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The Effect of Cell-Free Nontuberculous Mycobacterium Supernatants on Antibiotic Resistance and Biofilm Formation of Opportunistic Pathogens

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Abstract: The presence of nontuberculous mycobacteria in biofilms on the surface of medical devices may affect the opportunistic pathogens that are common inhabitants of such biofilms. This study assessed the effect of *Mycolicibacterium iranicum* cell-free supernatants on biofilm formation and antibiotic susceptibility of *Escherichia coli* and *Staphylococcus epidermidis* differing in the anti-hemoglobin activity level. The cell-free supernatants have been shown to stimulate biofilm formation and also help reduce susceptibility of opportunistic pathogens to a number of antibiotics.

Keywords: nontuberculous mycobacteria; opportunistic pathogens; biofilm formation; antibiotic susceptibility

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1. Introduction

Nontuberculous mycobacteria (NTM) are a group of gram-positive microorganisms whose natural habitats are soil and water, as well as aerosols generated above the earth's surface [1]. Among the NTM, there are saprophytic species, opportunistic and pathogenic species of animals and humans [2,3]. In recent years, the researchers' interest in this group of microorganisms has been increasing, which is associated with the spread of diseases caused by NTB worldwide [4]. The recorded increase in nontuberculous mycobacterial infections is associated firstly with the improvement of molecular diagnostic methods and their wider use in clinical practice, and secondly with an increase in patients with compromised immune system and hematological or oncological diseases [5–7]. Most diseases caused by NTM are associated with the ingress of these bacteria into the human body from the environment [8–10], but cases of iatrogenic infection of patients due to the presence of NTM in biofilms on the surface of implants, catheters, probes, and other medical devices have also been described [11–13]. It is known that one of the main mechanisms of virulence of opportunistic pathogens (*E. coli*, *S. epidermidis*, and other bacterial species) and the spread of nosocomial infections is the biofilm formation on the surface of medical devices and implants [14]. The process of bacterial biofilm formation is a complex phenomenon mediated by quorum-sensing molecules. Implant-related infections are among the most dangerous complications after surgery [15]. Therefore, the NTM presence in biofilms and, in particular, their effect on biofilm formation and antibiotic susceptibility in opportunistic microflora is of great practical interest. As it was shown earlier, adhesion and intraerythrocytic penetration of *E. coli* and *S. epidermidis* increase in the NTB presence [16]. Therefore, the objective of this study was to evaluate the effect of cell-free NTB supernatants on the antibiotic resistance and biofilm-forming ability of opportunistic pathogens—*E. coli* and *S. epidermidis*.

2. Results

2.1. The Effect of Cell-Free M. iranicum Supernatants on Biofilm Formation of E. coli and S. epidermidis

In this study, used *S. epidermidis* and *E. coli* cells were different in antihemoglobin activity (AntiHbA). The anti-hemoglobin activity of the strains characterizes their ability to inactivate hemoglobin in human blood by capturing heme iron. Briefly, AntiHbA was studied by a method based on the residual hemoglobin determination in a substrate after its interaction with a bacterial culture. Strains *S. epidermidis* IKVS 7 and *E. coli* IKVS 1 were characterized by high AntiHbA values (15.7 and 8.9 g/L, respectively), while *S. epidermidis* IKVS 13 and *E. coli* IKVS 18 showed low levels of this activity (2.0 and 0.7 g/L, respectively). AntiHbA levels had previously been determined [16] and were hence used for further study. Under the influence of NTMB supernatants in the studied cells of *S. epidermidis* and *E. coli*, the ability to form biofilms and the sensitivity to antibiotics was studied.

Under the effect of the cell-free supernatant of *M. iranicum* IKVS S1, an enhanced biofilm formation was observed in the strains of *E. coli* IKVS 1 and *E. coli* IKVS 18 relative to the control values by 31.0 ± 2.9% and 20.4 ± 2.6%, respectively (Figure 1). Table S1 presents the average OD values of the studied samples used in calculations of biofilm formation. Moreover, it should be noted that an evident 1.5-fold enhancement in biofilm formation was observed in the *E. coli* IKVS 1 strain exposed to the *M. iranicum* IKVS S1 supernatant vs. the strain of *E. coli* IKVS 18 (at $p \leq 0.05$). The *M. iranicum* IKVS S1 supernatant had a similar effect on the strains of *S. epidermidis* IKVS 7 and 13—the biofilm formation increased by 25.6 ± 2.6% and 28.7 ± 2.7%, respectively. However, no significant difference was observed between strains.

