.77
4.1 < x ≤ 5.1	2	4.26
5.1 < x ≤ 6.1	2	4.26
6.1 < x ≤ 7.1	1	2.13

Table 2. The Influence of Vaginal Epithelial Cells on Growth of Lactobacilli ^a

<i>Lactobacillus</i> spp.	Frequency Stimulation Effect	Frequency Inhibition Effect	Frequency Indifferent Effect
H ₂ O ₂ producing	79	5	16
non-H ₂ O ₂ -producing	46 ^b	8	46 ^b

^a Values are presented as %.

^b The differences between H₂O₂-producing and non-H₂O₂-producing lactobacilli were statistically significant.

Table 3. The Influence of Vaginal Epithelial Cells on Antibacterial Activity of Lactobacilli

<i>Lactobacillus</i> spp.	Against <i>E. coli</i>		Against <i>S. aureus</i>	
	Without CSE	With CSE	Without CSE	With CSE
H₂O₂ producing	28.6 ± 0.8	38.5 ± 1.9	27.0 ± 0.4	41.4 ± 1.8
Non-H₂O₂ producing	19.9 ± 1.6 ^a	25.3 ± 2.4 ^a	25.2 ± 2,8	33.4 ± 3.4 ^a

^a The differences between H₂O₂-producing and non-H₂O₂-producing lactobacilli were statistically significant.

Table 4. The Influence of Lactobacilli on Antibacterial Activity of Vaginal Epithelial Cells^a

Antibacterial Activity of Vaginal Epithelial Cells	Against <i>E. coli</i>	Against <i>S. aureus</i>
Without CSL	5.6 ± 0.7	7.6 ± 0.5
With CSL of non-H₂O₂-producing	9.1 ± 1.1	11.0 ± 0.9
With CSL of H₂O₂ producing	15.3 ± 1.8 ^b	30.7 ± 2.7 ^b

^a Abbreviation: CSL, culture supernatants from *Lactobacillus*.

^b The differences between H₂O₂-producing and non-H₂O₂-producing lactobacilli were statistically significant.

Table 5. The Influence of Lactobacilli on Antibacterial Activities Muramidase and Lactoferrin^a

Test Strains/ Antimicrobial Polypeptides	Minimum Bactericidal Concentration, mg/mL		
	Control	With CSL of H ₂ O ₂ producing	With CSL of non-H ₂ O ₂ -producing
<i>E. coli</i>			
Muramidase	107 ± 9.8	51 ± 4.6 ^b	98 ± 10.3
Lactoferrin	47.2 ± 3.6	28.6 ± 1.8 ^b	44.6 ± 7.8
<i>S. aureus</i>			
Muramidase	93.6 ± 9.8	73 ± 5.9 ^b	88 ± 9.4
Lactoferrin	63.9 ± 5.1	49.1 ± 2.3 ^b	58.5 ± 6.4
<i>L. gasseri</i>			
Muramidase	7.2 ± 1.8	25.2 ± 3.8 ^b	9.3 ± 3.6
Lactoferrin	623 ± 21.8	617 ± 19.7	609 ± 20.3

^a Abbreviation: CSL, culture supernatants from *Lactobacillus*.

^b The differences compared to controls were statistically significant.

5. Discussion

In this study, evidence was obtained that the protection of vagina is not only the sum of individual actions of lactobacilli and host defense factors, but is determined by the results of the interaction of the host and commensals. The involvement of H₂O₂-producing lactobacilli in the protection of vagina was due to their ability to effectively interact with the host. On one side, this is manifested in the stimulation of growth and the antagonistic activity of H₂O₂-producing lactobacilli during the interaction with cells of the host; on the other side, H₂O₂-producing lactobacilli are able to stimulate the epithelial cells secretion of antimicrobial substances as well as to increase the antibacterial activity of already synthesized factors of protection (muramidase and lactoferrin). Therefore, the frequency of the occurrence species of lactobacilli, producing H₂O₂ from the reproductive tract of healthy females is high. This

accords with the previously formulated hypothesis about the role of ecological benefits and harms of lactobacilli (18) in maintaining the health and development of infections of the female reproductive tract.

Information on which *Lactobacillus* species may effectively prevent infections is contradictory. There are specific species of lactobacilli which are more prevalent in healthy subjects, but may also be present in the microflora of infected females (19). The role of commensal is determined not only by their direct influence on pathogens and increasing antimicrobial effects of other components of the protection of the ecosystem of the vagina. Based on the foregoing, adequate characterization of vaginal resistance requires not only an investigation of the individual components of the ecosystem, but also accounting the results of their interactions. This problem can be solved by using

multi-component model systems, approximate to reality. It would be correct to take into consideration when searching for potential probiotic strains. We regret to note that in the previous researches (9, 20), they aimed at evaluating the role of some protection components of the vagina, but the authors did not consider the interaction of the components of the ecosystem.

The phenomenon of mutual stimulation properties of host and lactobacilli detected in our research allowed explaining why the presence of H₂O₂-producing lactobacilli provided effective protection of the vagina. However, the role of particular metabolites in this process remains unclear, which requires further research.

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Authors' Contributions

Study concept and design: Elena A. Kremleva, Andrey V. Sgibnev. Analysis and interpretation of data: Elena A. Kremleva, Andrey V. Sgibnev. Drafting of the manuscript: Elena A. Kremleva, Andrey V. Sgibnev. Critical revision of the manuscript for important intellectual content: Elena A. Kremleva, Andrey V. Sgibnev. Statistical analysis: Elena A. Kremleva, Andrey V. Sgibnev.

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All the authors confirmed that they had no relevant financial interests or financial conflicts within the past five years and for the foreseeable future. They had no financial interests related to the material in the manuscript.

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