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A PROBIOTIC BASED ON THE *Escherichia coli* ŽP STRAIN. I. EFFICIENCY ASSESSMENT OF THE CONJUGATIVE TRANSFER OF THE COLICIN E7 ACTIVITY GENE INTO AVIAN PATHOGENIC *E. coli* STRAINS in vitro AND in vivo

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Abstract

The protection of farm animals against infectious diseases is a priority in veterinary medicine. The wide spread of pathogenic and opportunistic bacteria resistant to antibiotics on poultry farms requires the development of modern methods to maintain the health of birds in industrial production. A promising direction to improve measures to prevent and limit the spread of pathogens resistant to antimicrobial agents is the use of targeted bacterial drugs — probiotics. Veterinary probiotics based on genetically modified microorganisms can be used for the treatment and prophylaxis of infectious diseases in farm animals. We demonstrated the antagonistic effect of the genetically modified *Escherichia coli* ŽP strain against agents of avian colibacillosis, the avian pathogenic *Escherichia coli* (APEC) in both in vitro and in vivo models. The *colE7* gene (colicin E7 synthesis gene) carried on a conjugative plasmid was efficiently transferred by conjugation in conditions of planktonic and biofilm growth in vitro. The *E. coli* ŽP strain was shown to actively colonize the intestine of rats and quails and to contribute to beneficial effects of the microbiota. Our aim was to evaluate the competitive ability of the *E. coli* ŽP strain as well as the efficiency of killing APEC based on the conjugative transfer of the *colE7* gene, encoding a DNasae, in vitro and in vivo. We used the ColE7-mediated kill—anti-kill system based on the Nissle 1917 probiotic strain, the genetically modified *E. coli* ŽP strain (killer donor) carrying the *colE7* gene on the conjugative plasmid pOX38a, as well as the *immE7* gene (colicin E7 immunity gene) on the chromosome. As control, the *E. coli* N4i strain without *colE7* gene on the conjugative plasmid pOX38 (control donor) was used. Six APEC strains with resistance to ampicillin isolated from organs of broilers with colibacillosis were used as recipients in performed conjugation assays. The phylogenetic group of used APEC strains was detected with quadruplex PCR. The conjugation transfer was conducted in Luria-Bertani (LB) medium in immunological polystyrene 96-walls flat bottom plates in plankton and in biofilm culture. The experiments with rats (Wistar line) and Manchurian quail (*Coturnix coturnix*) were conducted in the vivarium of the Wagner Perm State Medical University. The competitive ability of *E. coli* ŽP strain was confirmed in co-cultivation assays with APEC strains, including bacteriocin producers, in various models. Conjugative *colE7* gene transfer to APEC was detected in vitro in plankton and in biofilm: in experiments with the control donor strain *E. coli* N4i the conjugation frequency varied from 10^{-6} to10^{-2}. In vivo, it was shown that *E. coli* ŽP strain was able to effectively colonize the rat (line Wistar) and Manchurian quail (*Coturnix coturnix*) intestine and persist there at least for a month. Introduced *E. coli* ŽP cells increased the total amount

of commensal *Escherichia* in the intestine of the animals and reduced the growth of the pathogenic microbes without affecting the lactic acid bacteria. The transfer ability of the conjugative plasmid pOX38 in the intestinal tract of both animal species was demonstrated. The conjugation in the intestinal tract occurred with a high frequency, on average 10^{-2}. No transconjugants were detected in both in vitro and in vivo experiments with the *E. coli* ŽP strain harboring the conjugative plasmid pOX38a; the recipient cells that received the *colE7* gene via the conjugative transfer expressed it and were lysed due to the colicin DNase activity. In groups in which the strain ŽP was applied, the number of APEC recipients also decreased. The obtained results indicated that the *E. coli* ŽP strain was able to colonize the intestine of animals and had antibacterial activity against enteropathogens due to the conjugative mechanism of colicin gene transfer. As the ŽP strain was also effective on APEC cells that were resistant and tolerant to bacteriocins, it has the potential to become the basis for a highly effective new generation of probiotics.

Keywords: colicins, ColE7, conjugative transfer, kill—anti-kill system, antibiotic alternative, probiotics, avian pathogenic *Escherichia coli* (APEC), animal models

Poultry farming is one of the fastest growing segments of agriculture [1, 2]. To increase the efficiency of poultry production, various feed additives, such as synthetic hormones and antibacterial drugs, are widely used [3]. Long-term use of antibiotics led to the appearance of gram-positive and gram-negative microorganisms resistant to these substances, which become the main cause of death of young birds in poultry farms [4, 5]. In addition, infected poultry are a potential reservoir of pathogens of acute intestinal infections for humans [6]. In 2014, the World Health Organization (WHO) adopted a strategy to limit the use of antibacterial drugs for the prevention of infectious diseases in farm animals [7], and in 2017, Russia joined this program [8].