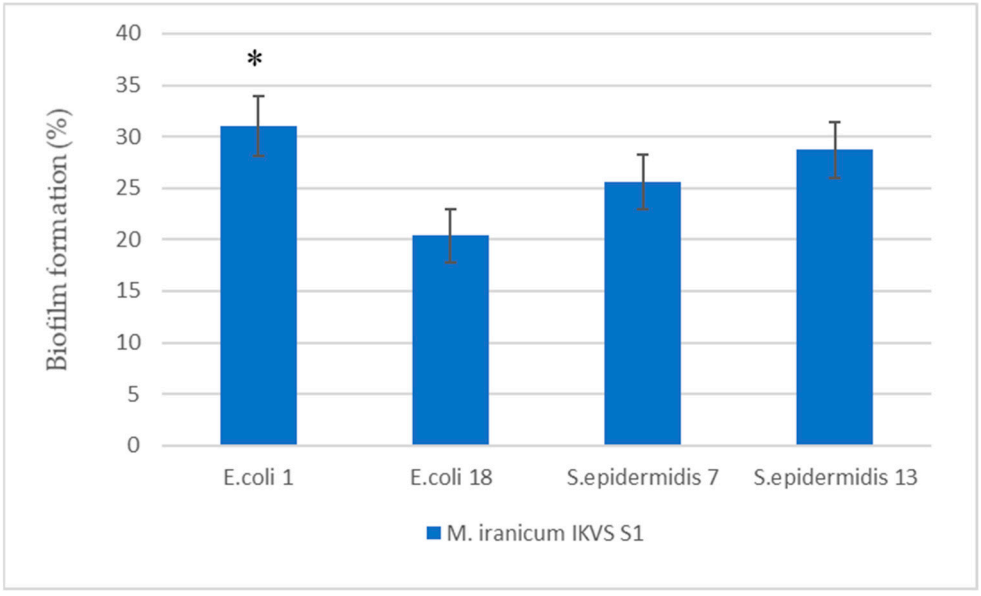

Figure 1. Enhancement of biofilm formation by *E. coli* and *S. epidermidis* under the influence of cell-free supernatants of *M. iranicum* IKVS S1. *—significance of differences between strains according to Student's *t*-test, $p < 0.05$.

The cell-free *M. iranicum* IKVS S2 supernatant affected the biofilm formation in opportunistic strains to a significantly lesser extent compared to *M. iranicum* IKVS S1 (Figure 2).

An increase in biofilm formation was observed in the strains of *E. coli* IKVS 1 and *E. coli* IKVS 18 relative to the control values by 16 ± 1.9% and 9.8 ± 1.3%, respectively. Moreover, an evident 1.6-fold enhancement in biofilm formation was observed in the *E. coli* IKVS 1 strain exposed to the *M. iranicum* IKVS S2 supernatant vs. the strain of *E. coli* IKVS 18 (at $p \leq 0.05$). The *M. iranicum* IKVS S2 supernatant had the least effect on the strains of *S. epidermidis* IKVS 7 and 13. An increase in biofilm formation relative to the control was

2.2 ± 0.73% and 7.6 ± 1.1%, respectively. If we compare the ability of the studied strains to form biofilms with each other, the *S. epidermidis* IKVS 13 cells showed a 3.4-fold increase in biofilm formation compared to *S. epidermidis* IKVS 7 (at $p \leq 0.05$) exposed to the *M. iranicum* IKVS S2 supernatant.

