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ORIGINAL ARTICLE

Anti-candida activity of *Lacticaseibacillus rhamnosus* R-2002 and its possible application in candidiasis prevention

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ABSTRACT

Background and Objectives: Probiotics are used for the treatment of yeast infections, they restore the balance in vaginal microbiome, adhere to epithelial cells, compete against pathogenic bacteria, acidify the environment, produce bacteriocins and modulate the immunity. The aim of the study was to investigate the anti-yeast activity (AYA) of the strain *Lacticaseibacillus rhamnosus* R-2002 against different *Candida* species.
Materials and Methods: From 20 strains of lactic acid bacteria examined, only *L. rhamnosus* R-2002 strain demonstrated beneficial properties against yeast. The effects of temperature and pH on AYA and its relation to cell wall were revealed by bi-layer agar assay. The connection of AYA to the cell wall was determined with the sonicated cells.
Results: R-2002 inhibited the growth of *C. albicans* ATCC 10291, *C. tropicalis* G 31 and *C. albicans* G4 (both isolated from vaginal samples). R-2002 maintained its AYA between a wide range of pH and its anti-yeast component/s are extracellular. The tested strain demonstrated stability against the high concentrations of progesterone and metronidazole, making it a suitable candidate for the mitigation of vaginitis.
Conclusion: The present study summarizes all the positive features of the strain R-2002 and its potential as a therapeutic agent in the treatment of candidiasis.

Keywords: Antifungal activity; *Lacticaseibacillus rhamnosus*; *Candida albicans*; Candidiasis; Bacteriocins

INTRODUCTION

Candida species are found in healthy human skin and various tracts (gastrointestinal and genital), they can also act as opportunistic pathogens, causing infections in mucosal layers (1). They are one of the main causes of fungal infection worldwide (2). Infectious properties of *Candida* are characterized by the transition from the single cell to mycelial form (3). There are five main species of *Candida* that cause

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infections: *C. albicans, C. glabrata, C. tropicalis, C. parapsilosis,* and *C. krusei.* Infections are very often caused by a combination of these species (4, 5).

The development of antifungal resistance of *Candida* species has increased the need for alternative treatment methods against *Candida* infections. The application of probiotic *Lactobacillus* species is a promising alternative for the treatment of infections (6).

Lacticaseibacillus rhamnosus (formerly *Lactobacillus rhamnosus*) belongs to the phylum Firmicutes, class Bacilli, order Lactobacillales, family *Lactobacillaceae,* genus *Lactobacillus.* Until 2020 this species was known as *Lactobacillus rhamnosus.* In 2020, based on the molecular-genetic analysis, Zheng et al. re-classified and renamed it as *Lacticaseibacillus rhamnosus* (7).

The strains of the species *L. rhamnosus* have been used as probiotic for a long time, especially in preventing and treating various gynecological diseases. The interest in this probiotic has not decreased, because different strains of this species may have different properties and activities.

L. rhamnosus R-2002 was isolated from traditional Armenian cheese Chanakh (8). *L. rhamnosus* R-2002 demonstrated maximum anti-bacterial activity against a wide spectrum of test organisms when it was grown in strictly anaerobic conditions, pH 4.77 for 9 hours, OD 0.305. The study of the viability of this strain in the stress conditions of gastrointestinal tract revealed the higher resistance of this strain compared to the others (9). Moreover, Bazukyan et al. demonstrated the inhibitory activity of *L. rhamnosus* R-2002 against the yeast *Debaryomyces hansenii* and molds *Penicillium aurantioviolaceum* and *Mucor plumbeus,* while anti-mold activity is non-distinctive for *L. rhamnosus* (10). Its maximum antibacterial activity was reached after 24 h, while its antifungal activity (AFA) was reached after 48h. Also, the bacterial components responsible for both antibacterial and AFA were sensitive to temperature, meanwhile they functioned at a wide spectrum of pH. Since the effect of various proteinases decreased the arbitrary units of antibacterial and antifungal components, the proteic nature of the active component was revealed (10).

The fungicide effect of this strain depends on the nature of the carbon source in the media. In the presence of some carbon sources, AFA can widen its spectrum of activity (11). *L. rhamnosus* species is often used in associations with other probiotic bacteria, however, this strain inhibits the growth of other bacteria in bacterial associations, resulting in decreased AFA (12).

