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An *in vitro* study of interactions of *Candida albicans* with *Klebsiella pneumoniae* and *Enterococcus faecalis* isolated from intestinal microbiome of HIV infected patients

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Abstract
The aim: *In vitro* identification of targets for antagonism factors in klebsiellas and enterococci for *Candida albicans* isolated from the intestinal microbiome of HIV infected patients.
Materials and methods. The tests were performed using 38 *Candida albicans* strains, 28 *Klebsiella pneumoniae* strains, and 30 *Enterococcus faecalis* strains isolated from the intestinal microbiome of 89 HIV infected children. The mean age of the patients was 24 ± 2 months; the group consisted of 49 (55%) boys and 40 (45%) girls. Microorganisms were isolated from the intestinal biotope using such selective media as HiChrome *Candida* Agar, HiChrome *Klebsiella* Selective Agar Base, and *Enterococcus* Agar; the study included identification of species. Model experiments were performed to study anti-catalase activity of *E. faecalis* exometabolites and the impact of *K. pneumoniae* on morphological transformation of *C. albicans* fungi.
Results. Klebsiellas decrease the intensity of germ tube formation in *C. albicans* by 58.7% ($p < 0.01$). When cocultured, 12.3% of the yeast cells produce germ tubes, while 29.8% of transformed cells was detected in the fungal monoculture. It has been found that exometabolites of 65.7% of *E. faecalis* strains decrease production of catalase in *C. albicans.* The initial catalase level in untreated cultures of *C. albicans* averages 1.02 μmol/min of optical density; after they are treated with *E. faecalis* exometabolites, the level decreases to 0.55 μmol/min, i.e. by 46.1% ($p < 0.05$).
Conclusions. *K. pneumoniae* and *E. faecalis* demonstrate antagonism of different intensity toward *C. albicans*. Morphological transformation and catalase production are targets for antagonism factors of facultative microbiota in *C. albicans*.

Keywords: *antagonism, Candida albicans, Klebsiella pneumoniae, Enterococcus faecalis, anti-catalase activity, morphological transformation*

Ethics approval. The study was conducted with the informed consent of the legal representatives of the patients. The research protocol was approved by the Ethics Committee of the Kemerovo State Medical University (protocol No. 5, January 31, 2019).

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Научная статья
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Исследование *in vitro* механизмов взаимодействия грибов *Candida albicans* с *Klebsiella pneumoniae* и *Enterococcus faecalis*, выделенных из кишечного микробиома ВИЧ-инфицированных пациентов

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Аннотация
Цель: определение *in vitro* мишеней для факторов антагонизма клебсиелл и энтерококков у грибов *Candida albicans*, выделенных из кишечного микробиома ВИЧ-инфицированных пациентов.
Материалы и методы. В экспериментах использованы 38 штаммов грибов *Candida albicans*, 28 штаммов *Klebsiella pneumoniae* и 30 штаммов *Enterococcus faecalis*, изолированных из кишечного микробиома 89 ВИЧ-инфицированных детей. Средний возраст пациентов составил 24 ± 2 мес, мальчиков было 49 (55%), девочек — 40 (45%). Микроорганизмы выделяли из кишечного биотопа с использованием селективных питательных сред HiChrome *Candida* Agar, HiChrome *Klebsiella* Selective Agar Base, Энтерококкагар; проводили видовую идентификацию. В модельных экспериментах изучена антикаталазная активность экзометаболитов *E. faecalis* и влияние *K. pneumoniae* на морфологическую трансформацию грибов *C. albicans.*
Результаты. Клебсиеллы на 58,7% снижают интенсивность образования ростовых трубок у *C. albicans* (p < 0,01). При совместном культивировании 12,3% дрожжевых клеток дают ростовые трубки, тогда как в монокультуре грибов обнаружили 29,8% трансформированных клеток. Установлено, что экзометаболиты 65,7% штаммов *E. faecalis* снижают продукцию каталазы у *C. albicans.* Исходный уровень каталазы у интактных культур *C. albicans* в среднем составляет 1,02 мкмоль/мин оптической плотности, после обработки экзометаболитами *E. faecalis* снижается до 0,55 мкмоль/мин, т.е. на 46,1% (p < 0,05).
Выводы. *K. pneumoniae* и *E. faecalis* проявляют антагонизм к *C. albicans* с разной степенью выраженности. Мишенями для факторов антагонизма факультативной микробиоты у *C. albicans* являются морфологическая трансформация и продукция каталазы.