Probiotics, the bacterial targeted drugs that play a leading role in replacing antibiotics, are promising in improving measures to prevent and limit the spread of pathogens resistant to antimicrobial agents [9, 10]. Probiotics are live cells of microorganisms of a single species or several species which are used as feed additives and act as a growth stimulant, and also beneficially affect the host physiological parameters and microbiota [11, 12]. In the first hours of life, the animal intestines are artificially colonized with bacterial strains and/or biocomplexes that provide an antagonistic effect against pathogenic or conditionally pathogenic microorganisms due to the competitive displacement by bacteria producing bacteriocins, the antibacterial substances which inhibit closely related microbial taxa. There are many reports on the positive results of using probiotics to prevent and treat infections in birds [1, 13-15]. Artificially constructed strains with multiple production of bacteriocins attract special attention [16-19]. For example, to restore intestinal competitiveness of probiotic strain *Escherichia coli* M17 which lost its antagonistic properties it was proposed to use recombinant plasmids for production of colicin E1 [20] or microcin C51 [21]. However, even these drugs may not be effective enough as bacteriocin-resistant bacteria appear [22] which possess modified surface receptors and translocation systems. Colicin delivery via horizontal *col*-gene transfer using conjugative plasmids may be an alternative approach [23] that allows us to target bacteriocin resistant/tolerant bacterial strains.

The antimicrobial bacterial kill—anti-kill system was tested with the reference (*E. coli* K-12 TG1) and uropathogenic (*E. coli* DL82) strains. Real-time PCR, a bioluminescent method, and flow cytometry confirmed the conjugative transfer of plasmid pOX38a carrying *colE7* to the recipient cell where transcription of *colE7* gene begins immediately and the synthetized bacteriocin kills the recipient [24].

This paper is the first report on the antagonistic effect of the genetically

modified *E. coli* ŽP strain against APEC (avian pathogenic *Escherichia coli*, the causative agents of escherichiosis in birds) in in vitro and in vivo experimental models. It was found that *in vitro* conjugative transfer of the colE7 gene to APEC cells is effective both under planktonic growth and biofilm development. In vivo, the strain *E. coli* ŽP was able to actively colonize the intestines of rats and Manchurian quail and persist there, contributing to the normalization of animal microbiota. Our results confirmed that the conjugative plasmid transfer from the used donor to APEC strains occured in the intestinal tract with a high frequency, and resulted in colicin acting also on cells that were resistant and tolerant to bacteriocins. This suggests the possibility of creating a highly effective probiotic drug of a new generation based on conjugative transfer of colicin synthesis genes.

The aim of our study was to evaluate the efficiency of APEC (avian pathogenic *Escherichia coli*) killing upon conjugative transfer of colicin E7 gene and the competitiveness of *Escherichia coli* ŽP strain in vitro and in vivo.

Materials and methods. ColE7-mediated kill anti-kill system based on a genetically modified Nissle 1917 probiotic strain, the *E. coli* ŽP pOX38a Gm^rCm^r strain (killer donor, KD) that carries the colicin E7 synthesis gene (*colE7*) with DNase activity on the conjugative plasmid and the colicin E7 immunity (*immE7*) gene on the chromosome, and the *E. coli* N4i pOX38 Gm^rCm^r strain (control donor, D) without *colE7* on the plasmid [25]. Recipients (R) were ampicillin-resistant *E. coli* strains ($n = 6$) isolated from the internal organs of infected broiler chickens (APEC). The strains had an individual genetic profile according to rep-PCR typing with ERIC 1R/ERIC 2 primers [26]. The phylogenetic affiliation of the isolates was determined by the multiplex polymerase chain reaction (quadruplex PCR) [27]. The primers used in this work were synthesized in Syntol LLC (Syntol, Russia). Amplification was run on a DNA Engine Dyad Thermal Cycler (Bio-Rad, USA). The bands were visualized using the Gel-Doc XR gel documentation system (Bio-Rad, USA).

The strains were deposited into the Ex culture collection at the Department of Biology, Biotechnical Faculty, University of Ljubljana (Univerza v Ljubljani, Slovenia).

APEC cultures were screened for sensitivity to bacteriocins using the BZB collection of indicator strains producing bacteriocins and microcins (University of Ljubljana) using the agar overlay method [22]. *E. coli* DH5α was used as the control (bacteriocin-sensitive) strain. The bacteriocin production by APEC strains and the sensitivity of *E. coli* ŽP to bacteriocins of the studied strains were assessed by a similar method [22].

Conjugative transfer was performed in Luria-Bertani (LB) medium (Amresco, USA) for 6 and 24 hours in plankton culture and biofilm in polystyrene flat-bottomed immunological 96-well plates (Lenpolymer, Russia).