The weakening of the immunity in organism can contribute to the development of candidiasis in women. It may be conditioned by physiological conditions, such as pregnancy or menstrual cycle. Increased progesterone level is associated with a high number of Firmicutes phylum representatives, while the decreased level of progesterone is correlated with high amount of Proteobacteria representatives (13). Metronidazole is very often applied for the treatment of vaginosis. However, it can also have negative effects on the whole vaginal microbiome, including lactic acid bacteria (LAB). High concentrations of metronidazole may completely inhibit the growth of lactobacilli (14).

L. rhamnosus species has been widely used against different *Candida* species and yeast infections. The action mechanisms against *Candida* are various and not fully elucidated yet. Some mechanisms involve the inhibition of biofilm formation and filamentation, as well as competition for adhesion sites of *Candida* (15). Additionally, the mutual growth of *Candida* and *L. rhamnosus* results in the decrease of proteinase synthesis, hemolysis, hyphae and biofilm production by *Candida* (16). Anti-candida effect of *L. rhamnosus* may also be conditioned by the blocking of adhesion sites and the reduction of nutrients (17). *Lactobacillus* representatives also regulate the reproduction and growth of pathogens through their metabolites that possess antagonistic properties, specifically, biosurfactants and bacteriocins (18). *L. rhamnosus* is involved in the regulation of the genes of *C. albicans* which are responsible for filamentation and adhesion (*als3* and *hwp1*) and transcription (*bcr1* and *cph1*) (19). Moreover, its supernatant was able to decrease the biomass and the metabolic activity of various *Candida* representatives (20).

Thus, the aim of this investigation was to determine the inhibitory effect of *L. rhamnosus* R-2002 against clinical *C. albicans* G4 and non-*albicans Candida* strains, as well as its resistance to progesterone and metronidazole, to reveal some properties of anti-fungal components.

MATERIALS AND METHODS

Objects. The objects of research are LAB strains kept at the Chair of Biochemistry, Microbiology and Biotechnology of Yerevan State University, which

were isolated from traditional dairy products of farms in different regions of Armenia, as well as from the gastrointestinal tract of bees. The strains were identified as *L. rhamnosus* R-2002 (accession number KY054594 and presented in gene library) deposited in Microbial Depository Center (WDM803) («Armbiotechnology» scientific and production center, RA National Academy of Sciences, Yerevan) under the number MDC9661, *Lactobacillus delbrueckii* subsp. *lactis* INRA-2010-4.2 and *L. delbrueckii* subsp. *bulgaricus* INRA-2010-5.2 presented, respectively, under the numbers MDC9632 and MDC9633 (21), *Enterococcus durans* (provided by the National Agricultural Research Institute, Nantes, France, INRA) (22), *Lactobacillus acidophilus* MDC 11227, *Lactobacillus paracasei* subsp. *paracasei* MDC 10888, *Lactobacillus buchneri* MDC 11229, *L. delbrueckii* subsp. *bulgaricus* (RIN-2003-Ls), *L. delbrueckii* subsp. *bulgaricus* BAM-2003-LB, *L. rhamnosus* INA-5.1, *L. rhamnosus* INA-21.1, *L. plantarum* INA-21.2, *Enterococcus hirae* INR-2010-Tsov-G-St, *E. faecium* KDS-1, *E. faecium* KDS-2, *Lactococcus cremoris* KDS-3, *Streptococcus thermophilus* KDS-4 (isolated from different Armenian dairy products), *L. helveticus* INRA-2010-H11, *L. acidophilus* MR-1980 (isolated from the feces of one-month-old baby).

All LAB strains were preserved in Man-Rogosa-Sharpe (MRS) broth in a freezer (at -30°C) with 20% glycerol and recultivated in MRS broth at 37°C during 24 hours for the consequent experiments.

Study of the anti-yeast activity of LAB against some *Candida* species. The anti-yeast activity of LAB was determined by the method described by Afzali et al. with slight modification (23). As a test organism *C. albicans* ATCC 10291 and other *Candida* species, isolated from the vaginal samples provided by Beglaryan and Erebuni medical centers were used. They were cultivated on Condalab *Candida* chromogenic agar (Laboratorios Conda, S.A., Spain) and identified according to the manufacturer's instructions.

First, LAB strains were cultivated in modified MRS medium containing 1.5% agar at 37°C for 48 hrs. After this incubation period, the surface of the plates was covered with 7 ml of pre-thawed and cooled till 50°C with Sabouraud nutrient medium, containing 0.9% agar. The yeast suspension, pre-cultivated in liquid Sabouraud medium at 37°C for 24 hours, was serially diluted to 106, and 0.5 ml from each dilution was poured onto the Sabouraud medium and spread over the entire surface. The yeasts were then incubated at 37°C for an additional 48 hours. A decrease in the CFU/ml of yeast compared to the control indicates the presence of AYA. As a control, the same system was used without the presence of LAB.