Ключевые слова: *антагонизм, Candida albicans, Klebsiella pneumoniae, Enterococcus faecalis, антикаталазная активность, морфологическая трансформация*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии законных представителей пациентов. Протокол исследования одобрен Этическим комитетом Кемеровского государственного медицинского университета (протокол № 5 от 31.01.2019).

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Introduction

The intestinal microbiome is an integrated system of interacting microorganisms; this system is capable of self-regulating by forming different types of microbial relationships [1, 2]. Microbial antagonism is one of the factors contributing to development of microbiocenosis [3]. Symbiont antagonism is characterized by production of antimicrobial substances [3], lytic enzymes (peptidase, amylase) destroying structures of microorganisms or molecules secreted by them [4, 5]. Some bacteria produce low molecular weight substances, which change growth properties and bac-

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teria persistence by affecting their genetic program [6, 7], inhibiting antioxidant systems of competitors [8, 9], activating metabolic shunts they need in their competition over food and iron sources [10].

Interactions between bacterial and fungal microbiomes in the intestinal biotope play a pivotal role in creation and maintenance of symbiosis and are associated with the risk of development of candidamycosis [3]. Bifidobacteria and lactobacilli create antagonistic relationships with fungi, which are aimed to prevent excessive fungal colonization of different biotopes [4, 11]. Some studies have demonstrated the mutual effect of virulence factors in opportunistic pathogenic bacteria and fungi on the macroorganism, including development of pathological processes [12]. In their competition over receptors present in the mucus membrane, micromycetes and opportunistic pathogenic bacteria enter into antagonistic relationships with the indigenous microbiota. However, when the population of opportunistic pathogenic bacteria reaches high levels of density, they become antagonistic toward fungi of the genus *Candida* [13].

Antagonism factors and targeted effect of opportunistic pathogenic bacteria from different taxonomic groups on fungi as well as the conditions required for implementation of antagonistic relationships and the factors of antagonism regulation in opportunistic pathogenic symbionts need further study. The mechanism of survival of fungi amidst "dual" antagonism (of indigenous and facultative bacteria) of intestinal symbionts remains unclear. Identification of bio-communicative mechanisms involved in prevention of development of endogenous candida infection is critically important for HIV infected patients.

The **aim** of the study was to identify *in vitro* targets for klebsiellas and enterococci antagonism factors in *Candida albicans* isolated from the intestinal microbiome of HIV infected patients.

Materials and methods

A total of 89 children diagnosed with HIV infection took part in the study; all of them were hospitalized to the respiratory infection department at the Kemerovo Regional Clinical Hospital for Infectious Diseases in 2019–2021; 10 (11%) children were hospitalized because of secondary bacterial diseases (pneumonia, tonsillitis) and 79 (89%) children were hospitalized with acute respiratory viral infections. The mean age of the patients was 24 ± 2 months; there were 49 (55%) boys and 40 (45%) girls. Most of the children (76.3%) had the 2nd stage of HIV infection (2A — 4.5%; 2B — 56.1%; 2C — 15.7%); 14.6% had the 3rd stage; 8.9% had the 4th stage. The stages of HIV infection are consistent with Pokrovsky's classification (2001), including the amendments adopted in 2006.[1]

The study was approved by the Ethics Committee of the Kemerovo State Medical University (minutes No. 5 of 31/1/2019). All the patients included in the study had their informed consent signed so that results of the study could be used for scientific purposes.