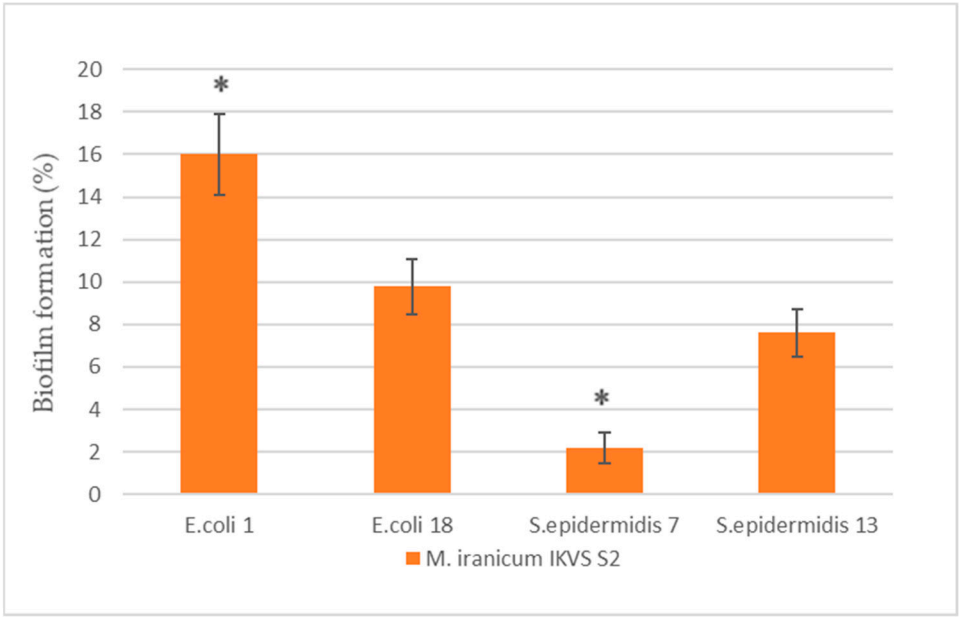

Figure 2. Enhancement of biofilm formation by *E. coli* and *S. epidermidis* under the influence of cell-free supernatants of *M. iranicum* IKVS S2. *—significance of differences between strains according to Student's *t*-test, $p < 0.05$.

In strains with high levels of AntiHbA (except for *S. epidermidis* IKVS 7), increased biofilm formation was observed under the influence of supernatants. We consider antihemoglobin activity as one of the pathogenicity factors. Strains characterized by a high level of this property contribute to the development of an infectious–inflammatory process, as shown in the works of [17]. It was previously shown that strains with high levels of AntiHbA are characterized by the presence of other pathogenicity factors (hemolytic, gelatinase, adhesive activity, etc.) [18]. This study revealed increased biofilm formation in strains with high levels of AntiHbA (with the exception of *S. epidermidis* IKVS 7), and the authors suggest that opportunistic microorganisms in the body can also lead to the development of an infectious process when influenced by NTM.

2.2. The Effect of the Cell-Free M. iranicum Supernatants on Antibiotic Susceptibility of the Strains of E. coli IKVS 1 and 18

Under the effect of the cell-free supernatants of *M. iranicum* IKVS S1 and *M. iranicum* IKVS S2, a decrease in antibiotic susceptibility was observed in the *E. coli* IKVS 1 strain (Table 1). According to the study results, a significant decrease (at $p \leq 0.05$) in the antibiotic susceptibility of *E. coli* IKVS 1 was observed under the effect of the *M. iranicum* IKVS S1 supernatant relative to the control to vancomycin (11.3 ± 1.7 vs. 7 ± 1.4) and cefotaxime (26 ± 2.1 vs. 21 ± 1.4), respectively. On the other hand, a 2.4-fold increase in antibiotic susceptibility to clindamycin was observed relative to the control; the strain growth restriction was 20.3 ± 1.7 vs. 8.6 ± 1.6 (at $p \leq 0.05$).

Table 1. Effect of cell-free supernatants of NTM *M. iranicum* IKVS S1 and IKVS S2 on the antibiotic susceptibility of the strains *E. coli* IKVS 1.

Antibiotic/Strain	*E. coli* IKVS 1 Control, mm	Strain Growth Inhibition under the Influence of Supernatant *M. iranicum*, mm	
		IKVS S1	IKVS S2
ceftriaxone	21.3 ± 2,4	21.6 ± 1.4	23 ± 1.4
cefepime	22 ± 2.4	18 ± 2.1	17 ± 1.4 *
gentamicin	23 ± 2.4	19 ± 1.4	11 ± 1.4 *
vancomycin	11.3 ± 1.7	7 ± 1.4 *	9 ± 1.3
clindamycin	8.6 ± 1.6	20.3 ± 1.7 *	13 ± 1.4 *
cefotaxime	26 ± 2.1	21 ± 1.4 *	20 ± 1.4 *
meropenem	23.3 ± 2.4	24 ± 1.4	20.3 ± 1.4
rifampicin	0	0	0
benzylpenicillin	2.6 ± 0.5	2.6 ± 1.3	2.6 ± 0.5
azithromycin	14.3 ± 1.9	11.6 ± 1.7	14.3 ± 1.9
ciprofloxacin	25.3 ± 2.1	24 ± 1.4	25.3 ± 2.1
clarithromycin	8.3 ± 1.6	7 ± 1.4	8.3 ± 1.6

Note *—significance of differences from control according to Student's *t*-test, $p < 0.05$.