Effect of temperature on the AYA of *L. rhamnosus* R-2002 against *C. albicans* ATCC 10291. LAB were grown in liquid MRS nutrient medium for 24 hours at 3°C. Then the cultural liquids were exposed to 45-75°C for 15 minutes. Residual AYA was studied in the above-mentioned way. The obtained data were compared with positive and negative controls. The culture grown at 37°C served as a positive control, and the CFU number of yeast grown without LAB was used as a negative control (10).

Effect of pH on the AYA of *L. rhamnosus* R-2002 against *C. albicans* ATCC 10291. LAB were grown in MRS nutrient medium, then the pH of the resulting culture was adjusted to pH 5-9 with 40% NaOH solution or concentrated HCl. Residual AYA was studied in the above-mentioned way. The CFUs of remaining viable yeasts were compared to the CFUs of pH-unaltered culture fluid-stressed yeast and with the control (10).

Study of extracellular and intracellular nature of *L. rhamnosus* R-2002 anti-yeast activity. The study of the connection between anti-yeast component/s and the cell wall was conducted using with the method described by Matevosyan et al. (10). *L. rhamnosus* R-2002 was grown in MRS nutrient medium for 48 hours at 37°C. Then the cells were separated from the culture fluid with centrifugation for 40 min at 3000 RPM: The cell-free supernatant was collected and dried in a vacuum oven at 37°C until the volume of the supernatant was decreased for 12.5 times. The cells were resuspended in 1 ml saline and frozen at -20°C for 12 hours. The cells were then disintegrated by sonication for 30 min at a sound frequency of 500 Hz and a power of 300 W (Emerson 40:0.15, Branson, Mexico). Cell debris was separated by centrifugation at 13 500 RPM for 5 min. The AYAs of the resulting culture fluid were concentrated, and the cell supernatant and cell debris were studied as described above. The obtained data were compared with the AYA of undegraded bacteria according to the generation of residual CFU of *C. albicans* ATCC 10291.

Determination of glycosyltransferase activity. The glycosyltransferase activity was determined with the native 12% polyacrylamide gel electrophoresis

method followed by incubation in 10% sucrose solution and testing the presence of polysaccharides with Schiff's reagent, as described before (24).

The synthesis of polysaccharides was tested with Congo red staining method too (25).

Effect of progesterone and metronidazole on growth and antibacterial activity. The effect of metronidazole on the growth was carried out through broth microdilution, according to the CLSI. Bacteria were cultivated in MRS broth, and the experiment was conducted according to the method described earlier (14). The pre-cultivation of bacteria was performed in MRS broth for 24 hours at 37°C. The cell concentration was adjusted to 10^8 CFU/ml. This suspension was then added to fresh MRS broth to achieve a concentration of 10^4 CFU/ml. Simultaneously, the appropriate concentration of metronidazole was added to the media from a concentrated stock. The mixture was evenly dispensed into sterile 96-Well Microtiter™ Microplates, which were then placed in a thermostat for incubation at 37°C for 48 hours. Samples were taken every 3 hours, and the optical density was measured with Thermo Scientific ALPS5000 Automated Microplate Plate Heat Sealer (USA). The final concentration of metronidazole ranged from 0.1 to 1 μg/ml.

The effect of progesterone was determined by applying the concentrations 1.4-333 ng/ml, which correspond to the various physiological conditions of a woman's organism (26).

Data processing. Mean values and standard deviations were determined after three independently repeated experiments and the obtained data were processed with the software Statgraphics (Statpoint Technologies, Inc; Warrenton, Virginina, USA). The statistical significance of collected data was determined with R Project for Statistical Computing version R 3.1.0 (The R foundation for Statistical Computing, Vienna, Austria); $P<0.05$. Besides, the received data were processed by a 2-way ANOVA Tukey's Statistical Test, using GraphPad Prism 8.0.2 software (GraphPad Software, USA).

RESULTS

The AYA of 20 different LAB strains were studied. The data are presented in Fig. 1.

Only *L. delbrueckii* subsp. *bulgaricus* RIN-2003-LS, *L. rhamnosus* R-2002 and *L. acidophilus* MDC 11227 possess AYA. And *L. buchneri* MDS 11229 only partially inhibits the growth of the representative of the genus *Candida*.

