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Antimicrobial activity of vaginal lactobacilli against *Gardnerella vaginalis* and pathogens

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Abstract

Bacterial Vaginosis (BV) is a vaginal inflammatory disease, related to the high percentage of recurrences and serious obstetric, gynecological and reproductive problems. This is the reason why microbiologists and gynecologists search for new methods in the treatment and prevention of this condition. In this regard, the characterization of the biological activity of vaginal lactobacillus, that have a key role in the healthy homeostasis of the vagina, is an approach that will help uncover the natural mechanisms of the protection of the urogenital tract. The aim of the present study is to identify in vitro activity of vaginal *Lactobacillus*, isolated from healthy Bulgarian women (volunteers in the study) in reproductive age. The tests with the panel of pathogenic microorganisms proved a broad spectrum of activity against *Escherichia coli* and other pathogens. The results, obtained with cell-free supernatants of *Lactobacillus* spp. strain VLb3, were quite promising and the VLb3 is the strain that inhibits the growth of *G. vaginalis*, one of the main pathogens associated with BV. The selected Bulgarian strains lactobacillus VLb1 and VLb7 produce thermostable metabolites other than lactic acid and active against *E. coli.* Additional biochemical characterization, however, is needed and it is still in progress. In conclusion, in the natural microbiota of healthy Bulgarian women there are strains that are perspective and could be developed as a bio-active agents and vaginal probiotics.

Keywords: Bacterial vaginosis, vaginal lactobacilli, antimicrobial activity.

Introduction

One of the most recent discoveries that has thrown some light on the normal vaginal flora has been performed more than 100 years ago by Albert Döderlein – a renowned German Ob/gyn specialist. At the end of the 19th Century, he noticed that there is a dominance of Gram - positive bacteria in the vagina that were named Döderlein's bacteria, in his honor (Döderlein A, 1892). In 1901, they were termed *Lactobacillus* by Beijerinck and he proposed the inclusion of a new genus. The Genus *Lactobacillus* currently includes more than 170 different species (Goldstein E. J. et al. 2015) and only a part of them are representatives of protective vaginal microbiota (Mastromarino P., al. 2013). In the vagina, they are in constant competition and permanently dominate another 50 aerobic bacteria (Lamont R. F., et al. 2011). This is the result of the combination of diverse defense mechanisms (Mastromarino P., al. 2013) such as the competition with other microorganisms for nutrients and for adherence to the vaginal epithelium;

reduction of the vaginal pH by the production of organic acids, production of antimicrobial substances (Aroutcheva *et al.*, 2001).

Lactobacilli produce a wide range of active metabolites that assist them in the rivalry with other microbial species for one and the same ecological niche. These are organic acids (lactic acid, acetic acid and other short-chain acids), hydrogen peroxide, carbon dioxide, low-molecular antimicrobial substances (reuterin), a number of bacteriocins and bacteriocin-like substances (Karaoúlu S, L. et al. 2003). Zamfir M. investigates a bacteriocin, synthesized from the *Lactobacillus acidophilus* IBB 801 (Zamfir M. et al, 2000), which was reported as the most important vaginal *Lactobacillus* spp. In addition, the lactobacilli can participate in the protection of the vaginal epithelium by the means of a number of other barrier mechanisms (auto-aggregation, adhesion, formation of co-aggregates with potential pathogens, formation of a protective biofilm, etc.). This is the reason why the *Lactobacillus* could be used as prophylactic and therapeutic agents (McLean N, 2000).

In the last years, there is a growing interest of the researchers on the topic: what is the antimicrobial activity of vaginal lactobacilli in terms of *Gardnerella vaginalis*, a typical representative of microorganisms - associated with bacterial vaginosis (BV), as well as with other uropathogens. For example, Aroutcheva A. et al. (2001) reported a bacteriocin, produced by *Lactobacillus acidophilus* 160, which inhibits the growth of *G. vaginalis* (Aroutcheva A. et al., 2001). Bacteriocin-production is a strain-specific characteristic and the finding of active strains is possible with the assistance of active screening of a number of strains. The search for active strains against *G. vaginalis* and other pathogens presents not only theoretical but also practical interest in terms of the problematic therapy of bacterial vaginosis.