The most evident significant decrease in the antibiotic susceptibility of the *E. coli* IKVS 1 strain exposed to *M. iranicum* IKVS S2 was detected to cefepime, gentamicin, and cefotaxime. A significant increase in antibiotic susceptibility was observed to clindamycin relative to the control—growth restriction was 13 ± 1.4 vs. 8.6 ± 1.6 (at $p \leq 0.05$).

Under the effect of supernatants of *M. iranicum* IKVS S1 and IKVS S2, a significant decrease in antibiotic susceptibility to ceftriaxone, cefepime, gentamicin, cefotaxime, azithromycin, and ciprofloxacin was observed in the *E. coli* IKVS 18 strain relative to control values (Table 2).

Table 2. Effect of cell-free supernatants of NTM *M. iranicum* IKVS S1 and IKVS S2 on the antibiotic susceptibility of the strains *E. coli* IKVS 18.

Antibiotic/Strain	*E. coli* IKVS 18 Control, mm	Strain Growth Inhibition under the Influence of Supernatant *M. iranicum*, mm	
		IKVS S1	IKVS S2
ceftriaxone	27 ± 1.4	17 ± 1.4 *	21 ± 1.4 *
cefepime	22.6 ± 2.1	17 ± 1.4 *	17 ± 1.4 *
gentamicin	20.6 ± 2.1	15 ± 1.4 *	13 ± 1.4 *
vancomycin	14.6 ± 1.8	15 ± 1.4	14.6 ± 1.04
clindamycin	0	0	0
cefotaxime	27 ± 1.4	18 ± 1.4 *	21.6 ± 1.4 *
meropenem	27 ± 1.4	23 ± 1.4	27 ± 1.4
rifampicin	0	0	0
benzylpenicillin	9.3 ± 1.6	8.3 ± 1.6	11.6 ± 1.8
azithromycin	16 ± 1.4	10.6 ± 1.9 *	12.3 ± 1.8
ciprofloxacin	29 ± 1.4	20.3 ± 1.04 *	23 ± 1.4 *
clarithromycin	0	0	0

Note *—significance of differences from control according to Student's *t*-test, $p < 0.05$.

Thus, the supernatants of *M. iranicum* IKVS S1 and IKVS S2 had an evident effect on the antibiotic susceptibility of both *E. coli* strains. It should be noted that, under the effect of the *M. iranicum* IKVS S1 supernatant, the strain *E. coli* with the higher anti-HbA values showed a lesser decrease in the susceptibility to antibiotics of the group of β-lactams, aminoglycosides, fluoroquinolones, and azalides compared to the strain *E. coli* characterized by the lower anti-HbA values. A similar trend was observed under the effect of the cell-free supernatant of *M. iranicum* IKVS S2—*E. coli* IKVS 1 with the higher anti-HbA values did not

change the susceptibility to ciprofloxacin (fluoroquinolone) and ceftriaxone (cephalosporin) in contrast to the *E. coli* IKVS 18 strain with the lower anti-HbA values. It is of particular interest to note that with the *E. coli* 1 strain, an increase in clindamycin susceptibility was observed under the effect of the cell-free *M. iranicum* supernatants. Moreover, an increase in susceptibility was higher under the effect of *M. iranicum* IKVS S1 compared to *M. iranicum* IKVS S2.

2.3. The Effect of the Cell-Free M. iranicum Supernatants on Antibiotic Susceptibility of the Strains of S. epidermidis IKVS 7 and 13

The *S. epidermidis* IKVS 7 strain showed a significant decrease in susceptibility to ceftriaxone, gentamicin, vancomycin, clindamycin, cefotaxime, meropenem, rifampicin, azithromycin, and clarithromycin when exposed to the cell-free supernatants of M. iranicum IKVS S1 and IKVS S2 relative to the control values (at $p \leq 0.05$) (Table 3).

Table 3. Effect of cell-free supernatants of NTM *M. iranicum* IKVS S1 and IKVS S2 on the antibiotic susceptibility of the strains *S. epidermidis* IKVS 7.