Since only the RIN-2003-LS, R-2002, and MDC 11227 strains exhibited AYA, further studies focused on these strains. AYA of those three strains was tested against other *Candida* species isolated from the vaginal samples (*C. tropicalis* G31, *C. glabrata* G 7 and *C. krusei* G11).

Although all 3 strains totally inhibited the growth of *C. albicans* ATCC 10291, only R-2002 and MDS 11229 demonstrated AYA against vaginal *C. albicans* G4 strain. RIN-2003-LS strain did not have any AYA against vaginal *C. albicans* G4 (Table 1).

None of the tested strains had AYA against two vaginal *Candida* species *C. glabrata* G41 and *C. krusei* G11. The only strain that inhibited the growth of *C. tropicalis* G31 was the strain R-2002, moreover, *C. tropicalis* G31 colonies were very small, which testifies once again that R-2002 has significant AYA against *C. tropicalis* G31. Therefore, all the subsequent studies were carried out with the strain *L. rhamnosus* R-2002.

The duration of LAB cultivation on the manifestation of AYA was studied. The obtained data are presented in Fig. 2.

The strain R-2002 shows the maximum AYA at the 48^{th} hour of cultivation and partial AYA at the 72^{nd} hour of cultivation. After 48 hours, the cells begin to die, therefore, AYA begins to decrease.

The temperature dependence of AYA was studied. The data are presented in Fig. 3.

The stability of AYA exhibited by *L. rhamnosus* R-2002 at different pH values was also studied. The obtained data are shown in Fig. 4.

The presence of substances with AYA in the cell-free culture fluid, in the supernatant of the crude lysate of ultrasound-disrupted cells, and in the cell wall remnants was studied. The obtained data are presented in Table 2.

The obtained data indicate that the substances possessing AYA are extracellular.

No glycosyltransferase activity was observed neither electrophoretically, nor by the staining method with Congo red (data not shown).

The effect of progesterone and metronidazole on the growth and antibacterial activity of *L. rhamnosus* R-2002 and *L. acidophilus* MR-1980 was also investigated. The results are presented in Figs. 5 and 6.

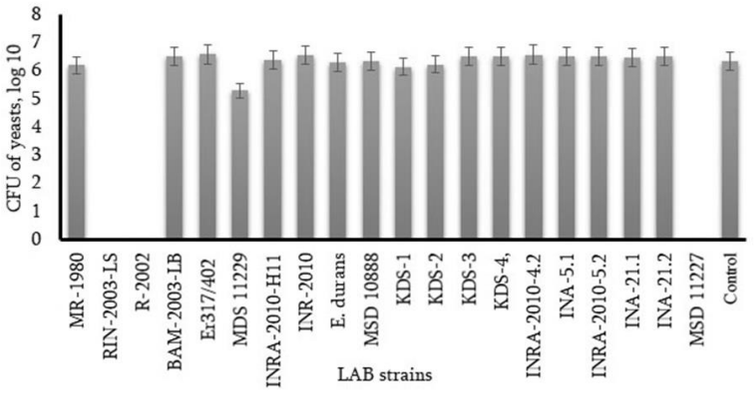

Fig. 1. The AYA of lactic acid bacteria against *C. albicans* ATCC 10291
*All the data presented in the figure are the mean of three independent experiments, P<0.0005

Table 1. Anti-yeast activity of LAB against some *Candida* species (CFU/ml)

	***C. tropicalis* G31**	***C. glabrata* G41**	***C. krusei* G11**	***C. albicans* G4**
Control	$5 \times 10^6 \pm 0.4^*$	$3 \times 10^7 \pm 0.4$	$4.6 \times 10^7 \pm 0.3$	$5.5 \times 10^6 \pm 0.1$
L. rhamnosus R-2002	$3 \times 10^4 \pm 0.3$	$3 \times 10^7 \pm 0.3$	$2 \times 10^7 \pm 0.5$	$1 \times 10^2 \pm 0.2$
L. delbrueckii subsp. *bulgaricus* RIN-2003-LS	$4.6 \times 10^6 \pm 0.2$	$5 \times 10^7 \pm 0.2$	$3 \times 10^7 \pm 0.4$	$4.5 \times 10^6 \pm 0.5$
L. buchneri MDS 11229	$5 \times 10^6 \pm 0.5$	$2 \times 10^7 \pm 0.1$	$4 \times 10^7 \pm 0.5$	$1 \times 10^2 \pm 0.2$