The aim of the present study is to identify *in vitro* activity of vaginal lactobacilli, isolated from healthy Bulgarian women (volunteers of the study) in reproductive age.

Materials and Methods

Microorganisms, nutrient medium and cultivation conditions

The investigations in the present research were conducted with 24 lactic-acid bacteria, isolated from vaginal swab samples, from healthy Bulgarian volunteers in reproductive age. They are selected after a survey with prophylactic examinations in Ob/Gyn Hospital "Dr. Shterev", Bulgaria.

The pure cultures are obtained of selective, for lactic-acid bacteria (LAB), culture medium MRS (*de Man, Rogosa & Sharpe*) agar (Merck, Germany) with pH 6.5. They were selected by a morphotype and in correspondence to the major characteristics of the genus *Lactobacillus*. After a revision of the purity of the vaginal LAB strains, they were preserved in MRS broth supplemented with glycerol (20% v/v) at - 20ºC until the start of the experiments.

The test–microorganisms, culture media and conditions, in assay for the determination of the spectrum of antibacterial activity of vaginal LAB strains, are presented in Table 1. They are stored as bacterial suspensions with 20% glycerol (v/v). For the *in vitro* experiments they have undergone twice pre-cultivation and were inoculated into the agar (inoculum 10^6-10^9 CFU/ml).

The assay for growth capability in acidic environment was carried out in four laboratory medium as followed: (i) modified MRS broth (experimental media) with pH 2.0, 3.0, 4.0, adjusted before the sterilization with lactic acid (Merck) and (ii) pH 6.5 (as a control) inoculated with 10^7 - 10^8 CFU/ml from over-night LAB cultures in MRS broth (pH 6.5), harvested (centrifugation at 3500 rpm, (Hermle, Germany) and washed cells with PBS 6.0 and equal aliquots are re-suspended in the MRS broth with corresponding pH 2.0; 3.0; 4.0; 6.5.

Inhibitory activity of vaginal lactobacilli

(1) Acidification ability of vaginal lactobacilli in laboratory conditions

A change in pH during the *Lactobacillus* cultivation is considered as an indicator of their ability to acidify the culture media. The pH was measured at the end of the growing exponential phase of each strain (36 h) with a pH meter (Hanna Instruments, HI2211) using a calibrated glass electrode. The experiments were performed in triplicate and the mean delta pH (pH 0h-pH 24h) ± s.d.

(2) Antibacterial activity

For the purpose of the investigation of antagonistic activity, double pre-cultivation of freshly thawed

aliquots of vaginal isolate stocks were performed, inoculated (with 1% v/v inoculum) in MRS broth (Merck, Germany) in anaerobic conditions (Anaerobic System GasPak®, BBL), with a subsequent cultivation with an application of 5% (v/v) and 10% (v/v) inoculum from an overnight culture in MRS broth.

The test-cultures were cultivated in the appropriate nutrient medium and temperature (Table 1).

Table 1. Test – microorganisms used to estimate the antibacterial activity of vaginal *Lactobacillus* strains

Test-microorganisms		Growth conditions:	
Name	Strain collection	T°C	Media
Escherichia coli	ATCC *25922*	37°C	MacConkey (Merck, Germany)
Escherichia coli	HB101, IMSA	37°C	Luria Bertran agar/broth
Listeria innocua F	82^T CIP, France	30°C	Elliker agar/broth
Bacillus subtilis	ATCC 6633	28°C	Nutrient agar/broth
Proteus vulgaris	*Д52*, IMSA	37°C	Nutrient agar/broth
Staphylloccocus aureus	ATCC 19636	37°C	Blood agar; Nutrient agar/broth
Klebsiella pneumoniae	*5214* CIP, France	37°C	Nutrient agar/broth
Salmonella typhimurium	160 IMSA	37°C	Nutrient agar/broth
Candida albicans	*562, NCIPD*	37°C	Sabouraud, agar/broth
Pseudomonas aeruginosa	*2* IMSA	37°C	Nutrient agar/broth
Gardnerella vaginalis	ATCC 14018	37°C	BHI with 20% horse serum agar
Yersinia enterocolitica	Serotype 3, IMSA	37°C	Nutrient agar/broth