The conjugation mixture contained 100-fold diluted night cultures (standardized to 2.0 according to the McFarland turbidity standard) of the donor and recipient in a 1:4 ratio. Pre-washed (0.89% NaCl) biofilms were destroyed by ultrasound (37 Hz, Elmasonic 30S, Elma Schmidbauer GmbH, Germany) in 100 μl of saline for 1 min (5 cycles). The colony forming units (CFU) were calculated from the counts on selective media (on LB medium supplemented with 50 μg/ml chloramphenicol and 50 μg/ml ampicillin for transconjugants, on LB medium with 50 μg/ml ampicillin for recipient cells, and on LB medium with 40 μg/ml gentamicin for donor cells). Conjugation transfer frequency (Y)

was estimated as the transconjugant cells (T) CFU to recipient cells (R) CFU ratio [28].

Biofilm biomass was determined as described by Merritt et al. [29] on a Benchmark Plus microplate reader (Bio-Rad, USA) at $\lambda = 570$ nm.

Bacterial growth in co-culture was assessed in a polystyrene flat-bottomed immunological 96-well plate on rich (LB) and minimal (M9) media at 37 °C from 1 to 3 days. Plating was performed from decimal dilutions of the bacterial suspension on the corresponding selective agars with antibiotics.

Tests on Wistar rats and Manchurian quail (*Coturnix coturnix*) were performed in a vivarium (Wagner Perm State Medical University). Stocking density, feeder space and watering space, temperature, humidity, lightness were within the recommended limits (VNITIP). Care for rats and poultry was carried out in accordance with GOST 34088-2017 [30].

In the first in vivo conjugative transfer experiment, 30-day-old male rats of 175.25±10.31 g weight were assigned into three groups, 10 animals per group. The rats were kept for 21 days in plastic cages (5 animals per cage) in a ventilated room at 21-23 °C and natural lighting, with access to food and water ad libitum. Live strains of the control donor (group I) and killer donor (group II) were added to water at 10^8 cells per rat for 7 days. Intestinal colonization was determined on day 3 and day 6 by plating feces on selective media. From day 8 to day 21, in both test groups, live culture of APEC strain resistant to ampicillin (the recipient) were added to water at 10^8 bacteria per rat. After 6 hours, water was changed and donor strains were added. The control group did not receive any of the *E. coli* strains. The presence of ampicillin-resistant recipients, control/killer donors, and transconjugants in the intestine was determined on days 10, 14, and 21.

In the second in vivo experiment, Manchurian quails of 114.0±7.50 g weight were assigned into three groups, 5 individuals each, and kept for 1 week under conditions similar to those in the first experiment. Live cells of the control donor (group I) and killer donor (group II) were administered via water (10^8 bacteria per quail) for 3 days, with daily control of intestinal colonization by plating feces on selective media. From day 2 to day 6, live cells of the ampicillin-resistant APEC strain (10^8 bacteria per quail) were administered in both experimental groups, after 6 h water was changed and donor strains were applied. The control group did not receive any *E. coli* strains. The presence in the intestine of ampicillin-resistant recipients and transconjugants was determined on days 2, 3, and 6.

In both experiments, the number of viable cells was calculated per 1 g feces, the conjugation frequency was determined similarly to the in vitro experiment.

Statistical analysis was performed using Microsoft Office Excel and STATISTICA 10 software (StatSoft, Inc., USA). The results are presented as arithmetic mean (M) and its error (±SEM). The significance of differences between the average values was assessed by Student's t-test at $p < 0.05$ and the relationship between the quantitative values by the linear Pearson correlation coefficient (r_p).

Results. The results of quadruplex PCR revealed that the APEC strains used in this study belonged to the phylogenetic groups B1, B2, and E. Colicinogenesis was detected in four cultures, one of them turned out to be insensitive to bacteriocin ColE7 (Fig. 1, Table 1). All APECs were resistant to ten or more bacteriocins, including those with pore-forming, DNase, rRNAse, and tRNAse action.

1. Characterization of *Escherichia coli* strains from the internal organs of infected broiler chickens (avian pathogenic *E. coli*, APEC)

APEC strain	Phylogroup	Bacteriocines	Resistance to colicins	Resistance to microcins
RB1	B1	No	A, B, D, E1, E3, E5, E7, Ia, Ib, K, N, M, S4	B17, C7, V
RB2	E	No	A, B, D, E1, E5, E7, Ia, Ib, K, N, M, S4	C7, V
RB3	E	Yes	A, B, D, E1, E2, E3, Ia, Ib, K, N, M, S4	B17, C7, V
RB4	E	Yes	A, B, D, E1, E, E5, E7, E8J, Ia, Ib, K, N, M	B17, C
RB5	B1	Yes	A, B, D, E1, E5, E6, Ia, Ib, K, N, M, S4	B17, C7, V
RB6	B2	Yes	A, B, D, Ia, Ib, N, M, S4	B17, C7, V