The tests were performed on 38 *C. albicans* strains, 28 *Klebsiella pneumoniae* strains, and 30 *Enterococcus faecalis* strains isolated from the intestinal biotope. The isolation of microorganisms was performed using selective and differential media. To extract *K. pneumoniae*, we used HiChrome *Klebsiella* Selective Agar Base (HIMEDIA); the specimens were cultured at 37ºC for 24 hours. The purple-magenta-colored colonies were reseeded on Kligler's medium (State Scientific Center of Applied Microbiology and Biotechnology (SSC AMB)) to accumulate pure culture and to do preliminary analysis of the biochemical properties. To isolate *E. faecalis,* we used the Enterococcus Agar medium (SSC AMB) for seeding, selected typical colonies in 24 hours, analyzed the morphology, and accumulated pure cultures. *C. albicans* fungi were isolated using the HiChrome *Candida* Agar (HIMEDIA); then we selected colonies corresponding to *C. albicans* by color using the differential scale in the manufacturer's instruction. To eliminate the risk of a false-negative result in the tests involving inhibition of fungal morphogenesis, all the strains were assessed for their ability to form germ tubes in the horse serum three hours after the culturing at 37ºC [14]. The final species-level identification of all microorganisms was performed using the VITEK 2 Compact analyser (BioMerieux). The tests were performed on *E. faecalis–C. albicans* pairs; each symbiont in the pair was obtained from the same patient to prevent the risk of the outsider phenomenon [15]. As a result, there were 26 pairs of symbionts. The tests were performed twice in 3 replications.

The effect of *K. pneumoniae* on the morphological transformation of *C. albicans* was assessed [14]. At first, *K. pneumoniae* cultures were grown in Mueller–Hinton broth (SSC AMB) for 18 hours at 37ºC; *C. albicans* fungi were grown on Sabouraud agar (SSC AMB) for 24 hours to match the completion of the exponential growth stage [16]. The *C. albicans* suspension was prepared in the sterile 0.9% NaCl solution with turbidity of 0.5 McFarland units, being equal to $1–5 \times 10^6$ CFU/ml) [17]. The *Klebsiellas* suspension was diluted 100 times to have the similar turbidity. The final concentration of *Klebsiellas* was 1 × 106 CFU/ml. 100 µl of *K. pneumoniae* and *C. albicans* suspension was placed into a tube with 0.5 ml of horse serum (Microgen NPO). Microorganisms were incubated at 37ºC. Three hours after, "crushed drop" smears were prepared and 100 cells of *C. albicans* fungi examined under a Carl Zeiss Primo-

[1] Decree No. 166 of the Ministry of Health and Social Development of the Russian Federation, adopted on 17/3/2006, The Instruction for Completing of Annual Form No. 61 of Federal Statistical Survey "Data on Cohorts of HIV-Infected Patients".

star microscope; the percentage of cells forming germ tubes was recorded. Untreated *C. albicans* cultures were used as control cultures, which were also assessed for their ability to form germ tubes in the protein-based medium.

The effect of *E. faecalis* exometabolites on the *C. albicans* catalase was assessed using the methods [9] including modifications, i.e. using stable ammonium molybdate instead of unstable potassium iodide. The two-day broth culture of *E. faecalis* was used to produce the supernatant by centrifuging the culture two times at 3000 rpm for 15 min. The supernatant liquid was separated from bacterial cells using membrane filters. *C. albicans* cultures were used to prepare suspension in the sterile 0.9% saline solution with turbidity of 0.5 McFarland units. Test samples were prepared by mixing 0.1 ml of *C. albicans* suspension**,** 2.6 ml of Sabouraud broth, and 0.3 ml of supernatant from broth cultures of *E. faecalis*. The values of catalase activity of fungal broth cultures not exposed to exometabolites of enterococci (0.1 ml of fungal suspension and 2.9 ml of broth) were used as reference variables. To measure the catalase activity, we added 1 ml of 0.0125 M solution of H_2O_2 to the test samples and to the reference samples of 0.2 ml; 10 minutes after, the reaction was discontinued by adding 1 ml of 4% ammonium molybdate solution. The non-inactivated H_2O_2 reacted with ammonium molybdate to produce colored complexes, optical density (OD) of which was measured with the SF 2000 spectrophotometer (OKB Spectr) at λ = 550 nm compared to the medium. The catalase activity was calculated using the formula [9]. The obtained results were compared with the catalase activity of *C. albicans* cultures, which were not treated with enterococcus supernatants.