**** Collections:** ATCC—American Type Culture Collection, Virginia, USA; CIP—Collection of Bacteries de l'Institute Pasteur, Paris, France; IMSA—Institute of Microbiology "Stephan Angeloff", Sofia, Bulgaria*

The inhibitory effect of vaginal strains on selected test-microorganisms was determined by the agar well-diffusion (Tagg and McGiven, 1971). Normally, 1.5% agar (Difco, USA) was used. The tests were performed with filtered (Millipore 0.45 µm) cell-free supernatants (CFS) – acid (non-treated) and neutralized with 5M NaOH to eliminate the effect of produced acids from exponential and/or stationary-phase LAB cultures of vaginal strains in MRS broth. In addition, 36-hours cultures of vaginal lactobacilli, in MRS broth were destroyed with autoclaving procedure at 121°C for 20 min, harvested by centrifugation at 5000 rpm (Hermle, Germany) and the supernatants was applied in *in vitro* tests.

The activity was reported in millimeters sterile zone, measured after 24 hours cultivation of the test-microorganism at 30°C or 37°C.

Results

In vitro antimicrobial activity of newly isolated vaginal lactobacilli from Bulgarian healthy women

In the present study 24 strains were tested for an inhibitory activity against a panel of 12 test-microorganisms, some of them closely related to the etiology of the bacterial vaginosis and/or other pathogens of the urogenital tract. The tests conducted with the agar-well diffusion tests (Tagg G. and McGiven, 1971) were performed with cell-free culture supernatants (CFS), obtained after 24 hour cultivation in MRS broth in anaerobic conditions. The summarized results are presented in Fig. 1.

It is widely known that the lactic acid bacteria inhibit the growth of pathogenic microorganisms through the production of different inhibiting substances (Arutcheva et al. 2001, Matu et al. 2010). This is the reason why the tests were performed in two variants: (i) with acidic pH (aCFS) and neutralized to the pH 6.0- 6.5 of the spent cultures (nCFS), with aim to eliminate the inhibitory effect of the produced lactic acid. All in vitro tests was repeated in triplicate. As expected with the assays with acid supernatants (no pH adjustment), a higher number of active strains and strong inhibition zones was detected (Fig. 1).

The most intriguing was the reported inhibitory effect of the acid CFS and neutralized CFS from 24-hour cultures of the strainVLb3 against *G. vaginalis* ATCC 14018. The zone of inhibition was with a diameter of 12±1 mm and it was clearly detected in both models of the study - with ~10^9 CFU/ml test-microorganism, inoculated in medium BHI, (as a model of Bacterial vaginosis) and with ~10^4 CFU/ml (like a post-therapeutic period or a status with low risk of vaginal infections).