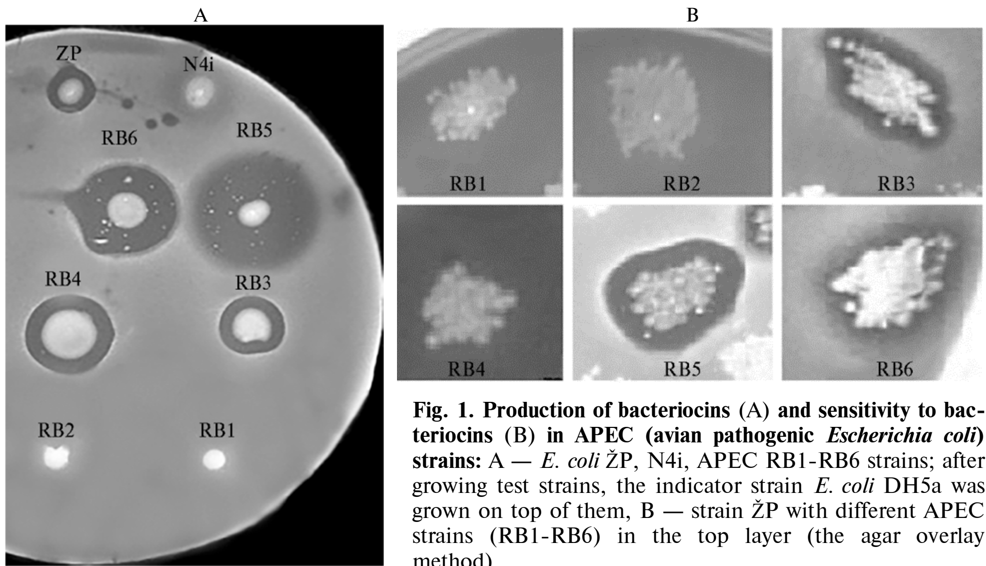

Fig. 1. Production of bacteriocins (A) **and sensitivity to bacteriocins** (B) **in APEC (avian pathogenic *Escherichia coli*) strains:** A — *E. coli* ŽP, N4i, APEC RB1-RB6 strains; after growing test strains, the indicator strain *E. coli* DH5a was grown on top of them, B — strain ŽP with different APEC strains (RB1-RB6) in the top layer (the agar overlay method).

In experiments with the control donor strain *E. coli* N4i, the frequency of conjugative transfer in 6 hours varied within 10^{-6}-10^{-2} and turned out to be higher in the biofilm than in plankton growth ($1.73\times10^{-2}\pm2.24\times10^{-2}$ vs. $2.27\times10^{-5}\pm2.40\times10^{-5}$, respectively), and after 24 hours it was comparable in both models ($4.45\times10^{-4}\pm5.46\times10^{-4}$ vs. $6.25\times10^{-3}\pm8.63\times10^{-3}$) (Table 2). A biofilm model was necessary because in vivo, including in the intestine, microorganisms exist mainly as part of attached communities. Upon a 24-hour exposure, the correlation between plasmid transfer in plankton and biofilm was r_p = 0.905 (p = 0.05).

In all variants of conjugation of the killer donor *E. coli* ŽP with recipient strains, transconjugants were not detected. That is, recipient cells that received *colE7* gene via conjugative transfer expressed it and were lysed due to the colicin DNase activity.

After 24 hours we also determined biofilms biomass in mixed cultures. For different strains, the OD_{570} value ranged from 0.100 to 0.187, in average 0.144±0.007. An inverse moderate relationship was revealed between the biofilm biomass and the frequency of conjugation (r_p = –0.630, p = 0.05).

It should be noted that after 24 hour incubation of conjugation mixture the average counts of control and killer donors were $2.22\times10^{7}\pm2.03\times10^{7}$ and $1.02\times10^{8}\pm7.69\times10^{7}$ CFU/ml in plankton, and $7.38\times10^{6}\pm2.33\times10^{6}$ and $9.89\times10^{6}\pm1.13\times10^{7}$ CFU/ml in biofilms, respectively, which may indicate a high

2. The number of donor, recipient, transconjugant cells (CFU/ml) **and the conjugation frequency in plankton growth and in biofilms upon conjugative transfer of plasmid pOX38 to APEC** (avian pathogenic *Escherichia coli*) **strains in vitro** (M±SEM)