Antibiotic/Strain	***S. epidermidis* IKVS 7 Control, mm**	**Strain Growth Inhibition under the Influence of Supernatant *M. iranicum*, mm**	
		IKVS S1	**IKVS S2**
ceftriaxone	21 ± 1.4	15.3 ± 1.4 *	17.3 ± 1.7
cefepime	0	0	0
gentamicin	20.3 ± 1.7	15 ± 1.4 *	16.3 ± 1.4
vancomycin	16 ± 1.4	11 ± 1.4 *	13.6 ± 1.2
clindamycin	25 ± 1.4	20.3 ± 1.4 *	16.3 ± 1.3 *
cefotaxime	25 ± 1.4	20.6 ± 2.08 *	19.6 ± 2.08 *
meropenem	32.3 ± 1.7	27.6 ± 1.7 *	28.3 ± 2.4
rifampicin	26.6 ± 1.04	20 ± 1.4 *	20.6 ± 2.08 *
benzylpenicillin	10 ± 1.4	9.3 ± 1.04	13.6 ± 2.08
azithromycin	22.6 ± 2.08	15.3 ± 1.4 *	16.3 ± 1.7 *
ciprofloxacin	23 ± 1.4	20.3 ± 1.7	17.6 ± 2.08 *
clarithromycin	21.6 ± 1.4	15.3 ± 1.7 *	17.6 ± 2.08

Note *—significance of differences from control according to Student's *t*-test, $p < 0.05$.

S. epidermidis IKVS 13 showed a significant decrease in susceptibility to ceftriaxone, gentamicin, clindamycin, cefotaxime, meropenem, rifampicin, azithromycin, ciprofloxacin, and clarithromycin when exposed to the cell-free *M. iranicum* IKVS S1 supernatant (Table 4), and to clindamycin, cefotaxime, meropenem, azithromycin, and clarithromycin when exposed to the *M. iranicum* IKVS S2 supernatant, relative to the control values (at $p \leq 0.05$).

Table 4. Effect of cell-free supernatants of NTM *M. iranicum* IKVS S1 and IKVS S2 on the antibiotic susceptibility of the strains *S. epidermidis* IKVS 13.

Antibiotic/Strain	***S. epidermidis* IKVS 13 Control, mm**	**Strain Growth Inhibition under the Influence of Supernatant *M. iranicum*, mm**	
		IKVS S1	**IKVS S2**
ceftriaxone	23 ± 1.4	15.6 ± 1.04 *	20.6 ± 2.08
cefepime	0	0	0
gentamicin	20 ± 1.4	15.3 ± 1.7 *	20.6 ± 1.4
vancomycin	13 ± 1.2	13 ± 1.2	13.6 ± 1.2
clindamycin	26 ± 1.4	19.6 ± 1.04 *	20.3 ± 1.7 *
cefotaxime	23.3 ± 1.7	17.3 ± 1.7 *	18.3 ± 1.7 *
meropenem	32 ± 2.08	27 ± 2.08 *	24 ± 2.08 *
rifampicin	24.6 ± 1.5	19.3 ± 1.7 *	22.6 ± 2.08

Table 4. *Cont.*

Antibiotic/Strain	*S. epidermidis* IKVS 13 Control, mm	Strain Growth Inhibition under the Influence of Supernatant *M. iranicum*, mm	
		IKVS S1	IKVS S2
benzylpenicillin	11.6 ± 1.04	10.6 ± 1.7	16 ± 2.08
azithromycin	22 ± 1.4	16.3 ± 1.7 *	14.3 ± 1.2 *
ciprofloxacin	25.3 ± 1.7	20.3 ± 1.7 *	25.3 ± 1.7
clarithromycin	23.3 ± 1.7	16.3 ± 1.7 *	16 ± 2.05 *

Note *—significance of differences from control according to Student's *t*-test, $p < 0.05$.

Thus, the most pronounced effect of reducing antibiotic sensitivity in *S. epidermidis* strains, regardless of the level of AntiHbA, was observed under the influence of the cell-free supernatant of *M. iranicum* IKVS S1: in *S. epidermidis* strains IKVS 7 and 13, there was a significant decrease in sensitivity to ceftriaxone, cefotaxime, miropinem, gentamicin, clindamycin, azithromycin, and clarithromycin. Differences in reduced antibiotic susceptibility between strains with different anti-HbA levels were insignificant. It was noted that benzylpenicillin susceptibility, under the effect of supernatants of *M. iranicum* IKVS S2 and IKVS S1 strains, did not change in both strains of *S. epidermidis*.