To perform the statistical analysis, we used the IBM SPSS Statistics/PS IMAGO software package («IBM/Predictive Solutions Sp z.o.o.»). The normality of data distribution was verified with the Shapiro–Wilk test. The comparative analysis was performed using non-parametric methods for assessment of statistical significance (the χ^2 and Mann–Whitney tests) [18]. The experimental data are presented as average values and standard deviation, the median, and interquartile range [the 25th and 75th percentiles]. In statistical hypothesis testing, the significance level was equal to or less than 0.05 [18].

Results

The tests showed that *K. pneumoniae* inhibited the ability of fungi to form germ tubes. When cocultured with *Klebsiellas*, on average, 12.3 [6.33; 15]% of yeast cells produced germ tubes, while the fungal monoculture contained 29.8 [25; 36,7]% of cells with blastospore transformation. Thus, the inhibition of morphological transformation of *C. albicans* in associations with *K. pneumoniae* accounted for 58.72% ($p < 0.01$).

Enterococcus supernatants affected *C. albicans* in different ways (Table 1). In 65.4% of cases, enterococci inhibited the catalase of micromycetes; in 19.2% of cases, the catalase activity in fungi did not demonstrate any changes after treatment with *E. faecalis* supernatants; only in 15.4% of cases, the catalase production increased. The initial catalase level in untreated *C. albicans* cultures averaged 1.02 [0.87; 1.13] µmol/min OD; after the treatment with *E. faecalis* exometabolites, it decreased to 0.55 [0.36; 0.73] µmol/min OD ($p < 0.05$).

On average, the catalase activity of *C. albicans* was inhibited by 46.1% ($p < 0.05$).

The obtained results demonstrate that antagonism of *Klebsiellas* and enterococci toward *C. albicans* has different target points, different intensity and is a product of competition in the multicomponent microbial community.

Discussion

As the number of HIV infected people is increasing, candidiasis has become a common concomitant condition; therefore, a special emphasis is placed on new approaches in prevention of the process progression and timely diagnosis of opportunistic mycosis prior to the onset of symptoms [19]. Using of biocoenotic relationships and factors making it possible to regulate biological properties of *C. albicans* in microbiocenoses and counteract the implementation of their pathogenic potential offers promising prospects [20, 21]. Regardless of the biotope, resident microbiota regulates the virulence of *C. albicans*. Lactic acid and bacteriocins produced by *Lactobacillus spp.* play an essential role in inhibiting the activity of proliferation genes, impeding the growth and production of hyphae in *C. albicans,* and decreasing the expression of hyphal wall proteins 1 (Als3 and Hwp1) [11, 12]. The anti-biofilm effect toward fungi is produced by oleic and pentadecanoic acids resulting from the metabolism of fatty acids of anaerobic bacteria [22]. As for *E. faecalis*, their antagonism is strongly associated with the secretion of bacteriocin (ENTV) inhibiting the development of hyphae and affecting the formation of *C. albicans* biofilms. Microorganisms of the genus *Bacteroides, Prevotella, Bifidobacterium* decrease the anti-complementary activity of fungi [23].

When describing the interaction between fungi and the opportunistic pathogenic microbiota, most studies focus on mutual enhancement of antagonism toward indigenous bacteria [23]. In bacterial and fungal associations, fungi tend to act as “helpers” of facultative microorganisms. *Candida* metabolites enhance the anti-lysozyme activity of *Staphylococcus aureus*, *Klebsiella spp*., *E. coli lac–/hly+*, and have a direct inhibitory effect on the anti-lysozyme factor of bifidobacteria [24]. The component of the cell wall — *C. albicans* b-1,3-glucan increases the antibiotic resistance of *St. aureus* [25]. In the meantime, there are data demonstrating that similar to indigenous microorgan-

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The influence of *E. faecalis* exometabolites on the catalase activity of *C. albicans* (*M* ± *SD*)