*All the data presented in the table are the mean of three independent experiments, P<0.0008

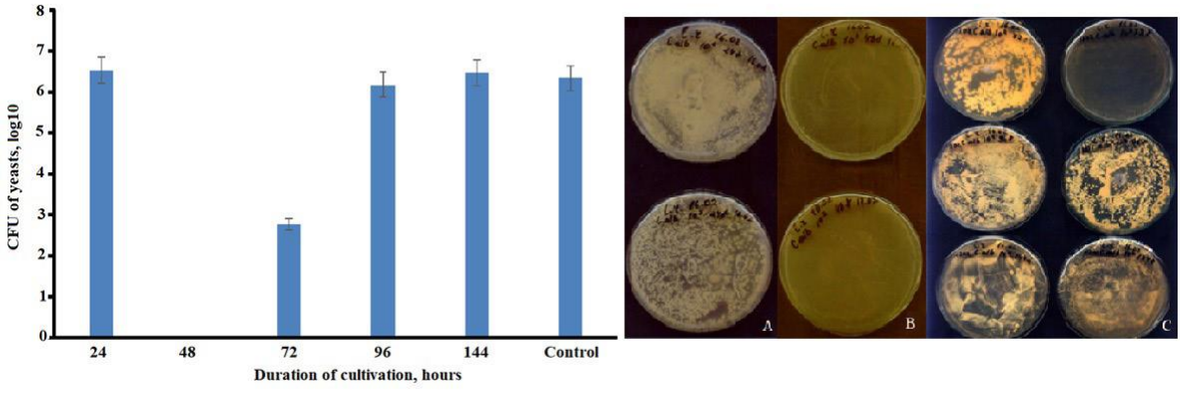

Fig. 2. The kinetics of the synthesis of the component/s with anti-yeast activity
Anti-yeast activity of R-2002 strain after A – 24 hours, B – 48 hours, C – 72, 96 and 144 hours, given in log10 of the CFU/ml of the yeasts.

The increase in progesterone concentration did not have a significant effect on the growth of *L. rhamnosus* R-2002 strain, while the growth of *L. acidophilus* MR-1980 growth was dramatically inhibited at progesterone concentrations above 67 ng/ml. The results show that *L. rhamnosus* R-2002 strain is stable to 1 μg/ml concentration of metronidazole, while *L. acidophilus* MR-1980 is sensitive to the tested antibiotic starting from 0.1 μg/ml. Moreover, different concentrations of progesterone affect the antibacterial activity of *L. rhamnosus* R-2002 strain. The increasing concentrations of progesterone (209 and 333 ng/ml) lead to a complete decrease in antibacterial activity (Fig. 6).

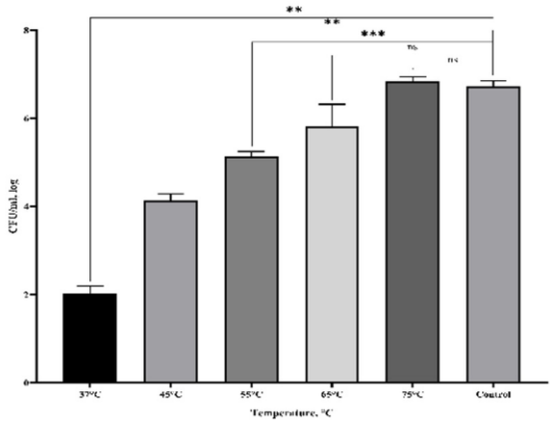

Fig. 3. The dependence of R-2002 strain on temperature.
** P value <0.008, *** P<0.0004, ns 0.1362-0.3236

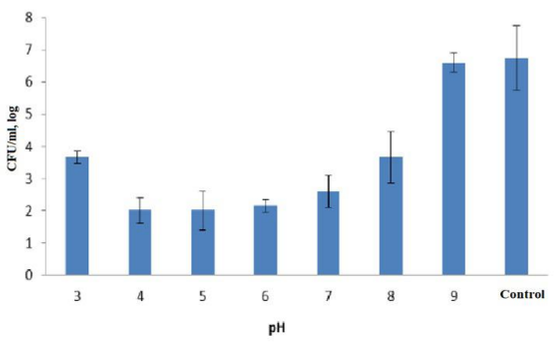

Fig. 4. The effect of pH on AYA of *L. rhamnosus* R-2002
* P value <0.035, ** P<0.0022, *** P<0.0005
AYA is maintained in the pH range of 4-7.