3. Discussion

Infections associated with medical devices are most often caused by biofilms formed by *S. epidermidis* and *E. coli* [19]. Such infections are more difficult to treat because microorganisms within biofilms actively exchange metabolites that enhance microbial survival, regulate biofilm formation and maturation, modulate the host immune response, and cause genetic changes [20–22]. The ability of bacterial metabolites to regulate biofilm formation has been described for a number of clinically or environmentally significant strains [23,24]. For example, *Yersinia enterocolitica* metabolites were shown to stimulate biofilm formation in *E. coli* strains with limited biofilm-forming ability [24]. The presence of *M. mucogenicum* in multispecies biofilms stimulated biofilm formation, but its cell-free supernatant reduced the ability to form biofilms in a number of bacteria, including *Staphylococcus* sp. [23]. In recent years, information has appeared in the literature about the presence of *M. iranicum* in biofilms on the surfaces of medical instruments [11]. *M. iranicum* in biofilms can interact with *S. epidermidis* and *E. coli* and affect the biofilm formation and/or the antibiotic susceptibility of opportunistic human microflora. Our study showed that *E. coli* and *S. epidermidis* exhibited a significant increase in biofilm formation when exposed to the cell-free *M. iranicum* supernatant. The most evident effect was observed under exposure to the *M. iranicum* IKVS S1 supernatant. Noteworthy is the fact that an increase in biofilm formation in *E. coli* IKVS 1 strain characterized by the higher anti-HbA levels was twice higher under the effect of the cell-free supernatants of both NTM strains compared to a similar strain with the lower values of anti-hemoglobin activity. With *S. epidermidis* strains differing in anti-HbA, significant differences in biofilm formation between strains were only observed under the effect of the *M. iranicum* IKVS S2 supernatant, while biofilm formation increased most significantly in the *S. epidermidis* IKVS 13 strain characterized by the lower anti-HbA values. The data obtained suggest the presence of a synergistic relationship between *M. iranicum* and *E. coli* and *S. epidermidis*; this type of relationship between microorganisms is species-specific. Therefore, the simulation of biofilm formation in some species requires intercellular contact, while for others, signaling molecules are sufficient for interspecies communication [25,26].

The ability to form biofilms is one of the leading virulence factors in bacteria and a factor contributing to the infection transition to a chronic form [14,27]. The formation of bacterial biofilms mostly leads to increased bacterial resistance to antibiotics [28–30]. Our study showed a significant decrease in susceptibility of *E. coli* and *S. epidermidis* to a number of antibiotics, when exposed to the cell-free *M. iranicum* supernatants. The most evident effect of reducing antibiotic susceptibility was observed in *S. epidermidis* strains,

regardless of the anti-HbA level, and in the case of *E. coli* strains, in a strain with the lower AntiHbA values. It is known that antibiotic resistance in biofilms and at the cellular level is ensured by different mechanisms. However, despite the differences, these processes are largely synergistic: traditional resistance mechanisms of planktonic cells can also act in biofilms, and biofilms enhance resistance at the cellular level [31–33]. The study results were also confirmed in the works of Zakirov I. I. et al. and Carcione D. et al. [33,34]. They show the mechanisms of antibiotic resistance of microorganisms and mark the important role of biofilms in the formation of resistance to antimicrobial drugs, as well as difficulties in the treatment of such diseases contributing to the infection-associated mortality.

The intensification of the process of biofilm formation of opportunistic bacteria and the increase in their resistance to a number of antibiotics under the exposure to *M. iranicum* discovered in this study could potentially affect the virulence of these strains, as well as their antibiotic resistance, which may be beneficial during the surface colonization and further contribute to a chronic course of the infectious process.

4. Materials and Methods

4.1. Biophysiochemical and Cultural–Morphological Properties of NTM Strains

Collection strains of nontuberculous mycobacteria IKVS S1 and IKVS S2 previously identified as *Mycobacterium iranicum* (IKVS S1) and *Mycobacterium rutilum* (IKVS S2) of the Institute of Cellular and Intracellular Symbiosis, Ural Branch of the Russian Academy of Sciences (ICIS, UrB RAS) were used in the work. Both isolates were isolated from the salty Lantsug River, which flows into the hyperhaline Lake Elton (Volgograd region, Russian Federation). We clarified the species identity of archival strains based on the 16S rRNA gene sequence. Genomic DNA from bacterial cultures was isolated by enzymatic lysis [35]. The fragments of the nucleotide sequences of the DNA samples were submitted to SINTOL (Russia) for further analysis. The obtained sequences of the 16S rRNA gene fragments of the studied strains were 407 nucleotides in length. They were compared with homologous sequences of the 16S rRNA gene in the BLAST U.S. database. National Library of Medicine NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 30 May 2023). Based on the alignment results in the BLAST program, the maximum similarity of the sequences of the studied isolates with the type strains—*Mycobacterium iranicum* strain M05 HQ009482.1 *and Mycobacterium iranicum* strain H39 KX390634.1, was established and their 100% coincidence confirmed (Table 5). As a result of recent revision of the genus *Mycobacterium* and based on phylogenetic and genomic analysis, *Mycobacterium iranicum* species were attributed to the new genus *Mycolicibacterium* under the new name of *Mycolicibacterium iranicum* [36], which allows the IKVS S1 and IKVS S2 isolates to be attributed to *Mycolicibacterium iranicum*.