Pair of symbionts	Initial catalase level, μmol/min OD, control	Catalase level after exometabolite treatment, μmol/min OD, experience	Change in catalase activity, %
Decrease in catalase activity			
1	1,09 ± 0,03	0,57 ± 0,02	47,7
2	1,26 ± 0,04	0,53 ± 0,02	57,9
3	0,56 ± 0,05	0,25 ± 0,03	55,4
4	1,42 ± 0,03	0,75 ± 0,02	47,2
5	1,28 ± 0,05	0,65 ± 0,03	49,2
6	0,93 ± 0,05	0,37 ± 0,04	60,2
7	1,07 ± 0,04	0,47 ± 0,02	56,1
8	1,05 ± 0,04	0,65 ± 0,08	38,1
9	0,87 ± 0,05	0,35 ± 0,02	59,8
10	1,41 ± 0,02	0,74 ± 0,03	47,5
11	1,14 ± 0,05	1,09 ± 0,01	4,40
12	1,11 ± 0,05	0,75 ± 0,03	32,4
13	0,90 ± 0,07	0,72 ± 0,02	20,0
14	0,86 ± 0,04	0,43 ± 0,02	50,0
15	0,77 ± 0,03	0,33 ± 0,02	57,1
16	0,87 ± 0,03	0,23 ± 0,02	73,6
17	1,06 ± 0,03	0,26 ± 0,05	75,5
Increased catalase activity			
18	0,57 ± 0,31	1,17 ± 0,07	105,3
19	0,78 ± 0,08	1,16 ± 0,04	48,7
20	0,74 ± 0,07	1,09 ± 0,03	47,3
21	0,13 ± 0,02	0,57 ± 0,09	338,5
Catalase activity did not change			
22	0,78 ± 0,01	0,78 ± 0,03	0,00
23	1,17 ± 0,05	1,18 ± 0,03	0,00
24	0,98 ± 0,03	0,97 ± 0,03	0,00
25	0,56 ± 0,11	0,45 ± 0,01	0,00
26	0,42 ± 0,02	0,43 ± 0,02	0,00

isms, opportunistic pathogenic bacteria display antagonism toward fungi in high-density populations [22, 26].

Some molecular mechanisms of intermicrobial interactions of *C. albicans* with *K. pneumoniae* and *E. faecalis* isolated from HIV infected patients have been studied. By and large, the studied bacterial species demonstrate antagonism toward *C. albicans,* showing the consistency with the data provided by researchers from other countries [13].

It has been found that *K. pneumoniae* have an effective biopotential for regulation of the population size of *C. albicans*. The morphological transformation of *C. albicans* is the target for *K. pneumoniae*. Fungal morphogenesis into the hyphal form is seen as the pathogenicity factor for micromycetes, as they have a wider range of adhesins for mucosal surfaces, increased propagation in tissues, and the increased number of phospholipases, which concentrate on the termini of hyphal elements [27].

Antagonism toward fungi has been demonstrated not only by *K. pneumoniae*, but also by *E. faecalis*. *E. faecalis* inhibited catalase, which is a powerful antioxidant enzyme in microorganisms with aerobic respiration [28, 29]. Changes in the activity or inhibition of enzymes in antioxidant systems of microorganisms can result in accumulation of toxic forms of oxygen, thus affecting the permeability of a membrane, intake rate of nutrients and, eventually, the proliferation rate of microorganisms [30, 31].

Conclusion

The obtained results support the data on the role of facultative bacteria in functioning of the intestinal microbiome and demonstrate their regulating effect on

C. albicans. The antagonism of facultative bacteria toward *C. albicans* is based on the inhibition of morphological transformation and catalase production, opening up promising opportunities for methods aimed at prevention of candidiasis and involving enhanced antagonism of not only resident, but also transient microbiota. The results of tests and approaches to exploration of bacterial and fungal relationships have significant research and practical potential, making it possible to perform *in vitro* modeling of the process aimed at control of biological properties of *Candida* fungi by using the antagonism factors in the intestinal bacterial microbiota.

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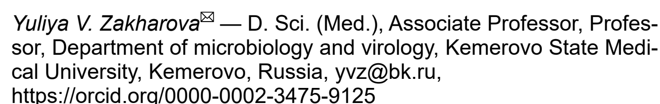

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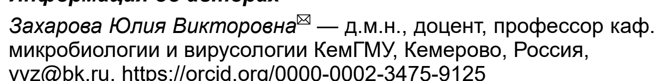

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