DISCUSSION

The AFA of LAB strains stored in the Department of Biochemistry, Microbiology and Biotechnology of the Faculty of Biology of Yerevan State University has previously been studied (4, 6). However, previous studies did not reveal any AYA of these LAB strains. Matevosyan et al. used the method of well diffusion in agar, where the AYA of LAB was studied after growing for 24 hours (10). However, they showed that these strains have AFA when mycelial fungi, such as *Trichoderma viride*, *Penicillum aurantioviolaceum*, *Geotrichum candidum*, *Aspergillus flavus*, *Mucor plumbeus* were used as test organisms (12). Moreover, this group of authors suggested that the AFA is manifested after cultivation for 48 hrs (12). For this purpose, we developed a modification of the method of agar layers, which made it possible to study LAB AYA after growing them for 48 hrs. The specificity of this modification is the use of MRS nutrient medium for the growth of LAB, which was then covered with a second layer of the nutrient medium containing 0.9% Sabouraud agar for the growth of yeasts. This concentration of agar was used for easy diffusion of the substance(s) possessing AYA. From the investigated 20 LAB strains only *L. delbrueckii* subsp. *bulgaricus* RIN-2003-LS, *L. rhamnosus* R-2002 and *L. acidophilus* MDC 11227 showed AYA after 48 hrs of cultivation. It is also reported that strains of LAB isolated from natural honey from Malaysia, Su-

Table 2. Binding of *L. rhamnosus* R-2002 anti-candida substances to the cell wall

Sample	Crude lysate supernatant	Cell wall remnants	10× dense cell-free culture fluid	Control
Candida G4 CFU/ml	$3 \times 10^{6\,ns}$	$2 \times 10^{6*}$	0***	5.5×10^{6}

*All the data presented in the Table 2 are the mean of three independent experiments, P value <0.0008, ns <0.5744, * <0.0382, ***<0.0006

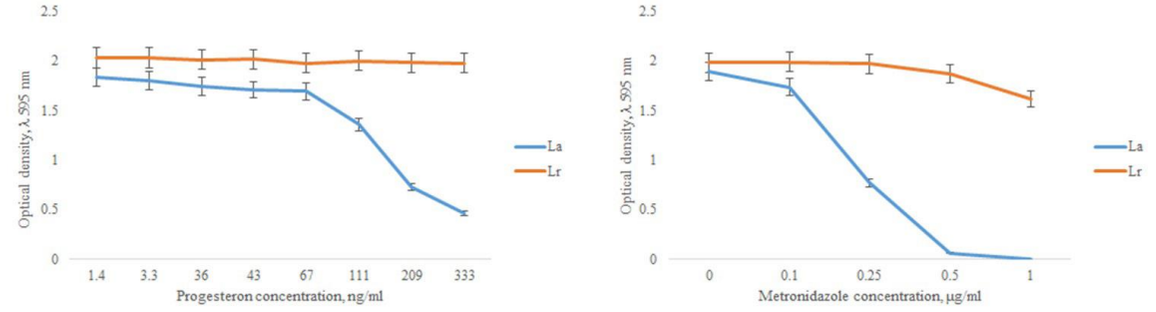

Fig. 5. The effect of different concentrations of progesterone and metranidazole on the growth of LAB
La- *L. acidophilus* MR-1980, Lr- *L. rhamnosus* R-2002

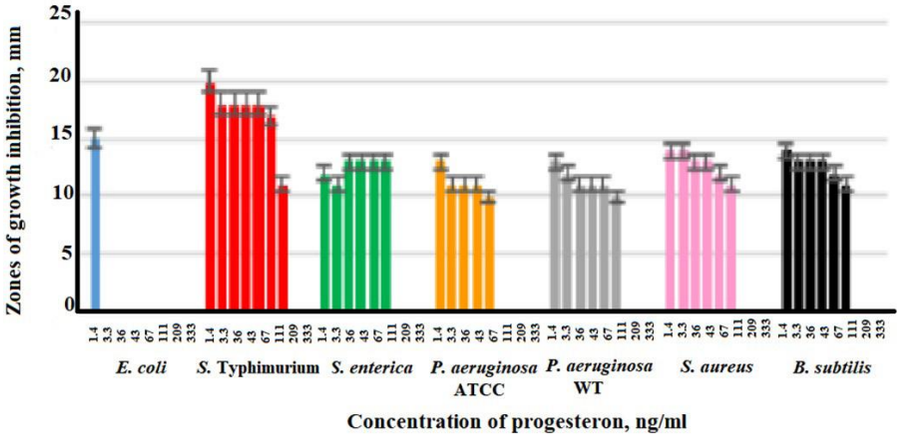

Fig. 6. The effect of different concentrations of progesterone on the antibacterial activity of *L. rhamnosus* R-2002