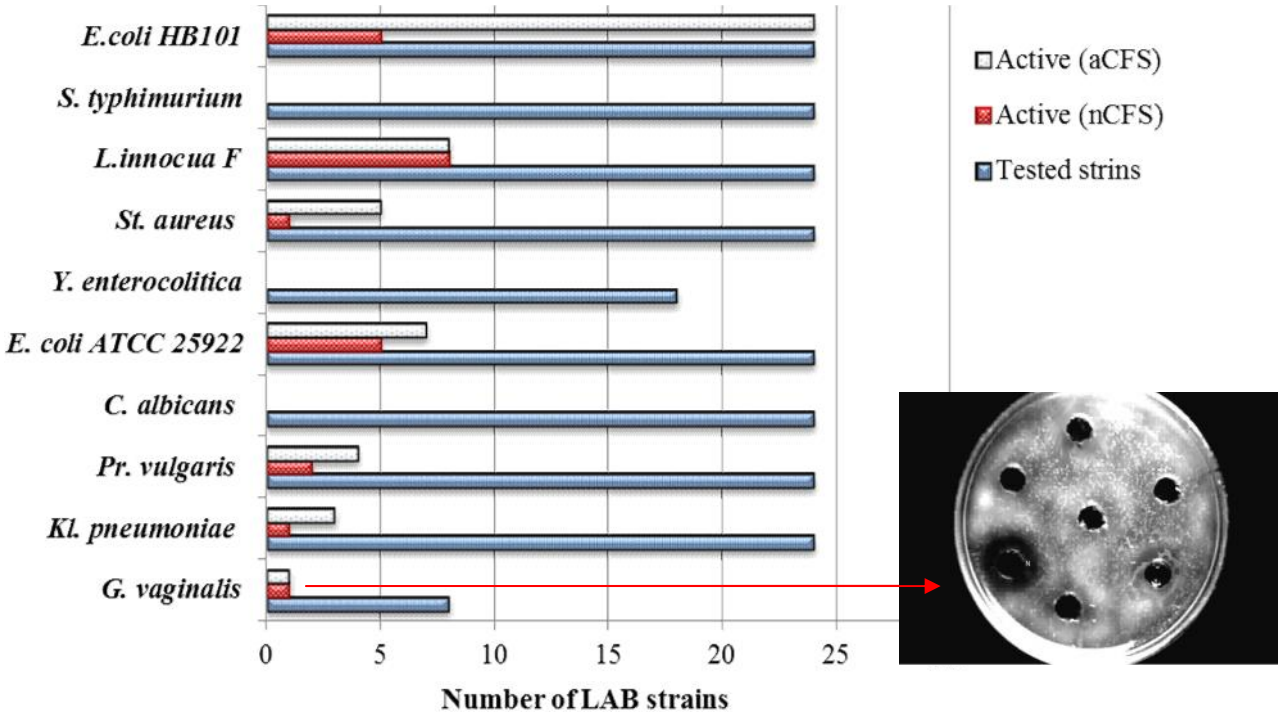

Fig. 1. Summary of results from *in vitro* tests for antagonistic activity of newly isolated vaginal lactobacilli from Bulgarian women.

It should be noted that a relatively high percentage of the active strains was observed against *E. coli* ATCC 25922 (Fig. 1) in the both variants, with acid and with neutralized CFS (Fig. 2 A, B). Likewise, the VLb1 strain showed a broad spectrum of activity against Gram (-) *E. coli* and Gram (+) *Bacillus subtilis* ATCC 6633. Such strong inhibitory effect against of *B. subtilis* was estimated with VLb1 and VLb7 36-hours cultures, which were destroyed by autoclaving at 121^0C for 20 min. (Fig. 2C and D). To our regret, these strains (VLb1, VLb7) as well as the others from the tested isolates did not show any activity against *Yersinia enterocolitica, Salmonella typhimurium* and *Candida albicans* in triple repeated tests. At once, eight out of tested 24 vaginal lactobacilli was able to inhibit the growth of food-associated *Listeria innocua* F (Fig.1). *L. innocua* F was used as a laboratory test – microorganism of the causative agent of the lysteriosis – *Listeria monocytogenes*, which may cause severe invasive disease in pregnant women and newborns (A. Benshushan et al. 2002). The results of agar-diffusion *in vitro* tests with vaginal lactobacilli showed a promising spectrum of activity. The inhibition of uropathogens is due to the produced lactic/other organic acids in combination with thermostable active metabolite(s) (strains VLb1 and VLb7) and bio-peptides or bacteriocin-like substances (BLIS) (Fig. 1 and Fig. 2B). However, additional tests are needed to estimate the nature of produced antagonistic substances. The pre-selected active strains should be characterized additionally in order to estimate them as putative probiotics.

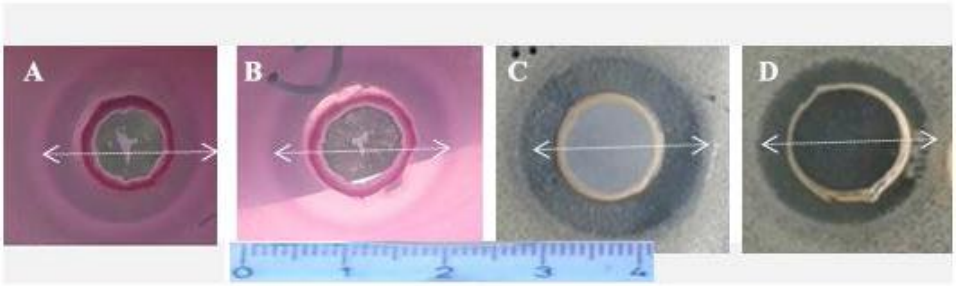

Fig.2. *In vitro* anti-bacterial activity of: vaginal strainVLb3 against *Escherichia coli* HB101 of detected with neutralized nCFS (A), and with acid aCFS (B); and against *Bacillus subtilis* ATCC6633 –autoclaved cultures of the strain VLb1 (C) and strain VLb7 (D).