APEC strain	Group I, *E. coli* N4i (control donor)				Group II, *E. coli* ŽP (killer donor)			
	D	R	T	Y	KD	R	T	Y
Plankton growth								
RB1	$1.04\times10^{7}\pm1.13\times10^{6}$	$1.70\times10^{8}\pm2.50\times10^{7}$	$2.10\times10^{5}\pm7.25\times10^{4}$	$1.33\times10^{-3}\pm6.22\times10^{-4}$	$3.14\times10^{7}\pm2.13\times10^{6}$	$9.13\times10^{7}\pm3.75\times10^{6}$	0.00	0.00
RB2	$1.76\times10^{6}\pm6.63\times10^{5}$	$1.16\times10^{8}\pm1.13\times10^{7}$	$2.62\times10^{6}\pm2.21\times10^{6}$	$2.46\times10^{-2}\pm2.13\times10^{-2}$	$8.43\times10^{7}\pm5.08\times10^{7}$	$3.86\times10^{7}\pm3.63\times10^{6}$	0.00	0.00
RB3	$5.71\times10^{7}\pm2.79\times10^{7}$	$5.50\times10^{7}\pm2.25\times10^{7}$	$2.16\times10^{5}\pm2.01\times10^{5}$	$2.92\times10^{-3}\pm2.46\times10^{-3}$	$1.70\times10^{8}\pm3.40\times10^{7}$	$6.80\times10^{7}\pm1.45\times10^{7}$	0.00	0.00
RB4	$1.74\times10^{6}\pm5.63\times10^{5}$	$3.65\times10^{8}\pm1.56\times10^{7}$	$1.84\times10^{5}\pm1.66\times10^{5}$	$5.03\times10^{-4}\pm4.55\times10^{-4}$	$2.03\times10^{7}\pm1.45\times10^{7}$	$1.59\times10^{8}\pm3.63\times10^{7}$	0.00	0.00
RB5	$2.36\times10^{7}\pm7.63\times10^{6}$	$2.89\times10^{8}\pm4.88\times10^{7}$	$1.13\times10^{4}\pm1.25\times10^{3}$	$3.94\times10^{-5}\pm2.31\times10^{-6}$	$6.83\times10^{7}\pm4.75\times10^{7}$	$1.88\times10^{8}\pm3.25\times10^{7}$	0.00	0.00
RB6	$3.88\times\pm1.13\times10^{7}$	$2.79\times10^{7}\pm1.46\times10^{7}$	$2.80\times10^{5}\pm2.20\times10^{5}$	$8.13\times10^{-3}\pm3.63\times10^{-3}$	$2.35\times10^{8}\pm1.84\times10^{8}$	$3.58\times10^{7}\pm2.34\times10^{7}$	0.00	0.00
Biofilm								
RB1	$1.15\times10^{7}\pm6.15\times10^{6}$	$1.15\times10^{7}\pm1.04\times10^{6}$	$5.13\times10^{2}\pm3.63\times10^{2}$	$4.89\times10^{-5}\pm2.06\times10^{-5}$	$7.94\times10^{6}\pm1.29\times10^{6}$	$3.04\times10^{7}\pm2.67\times10^{7}$	0.00	0.00
RB2	$4.07\times10^{6}\pm3.81\times10^{6}$	$7.76\times10^{6}\pm3.56\times10^{6}$	$7.38\times10^{3}\pm6.88\times10^{3}$	$1.60\times10^{-3}\pm3.42\times10^{-4}$	$3.43\times10^{7}\pm2.22\times10^{7}$	$3.03\times10^{7}\pm2.62\times10^{7}$	0.00	0.00
RB3	$5.32\times10^{6}\pm3.93\times10^{6}$	$4.78\times10^{6}\pm8.75\times10^{5}$	$1.13\times10^{3}\pm7.75\times10^{2}$	$2.13\times10^{-4}\pm1.23\times10^{-4}$	$4.70\times10^{6}\pm2.08\times10^{6}$	$5.25\times10^{6}\pm2.00\times10^{6}$	0.00	0.00
RB4	$8.16\times10^{6}\pm3.44\times10^{6}$	$3.96\times10^{7}\pm7.56\times10^{6}$	$2.49\times10^{4}\pm1.91\times10^{4}$	$5.58\times10^{-4}\pm3.76\times10^{-4}$	$1.48\times10^{6}\pm3.25\times10^{5}$	$2.83\times10^{7}\pm2.50\times10^{7}$	0.00	0.00
RB5	$7.43\times10^{6}\pm3.07\times10^{6}$	$2.02\times10^{7}\pm1.73\times10^{7}$	$2.50\times10^{1}\pm2.50\times10^{1}$	$8.47\times10^{-6}\pm8.47\times10^{-6}$	$1.55\times10^{6}\pm3.00\times10^{5}$	$2.96\times10^{6}\pm1.16\times10^{6}$	0.00	0.00
RB6	$7.82\times10^{6}\pm3.20\times10^{6}$	$2.01\times10^{7}\pm1.75\times10^{7}$	$1.10\times10^{3}\pm1.00\times10^{2}$	$2.40\times10^{-4}\pm2.13\times10^{-4}$	$9.38\times10^{6}\pm3.63\times10^{6}$	$7.74\times10^{6}\pm5.26\times10^{6}$	0.00	0.00

Note. D — control donor. KD — killer donor, R — recipient, T — transconjugant, Y — frequency of conjugation transfer.

competitiveness of donors in polymicrobial communities. This assumption was verified in experiments with the co-culture of the killer donor and APEC strains.

Growth of cultures of the killer donor *E. coli* ŽP and three APEC strains, RB2 (not producing bacteriocins and insensitive to ColE7), RB3 (producing bacteriocins and sensitive to ColE7) and RB4 (producing bacteriocins and insensitive to ColE7) on rich (LB) and minimal (M9) media are shown in Figure 2.