dan and Saudi Arabia including *L. plantarum* HS, *L. curvatus* HH, *P. acidilactici* HC, *P. pentosaceus* HM demonstrate AYA against pathogenic species belonging to the genus *Candida: C. glabrata* ATCC 2001, *C. parapsilosis* ATCC 2201, *C. tropicalis A*TCC 750, *C. krusei, C. albicans* (25). Interestingly, the cultivation up to 72 hrs led to the decrease of AYA of the studied strains. It is necessary to mention that an interesting effect of LAB AYA was observed during the experiments. When the amount of *Candida* G4 exceeds 10^6 CFU/ml, the LAB AYA is not observed, but when the number of yeasts is diluted to 10^4, AYA is observed completely, therefore, well diffusion method is not appropriate for the determination of AYA. In contrast, double layer diffusion method with diluted *Candida* can be used to testify AYA. One possible explanation for this is the quorum-sensing mechanism. However, multiple experiments are still needed to confirm this theory.

Our studies suggest that the anti-fungal component/s of the strain is not thermostable and AYA decreased significantly at 45°C and disappeared completely at 75°C. The low thermostability of *L. rhamnosus* R-2002 AYA limits its application. Our results are consistent with the experimental data of other authors. It was shown by Matevosyan et al. that processing the culture liquid of *L. rhamnosus* R-2002 at different temperature conditions (45°C-80°C) leads to a sharp decrease in AFA against mycelial fungi (12). So R-2002 loses its AFA against *P. aurantioviolaceum* after 15 min of treatment at 60°C, while it is unstable against *M. plumbeus* even at 45°C (12). At this point our received data differ from the similar studies performed by other authors. In one study it was shown that the AFA of LAB strains is retained under heat treatment, refrigeration and deep freezing. Similarly, Liu et al. showed that filtered culture fluid of *L. pentosus* 86 retained its AFA after heat treatment at 100°C for 30 min (27), and Rouse et al. found stability of AFA in concentrated *L. plantarum* in the supernatant, after subjecting the latter to heat treatment at 80°C for 1 h or 121°C for 15 min (28). Miescher et al. showed that the treatment of *L. paracasei* subsp. *paracasei* strain supernatant at 100°C for 10 min had no effect on AFA (29). Similar data were obtained in another work with heat treatment of *L. plantarum* supernatant liquid. Meanwhile, it is well known that Class III bacteriocins are very sensitive to the temperature and show a loss of AFA at 100°C (30). Thus, when applying the active compounds of *L. rhamnosus* MDC R-2002 to suppress the growth of yeasts in medicine, in no case should it be treated with temperature.

Because LAB produce organic acids, they may also activate the synthesis of other antifungal agents, such as peptides, by lowering pH (12). Our experiments were carried out in the conditions of a wide range of pH and according to obtained data, *L. rhamnosus* MDC R-2002 maintains AYA at pH spectrum 4-7. These data do not agree with the study of AFA of the same strain, according to which *L. rhamnosus* R-2002, cultivated for 48 h at 37°C retains its AFA against *P. aurantioviolaceum* and *M. plumbeus* at a wide range of pH values 3-10. Bazukyan et al. showed that the optimal pH spectrum of AFA of LAB against some molds is wider than AYA against some yeasts, which means that the mechanism of action may be different (10). Studies of pH-dependent

AFA have also been performed by other authors, resulting in conflicting data. It was shown that when *L. acidophilus, L. amylovorus, L. brevis* and *L. coryniformis* subsp. *coryniformis* strains supernatant pH value was corrected to 5.0; 5.5 and 6.0, the AFA was suppressed (31). Another study found that the AFA of *L. plantarum* was maintained at pH values of 6.0 and 7.0, suggesting that the AFA depends on both dissociated and undissociated organic acids. *L. lactis* subsp. *diacetylactis* was able to synthesize significant amounts of antifungal agent over a narrow range of pH values (5.5-7.0), although maximal synthesis was observed at pH 6.8 (32).