Acid resistance and the acid-formation role of the newly isolated vaginal lactobacillus strains – experimental *in vitro* studies:

In order to test the capability of the investigated *Lactobacillus* strains for resistance to low pH, a prerequisite for the application of therapeutic effect in a vaginal environment, we tested additional factors affecting the biological potential of a group of vaginal *Lactobacillus* strains.

For this purpose 8 strains were pre-selected from the group of 24 isolates from healthy women in reproductive age who had never experienced bacterial vaginosis. This was the method to test the ability of lactobacilli, as a protective component of the normal vaginal flora, to acidify the medium, measured by decline of the pH. On the first stage, they were cultivated in MRS broth (pH 6.5) with indicator bromocresol purple that changes its color from red-violet (in the range = 6.8-5.2 pH) to yellow (decrease in pH below 5.2). We observed that all tested strains were capable to acidify the medium, as a result of the metabolism of carbohydrates, where the reporting of the change of the color of the indicator medium that proves the process, was accomplished in some strains even on the 24 hour (i.e. at the exponential phase) and in other strains on the 42-48 hours (data not shown). A more precise evidence of the accumulation of the lactic acid was obtained by re-culturing the LAB cultures in the same medium and recording the decrease in pH in the medium during the fermentation at 24h and 48h (Fig. 3). In triplicate repeated experiments was a marked decrease of pH values below 3.7 - 4.0, even in the late stationary phase of the LAB cultures (Fig. 3).

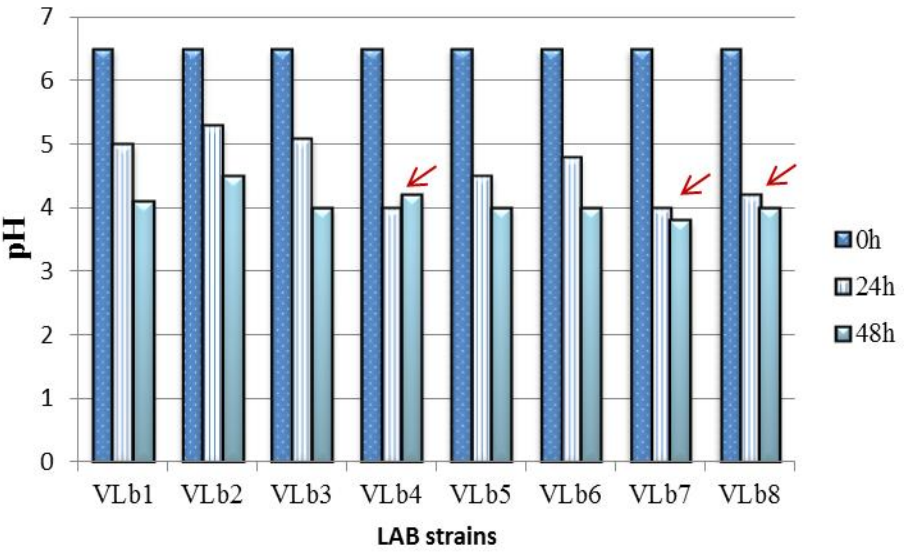

Fig. 3. Acidifying activity of selected vaginal LAB strains, during the cultivation in MRS broth (initial pH 6.5) at 37°C

In order to make our research closer to the *in vivo* conditions, we have additionally monitored the growth of the selected active LAB strains in acid pH conditions. For this reason, the growth of the *Lactobacillus* cultures was reported in parallel and they were inoculated with the same initial inoculum (~10^5 CFU/ml), in a MRS broth with initial pH-6.5; and adjusted pH before the experiments to the pH 4.0, pH 3.0 and pH 2.0 (Fig. 4).