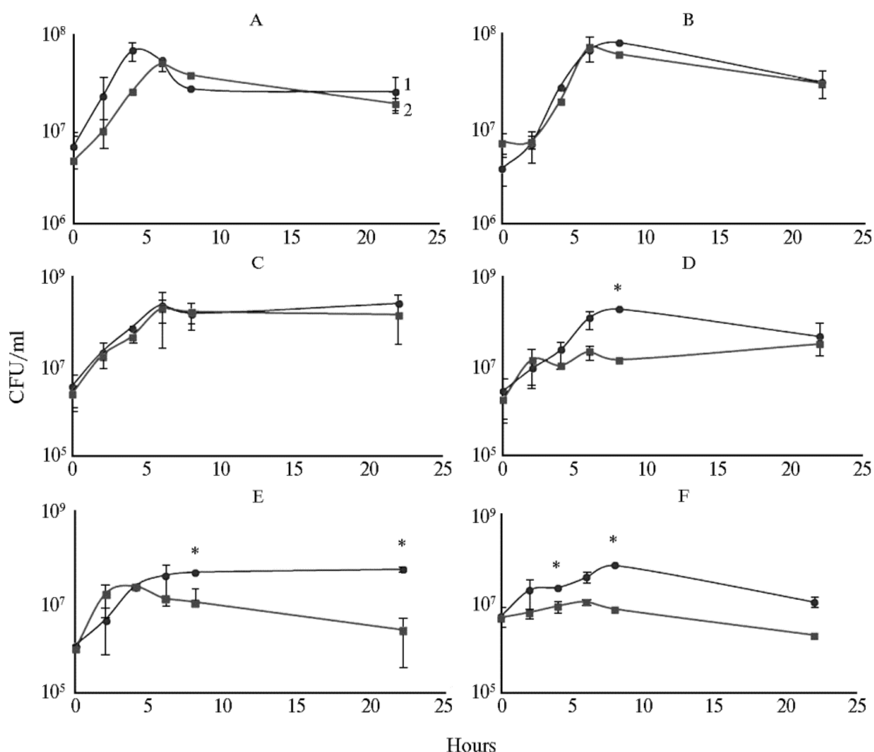

Fig. 2. Growth of APEC (avian pathogenic *Escherichia coli*) **strains in co-cultures with *E. coli* ŽP in rich (LB) medium** (left) **and minimal** (M9) **medium** (right)**:** 1 — recipient (APEC), 2 — killer donor (ŽP); A, B — strain RB2, C, D — strain RB3, E, F — strain RB4. Asterisks (*) indicate statistically significant differences ($p \leq 0.05$) between the growth of the recipient and the killer donor in the corresponding periods.

The dynamics of competition between *E. coli* ŽP and APEC strains in a rich medium (see Fig. 2, A, B, D) differed significantly. In the absence of antagonism between the strains (RB2 recipient), immediately after inoculation, the number of cells of both cultures increased, and a slight change in the ratio of bacteria of competing strains was most likely due to the advantage in the growth rate of the natural strain RB2. After 5 hours of co-culture, the amount of *E. coli* ŽP cells was stable. There was a tendency toward a decrease in the abundance of *E. coli* RB2 bacteria due to their lysis resulting from conjugative plasmid transfer, which was quite effective at a frequency of $2.46 \times 10^{-2} \pm 3.02 \times 10^{-2}$. In co-culture of *E. coli* ŽP and RB4, the number of *E. coli* ŽP cells towards the stationary phase, on the contrary, decreased. Apparently, at this stage the antagonistic activity of the APEC strain was manifested due to the synthesis of bacteriocin, which is induced by an increase in the culture density, starting from the end of the logarithmic growth. Considering that the bacteria *E. coli* RB4 are insensitive to ColE7 and the conjugation rate was low, the cell ratio after 24 hours in this pair is explainable. Interestingly, in a co-culture of *E. coli* ŽP and RB3, in which RB3 also produces bacteriocins but is sensitive to ColE7, antagonistic activity

3. The number of donor, recipient, transconjugant cells (CFU/ml) **and the conjugation frequency in intestines of Wistar rats and Manchurian quails upon conjugative transfer of plasmid pOX38 to APEC** (avian pathogenic *Escherichia coli*) **strains in vivo** (M±SEM)