Thus, the effect of pH depends on many factors such as environment, the duration of cultivation, temperature conditions, test organism etc.. Therefore, the explanation of the nature and the localization of anti-yeast component/s is also very essential. Our studies indicate that anti-yeast component/s of *L. rhamnosus* R-2002 are extracellular, as the dense cell-free cultural liquid was able to inhibit the growth of yeasts. These data are again different from previously obtained data. Matevosyan et al. showed that the supernatant liquid did not show antifungal activity (10), but a partial inhibition of the growth of *P. aurantioviolaceum* and *M. plumbeus* mold fungi was observed (12). The study of the AFA of the concentrated supernatant (pH 6.5) 10× by evaporation and 22.5× by lyophilization drying did not give any positive results. It was shown that LAB strains display antifungal properties through mechanisms related to their cell surface proteins (33).

It is well known that LAB can synthesize exopolysaccharides (EPSs). EPS can be responsible for antagonistic, anti-inflammatory, immunomodulatory antioxidant activities and the adhesion of LAB on mucous layers, as well as for biofilm formation (34). One of the EPS is polyglycans, for the synthesis of which glycosyltransferases are responsible. It was shown that the LAB glycans compete with *Candida* glycans for the receptors on the epithelial cells' surface. This is the main mechanism which explains the inhibition of *Candida* hyphae formation by LAB (35). Although, the presence of glycosyltransferases in *L. rhamnosus* R-2002 strain was not detected by us. It can be concluded that the substance responsible for anti-candida activity of this strain is not EPS. Therefore, it can be assumed that some other mechanisms, conditioning anti-candida activity of the strain, may be involved for this particular LAB strain. Further studies are needed to fully understand and describe those mechanisms.

Since the vaginal microbiome can vary depending on different factors, such as pregnancy, age, etc., the effect of different concentrations of progesterone on the growth of *L. rhamnosus* R-2002 and *L. acidophilus* MR-1980 strains was studied too. As can be seen from the Fig. 5, the increase in progesterone concentration did not have a significant effect on *L. rhamnosus* R-2002 strain, while *L. acidophilus* MR-1980 growth was notably inhibited at progesterone concentrations above 67 ng/ml. These concentrations are typical for the period of the first trimester of pregnancy. Since high concentrations of progesterone did not affect the growth of the *L. rhamnosus* R-2002 strain, it was interesting to study the effect of progesterone concentrations on the antibacterial activity of this strain, and as can be seen from Fig. 6, increasing concentrations of progesterone (209 and 333 ng/ml) leads to a complete decrease in antibacterial activity. Although high concentration of progesterone, which is observed in the third trimester of pregnancy, does not suppress the growth of *L. rhamnosus* R-2002, however, since the antibacterial activity decreases, this strain cannot be a proper barrier against pathogens. In contrast, Sovijit et al. indicated that certain concentrations of progesterone stimulate the in vitro growth of *L. reuteri* strain (26), therefore the stimulating or inhibitory effect of progesterone is strain-specific and also depends on the concentration of the hormone. We agree with the statement that the effect of progesterone is dose-dependent, however, the concentrations used in the studies were different: in our study, physiological concentrations of progesterone were used, while Sovijit et al. applied low concentrations for mice.

Since metronidazole is a widely used antibiotic for the treatment of yeast infections, it was a challenge to investigate, whether this antibiotic, which is used against eukaryotes, can also suppress *L. rhamnosus* R-2002 strain. Our data indicate that high doses of metronidazole affect the two studied strains, similar results were obtained by Simoes et al., who showed that the concentrations of metronidazole between 1000 and 4000 μg/ml partially inhibited the growth of various *Lactobacillus* representatives (*Lactobacillus casei, L. acidophilus* and *L. jensenii*) (14), while the concentrations ≥5000 μg/ml entirely suppressed the growth of LAB. In case when the treatment is implemented with metronidazole concentrations less

than 1000 µg/ml, *L. rhamnosus* R-2002 strain is a suitable candidate for the combined administration, which may be even better than applying higher concentrations of metronidazole only.

CONCLUSION

Given all the received data from this study, from the investigated strains of LAB, *L. rhamnosus* R-2002 strain possesses the best characteristics to have beneficial effects against the prevention and treatment of candidiasis and similar yeast infections caused by *C. albicans*. Due to its properties, particularly the wide spectrum of pH, extracellular nature of anti-yeast component/s, it has the potential to successfully antagonize pathogenicity caused by *C. albicans*, prevent, ameliorate the symptoms and treat vulvovaginal candidiasis.

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