The data from the change in the optical density (λ=600 nm) proved that there is a known pH-dependent inhibition of bacterial growth (Fig. 5). The tested lactobacilli maintained their vitality over a wide pH range: from 2.0 to 6.5 and active accumulation of biomass was also recorded. What was intriguing was the result that showed that they have not only survived but they also actively accumulate biomass in nutrient medium with pH 4.0 (Fig. 5.0) and up to pH 6.5. This parameter makes them emerge as potential probiotics for local/topical application. The obtained results showed that more than 50% of the group survive and growth at low pH – 3.0 and two of them in pH – 2.0 (Fig. 5).

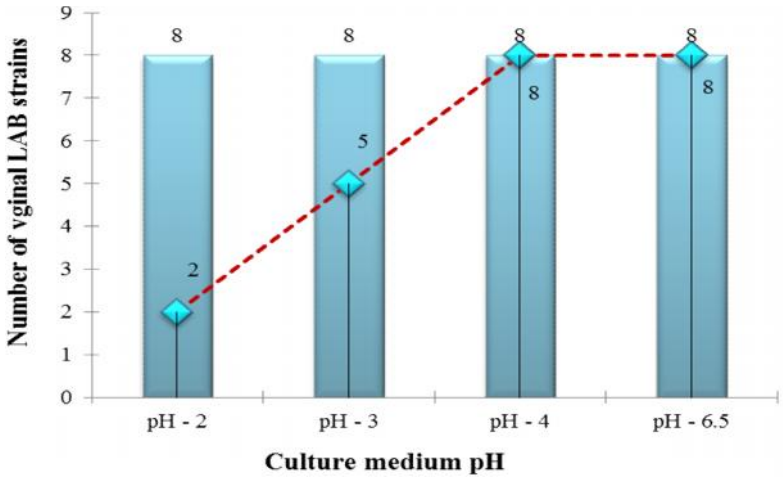

Fig. 4. Determination of the ability of eight vaginal *Lactobacillus* strains to grow at different pH of the culture medium.

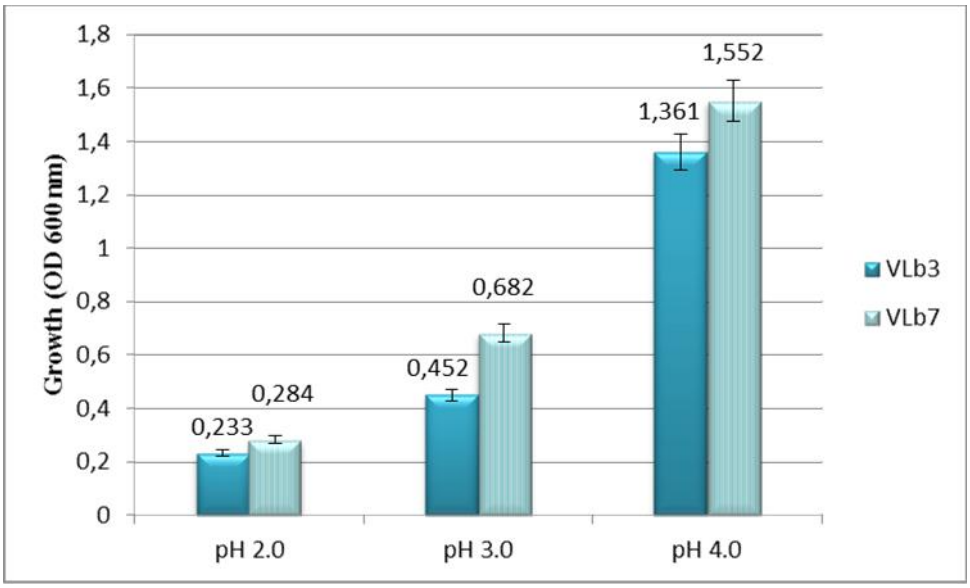

Fig. 5 Vaginal *Lactobacillus* spp. growth at low pH in a laboratory model system

Discussion

The vagina is a complex system with more than 50 anaerobic species which compete for nutrients. Our hypothesis is that during the struggle for existence and utilization of one and the same nutrient substrate, it is likely that the competitors will develop the ability to synthesize bacteriocins or other antagonistic substances. The bacteriocin production is one of the mechanisms which allow the domination of the species within the ecosystem or biocenoses. Usually the antagonistic activity is associated with the competitive fight, but there are numerous cases when it is directed against microorganisms belonging to a niche producer strain. Moreover, sometimes bacteriocins can perform a function other than an antibacterial one.