Days	Group I, *E. coli* N4i (control donor)				Group II, *E. coli* ŽP (killer donor)			
	D	R	T	Y	KD	R	T	Y
				Rats				
3	$3.50\times10^{4}\pm2.15\times10^{3}$	nd	nd	nd	$1.50\times10^{4}\pm1.25\times10^{3}$	nd	nd	nd
6	$7.21\times10^{5}\pm5.29\times10^{4}$	nd	nd	nd	$2.34\times10^{5}\pm1.74\times10^{5}$ [a]	nd	nd	nd
10	$2.96\times10^{6}\pm2.01\times10^{6}$ [a]	$3.54\times10^{3}\pm1.23\times10^{3}$	$2.15\times10^{2}\pm1.75\times10^{2}$	$6.07\times10^{-2}\pm5.58\times10^{-2}$	$5.97\times10^{5}\pm2.58\times10^{5}$ [a]	$2.19\times10^{3}\pm1.25\times10^{3}$	0.00	0.00
14	$1.26\times10^{7}\pm8.64\times10^{6}$ [a]	$5.21\times10^{5}\pm2.13\times10^{4}$	$4.11\times10^{4}\pm4.00\times10^{3}$	$7.87\times10^{-2}\pm8.32\times10^{-3}$	$5.64\times10^{7}\pm8.96\times10^{6}$ [a]	$4.22\times10^{4}\pm2.36\times10^{4}$	0.00	0.00
21	$7.25\times10^{7}\pm2.35\times10^{7}$ [a]	$8.69\times10^{6}\pm5.55\times10^{6}$ [a]	$6.15\times10^{4}\pm1.02\times10^{3}$	$7.08\times10^{-3}\pm9.64\times10^{-4}$	$2.23\times10^{7}\pm1.00\times10^{7}$ [a]	$5.91\times10^{4}\pm4.98\times10^{3}$ [b]	0.00	0.00
				Manchurian quails				
1	$8.00\times10^{3}\pm5.21\times10^{3}$	nd	nd	nd	$5.04\times10^{4}\pm1.22\times10^{4}$	nd	nd	nd
2	$2.70\times10^{6}\pm2.01\times10^{5}$ [a]	$5.00\times10^{5}\pm4.55\times10^{5}$	$5.00\times10^{3}\pm1.12\times10^{3}$	$1.00\times10^{-2}\pm1.12\times10^{-1}$	$8.12\times10^{5}\pm2.22\times10^{5}$	$4.68\times10^{4}\pm2.25\times10^{4}$	0.00	0.00
3	$4.45\times10^{6}\pm3.05\times10^{6}$ [a]	$3.05\times10^{6}\pm2.41\times10^{5}$	$1.05\times10^{4}\pm1.11\times10^{4}$	$3.44\times10^{-3}\pm2.46\times10^{-3}$	$1.74\times10^{6}\pm1.96\times10^{6}$ [a]	$7.39\times10^{5}\pm5.57\times10^{5}$	0.00	0.00
6	$6.42\times10^{7}\pm6.16\times10^{7}$ [a]	$9.12\times10^{6}\pm7.15\times10^{6}$ [a]	$4.18\times10^{5}\pm4.00\times10^{5}$	$4.58\times10^{-2}\pm2.12\times10^{-1}$	$2.57\times10^{7}\pm3.01\times10^{7}$ [a]	$8.29\times10^{5}\pm5.55\times10^{5}$ [b]	0.00	0.00

N o t e. D — control donor. KD — killer donor, R — recipient, T — transconjugant, Y — frequency of conjugation transfer, nd — not detected.

[a] Statistically significant differences ($p \le 0.05$): D: value compared with the day 3 value for rats and day 1 for quails; R: value compared with day 10 value for rats and day 2 value for quails; KD: data compared with day 3 value for rats and day 1 value for quails).

[b] Statistically significant differences ($p \le 0.05$) between the group with the control donor and the group with the killer donor.

between the strains did not appear.

On M9 medium (see Fig. 2, B, D, E), the ratio between *E. coli* ŽP and RB2 did not change, while APECs producing colicins (RB3 and RB4) suppressed the growth of the killer donor at the end of the logarithmic—the beginning of the stationary phase of growth. However, the absence of significant differences between associates at the end of culture, apparently, can be associated with ineffective conjugation of *E. coli* ŽP and low colicin synthesis by recipients (RB3 and RB4) when grown on a poor culture medium.

In our preliminary studies of in vivo conjugative plasmid transfer, *E. coli* bacteria resistant to ampicillin, chloramphenicol, and gentamicin were not found in the intestines of rats and quails. In group I of rats, on day 3 the number of viable cells of the control donor and recipient averaged $3.50\times10^4\pm2.15\times10^3$ and $3.54\times10^3\pm1.23\times10^3$ CFU/g, respectively (Table 3). *E. coli* Amp^rCm^r transconjugants were detected on day 3 after the administration of the recipient, on average, their number was $2.15\times10^2\pm1.75\times10^2$ CFU/g, the frequency of conjugation was $6.07\times10^{-2}\pm$ 5.58×10^{-2}. In this group, an increase in the cell counts of both the donor and the recipient was observed throughout the experiment. In group II, the number of viable cells of the killer donor and the recipient on day 3 was $1.50\times10^4\pm1.25\times10^3$ and $2.19\times10^3\pm1.25\times10^3$ CFU/g, respectively. Further, the abundance of *E. coli* ŽP increased to 10^7 CFU/g, while the number of recipient cells after the first administration increased insignificantly. Transconjugants in this group were not found in any assayed time point. In the group of animals that did not receive donor strains, *E. coli* resistant to ampicillin, chloramphenicol and gentamicin were not detected in any assayed time point.