Table 5. The results of sequence alignment of the studied IKVS S1 and IKVS S2 strains using the 16S rRNA gene fragment in the BLAST program.

Nº	Typical Strain	Similarity, %
1	MK493230.1 *Mycolicibacterium iranicum*	407/407(100%)
2	MH542421.1 *Mycolicibacterium iranicum*	407/407(100%)
3	MK890472.1 *Mycolicibacterium* sp.	407/407(100%)
4	MK890466.1 *Mycolicibacterium* sp.	407/407(100%)
5	LC458852.1 *Mycolicibacterium iranicum*	407/407(100%)
6	MH581232.1 *Mycolicibacterium* sp.	407/407(100%)
7	MK811419.1 *Mycolicibacterium iranicum*	407/407(100%)
8	KX390634.1 *Mycobacterium iranicum* strain H39	407/407(100%)
9	HQ009482.1 *Mycobacterium iranicum* strain M05	407/407(100%)
10	KU861842.1 *Mycobacterium iranicum* strain Y31	407/407(100%)

The sequences of the studied strains of *Mycolicibacterium iranicum* IKVS S1 (accession number OR058793) and IKVS S2 (accession number OR058794) are found in the BLAST U.S.

database. National Library of Medicine NCBI (https://www.ncbi.nlm.nih.gov/nuccore/?term=OR058793:OR058794[accn] (accessed on 30 May 2023)).

Mycolicibacterium iranicum IKVS S1 strain is an aerobe, immobile, and does not form spores. The cells are rod-shaped, sometimes curved, and eventually shortened to coccoid ones. According to the Runyon classification, the strain belongs to group IV of fast-growing NTM—visible growth was observed on days 3–4. The strain is scotochromogenic, the pigment is bright orange, gram-positive, catalase-positive, urease-positive, has an oxidative metabolic pattern, and produces lipase. It grows on standard culture media—1.5% meat infusion agar, Czapek medium. The strain is a moderate halophile capable of growing on media containing up to 10% sodium chloride. Growth temperature: 23–40 °C.

When cultivating on Czapek medium at a temperature of 25 °C for 3 days, IKVS S1 strain forms the convex, flat-topped, isolated, paste-like colonies colored orange with an uneven edge on the medium surface. It does not form aerial mycelium.

Mycolicibacterium iranicum IKVS S2 strain is an aerobe, immobile, and does not form spores. The cells are rod-shaped, sometimes curved, and eventually shortened to coccoid ones. According to the Runyon classification, it belongs to group IV of fast-growing NTM—visible growth is observed on days 3–4. Scotochromogen, orange pigment. The strain is gram-positive, catalase-positive, urease-positive, has an oxidative metabolic pattern, and produces lipase. It grows on standard culture media—1.5% meat infusion agar, Czapek medium. The strain is a moderate halophile capable of growing on media containing up to 7% sodium chloride. Growth temperature: 23–40 °C; optimal temperature: 37 °C.

When cultivating on Czapek medium at a temperature of 25 °C for 3 days, it forms the convex, flat, isolated, mucoid colonies colored orange with an uneven edge on the medium surface. With further cultivation, mucous "crowding" growth is observed.