The research study, performed with the tested strains, showed antagonistic activity both for *G. vaginalis* and for other pathogenic microorganisms. Presumably, this is due to the synthesis of the lactic acid that is accumulated rapidly, this leads to a rapid decrease of the pH levels and then to a suppression of diverse pathogens. The inhibitory effect of organic acids is mainly caused by undissociated form of the molecules (Šuškoviš J. et al. 2010). The organic acids are the end product of the fermentation of LAB. Their formation and the pH reduction prolongs the lag phase of the sensitive microorganisms. It is a well-known fact that

the antibacterial activity emerges at higher levels of pH when it is derived from organic acids in comparison with the one, derived from the mineral acids. The toxic effects of lactic/acetic acid consist of the reduction of intracellular pH and dissipation of the membrane potential (Lorca, G.L. & G.F. de Valdez, 2009).

The monitoring of the activity of the neutralized supernatants eliminates as a potential inhibitory agent the produced lactic acids. In these cases, we can presume the synthesis of the bacteriocin - like substances and hydrogen peroxide (H_2O_2). One additional test (data not shown) with catalase treatment of the the active CFS, against the Gram negative bacteria, let us suggest the synthesis of the BLIS substances. Certainly, it is required more studies with a set of proteolytic agents for a precise nature evaluation of the antibacterial components to be selected.

Our results are to support the reported activity of vaginal lactobacilli from Kenyan women (Matu *et al*., 2009). Dasari S. *et al.* (2014) were isolated vaginal lactobacilli- producers of antimicrobial compounds, able to inhibit the growth of cervical pathogens (Dasari S. *et al.* 2014).

The search for antagonistic activity against selected pathogens and against fungi is justified by the vaginal isolates in terms of the fact that they are inhabitants of the same ecological niche. This is the reason why our screening studies include a wider range of test-microorganisms, along with *Listeria innocua F* (Fig. 1). During the screening with the test *E. coli* HB101 all acid supernatants show an inhibitory effect (Fig. 1), that proves the potential inhibitory effect of the produced lactic acid during the cultivation. In the same time the low pH do not influenced dramatically the viability of vaginal lactobacilli (Fig. 3). Their role as a protective element is well expressed on different levels - from the maintenance of an acidity of the vagina, the synthesis of hydrogen peroxide, bacteriocins or BLIS and other antimicrobial substances, to direct antagonism and a competition with undesirable pathogens. In the scientific literature there is a lot of data that proves their role as natural antagonists. Lactobacilli, with antagonistic activity (Fig. 1) could be a beneficial competitor of the other microorganisms that colonize the vagina. They can play a central role in improvement and maintaining the internal microbial balance and to provide the effective protection of the urogenital tract. Promising are results with high acid resistance of newly isolated strains (Fig. 4 and 5), together with capacity to acidify actively the environmental. Thus, they will survive well in acidic environment and contribute to the mild acidity – pH ~4.5 (Fig. 3), which correspond to the healthy vagina. Therefore, the detection and characterization of new strains with biological activity has an important practical and fundamental significance. It help the discovery of new methods for protection from the development of pathogenic and putrefactious microorganisms and to protect human health naturally.

Established by our team anti-*G. vaginalis* active strain VLb3, is a natural Bulgarian vaginal isolate and it can be assumed that it expresses *in vivo* the same activity. It contains other biological properties such as fast acidifying capacity (Fig. 3) and high acid resistance (Fig. 5). Its full list of functional characteristics would be of interest in connection with the possible application of BLIS-producing strains in the prevention of certain diseases of the urogenital tract and also as a potential alternative of the settlement of the increasing drug resistance. A more detailed development of this strain could turn out to be one a promising start of the application of bacteriocionogenic strain for therapeutic or prophylactic purposes.

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