4.2. Characteristics of Opportunistic Pathogens

To study the effect of NTM supernatants on opportunistic microflora, archival strains of opportunistic bacteria such *Staphylococcus epidermidis* (IKVS 7, IKVS 13) and *Escherichia coli* (IKVS 1, IKVS 18), differing in the level of anti-hemoglobin activity (anti-HbA), were selected. The strains of *S. epidermidis* IKVS 7 and *E. coli* IKVS 1 were characterized by higher anti-HbA (15.7 and 8.9 g/L, respectively), while *S. epidermidis* IKVS 13 and *E. coli* IKVS 18 strains were characterized by a lower activity (2.0 and 0.7 g/L, respectively). Anti-hemoglobin activity of strains characterizes their ability to inactivate hemoglobin in human blood by capturing heme iron. Anti-HbA was studied using a method based on the determination of residual hemoglobin in the substrate after interaction with a bacterial culture. The method for determining this property is detailed in previous works [16,37]

4.3. Receiving the Cell-Free M. iranicum Supernatant

To obtain *M. iranicum* cell-free supernatant, IKVS S1 and IKVS S2 were cultivated in meat-peptone broth (MPB) for 48 h at 21 °C, and then obtained the supernatant by centrifugation at 9000 g/rev for 20 min and were passed through sterile filters (30 mm, PVDF membrane, 0.22 μm, Jet Biofil (Guangzhou, China)).

4.4. Determination of Bacteria Antibiotic Susceptibility

Antibiotic susceptibility testing was performed using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar according to EUCAST 2021 guidelines [38]. For this purpose, 24 h agar cultures of *S. epidermidis* and *E. coli* were suspended in 2 mL of sterile saline to prepare a density of 0.5 according to the McFarland turbidity standard. To assess the effect of nontuberculous Mycobacterium supernatants on the antibiotic susceptibility of opportunistic pathogens, test and control samples were prepared. In test samples, sterile cell-free supernatant of *M. iranicum* IKVS S1 or IKVS S2 in a volume of 2 mL was added to 1 mL of a suspension of the studied opportunistic pathogens; in control samples, 2 mL of MPB was added to 1 mL of a suspension of the studied opportunistic pathogens. Test and control samples were incubated for 24 h at 37 °C. Then, 0.1 mL of bacterial suspension was

applied to the surface of the Mueller–Hinton medium (HiMedia, Mumbai, India) in a Petri dish and distributed evenly with a spatula. The discs containing antibiotics were placed on different areas of the inoculated agar using forceps. The inoculated dishes were placed in a thermostat for 24 h at 37 °C, and then the results were assessed by measuring the inhibition zone (in millimeters) of the studied microorganisms. The following discs were used for this study: ceftriaxone (30 µg), cefepime (30 µg), gentamicin (10 µg), vancomycin (30 µg), clindamycin (2 µg), cefotaxime (30 µg), meropenem (10 µg), rifampicin (5 µg), benzylpenicillin (10 units), azithromycin (15 µg), ciprofloxacin (5 µg), clarithromycin (15 µg). All experiments were performed in triplicate.

4.5. Determination of the Biofilm Formation Intensity

The intensity of biofilm formation by opportunistic bacteria exposed to NTM supernatants was determined by the crystal violet binding degree in sterile 96-well plates. For this purpose, a suspension of the studied microorganisms—*S. epidermidis* (IKVS 7, IKVS 13) and *E. coli* (IKVS 1, IKVS 18)—at a concentration of 10^9 CFU/mL was prepared from 24 h agar cultures. The resulting suspension was added in a volume of 100 µL into each well in three replicates. Then, 100 µL of sterile cell-free supernatant of *M. iranicum* IKVS S1 or IKVS S2 was transferred to the test wells. As a control, 100 µL of a suspension of the studied microorganisms—*S. epidermidis* (IKVS 7, IKVS 13) and *E. coli* (IKVS 1, IKVS 18)—and 100 µL of MPB were also transferred to the control wells. The plate was incubated for 48 h at 37 °C. After 48 h, MPB was removed from the test and control wells and the wells were washed several times with sterile distilled water. The wells were stained with 0.1% crystal violet solution for 10 min, washed three times with distilled water, and air dried. Then, 95% ethanol was added to the dry wells and incubated for 15 min, 125 µL of alcohol was transferred into the wells of a clean plate, and the optical density was measured at 540 nm wavelength [39].

Biofilm formation calculation formula:

$$\text{Biofilm formation enhancement rate (\%)} = ((OD_{540test}/OD_{540control}) - 1) \times 100\%,$$

where

$OD_{540test}$ = optical density in the test;

$OD_{540control}$ = optical density in the control.

All experiments were performed in triplicate, and the results are expressed as the mean ± SD. Student's *t*-test was performed to determine the significance of the results obtained. Statistical analysis was performed using XLSTAT Statistical Analysis Software 2016 (2020.4.1.1018).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/bacteria2040013/s1, Table S1: Average optical densities of test and control samples.

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