Given the results on colonization of rat intestines with donor strains in a preliminary experiment, in the model with Manchurian quail, the frequency of conjugative transfer in vivo was followed for 6 days. Just 2 days after the administration of the bacterial suspension, the number of cells of the donor and killer strains of *E. coli* in the poultry intestine was $2.70\times10^6\pm2.01\times10^5$ and $8.12\times10^5\pm2.22\times10^5$ CFU/g, respectively. The frequency of conjugative transfer in group I (control donor) remained at 10^{-2}-10^{-3} level for 6 days of observation. Similar to the experiment with the rat model, transconjugants in group II (killer donor) were not detected. In the control, *E. coli* bacteria resistant to ampicillin, chloramphenicol, and gentamicin were also not detected.

Development of new methods and means of specific prophylaxis and/or therapy of bacterial infections in farm animals is actively carried out in Russia and abroad [31]. Maintaining effective symbiosis between the poultry organism and the intestinal microbiota is a necessary component of a successful feed strategy and the livestock safety and wellness. Highly effective probiotic preparations are being developed to control causative agents of escherichiosis, salmonellosis, campylobacteriosis and other intestinal infections in poultry. Evidence is provided that bacteriocins can replace antibiotics in animal husbandry.

The search for microbial producers of bacteriocins that can be used as probiotics, is constantly conducted by researchers. Torshizi et al. [32] screened lactobacteria isolated from chicken intestines and found two isolates (*Lactobacillus fermentum* and *Lactobacillus rhamnosus*) capable of inhibiting *Escherichia* growth in vitro. Ogunbanwo et al. [33] studied the potential therapeutic efficacy of the bacteriocinogenic strain *Lactobacillus plantarum* in experimental *E. coli* infection of broiler chickens. The strain *E. coli* S5/98 producing microcin B 5/98 with a wide range of antagonistic activity against *Escherichia*, *Salmonella*, *Klebsiella*

bacteria, which was isolated from feces of adult pigs, is already used to produce liquid and dry form of Microcycol probiotic [34]. Tests on broiler chickens showed that Microcycol can effectively control the intestinal microbiota balance, increase nonspecific resistance, preservation and productivity of poultry, and also improve meat quality [35]. Colicins, a class of bacteriocins produced by *E. coli* and acting against closely related taxa, were studied as a possible alternative to antibiotics. Colicin E1 inhibited growth of enteropathogenic *E. coli* strains (ETEC) [36], and, when added to feed, reduced the frequency and the severity of experimental diarrhea caused by ETEC [37]. These results show that the use of bacteria producing bacteriocins or pure bacteriocins in animal rearing can positively affect the safety of livestock and poultry products which are the main source of diarrhea strains of *E. coli* causing escherichiosis and toxicoinfections in humans.

It is believed that biological products for medical and veterinary use based on recombinant microorganisms with proven safety can be targeted and effectively used for the treatment and prevention of various diseases [16]. To date, the use of combined probiotic preparations combining several cultures with different properties or artificially constructed strains with multiple production of bacteriocins is approved. Members of the natural gastrointestinal microbiota, such as lactobacteria (*Lactobacillus* and *Bifidobacterium*) and *Escherichia*, e.g. *E. coli* Nissle 1917 are the source for developing novel probiotics [38]. Being commensal microorganisms, they usually have a therapeutic effect on their own and are excellent for use in engineering synthetic biology [39, 40]. pColap and pPAL3 vectors, *E. coli* M17 and natural producer *E. coli* S5/98 were used to create recombinant *E. coli* M17 strains pPAL4 which produces microcin B and colicin E1 and pPAL5, synthesizing microcin but resistant to colicin E1 [35]. Romacol, a probiotic based on a genetically engineered strain *E. coli* M17 (p74), which produces C51 microcin, has been proposed [21].

E. coli ŽP is promising as the basis for a probiotic preparation due to the possibility to affect enterobacteria which are resistant to antibiotics and bacteriocins and circulate in poultry and livestock farms. Incorporation of *E. coli* ŽP into gut microbiota will increase the total level of commensal *Escherichia* in the intestines of animals, while inhibiting pathogenic representatives of this species without a noticeable effect on lactobacteria and bifidobacteria. The effectiveness of the strain is determined by an alternative system of colicin delivery, which ensures its high competitiveness in various ecological niches where bacteriocin producers can also be present.

Thus, in co-cultures, the interaction between *Escherichia coli* ŽP and APEC (avian pathogenic *E. coli*) strains is determined not only by the growth rate and sensitivity to bacteriocins, but also by conjugative plasmid transfer. The combination of these factors will determine the dynamics of competitiveness of a new probiotic strain *E. coli* ŽP and heterogeneous APEC in gastrointestinal tract of birds. Our experiments confirmed that *E. coli* ŽP strain is able to effectively colonize the intestines of rats and Manchurian quail and persist for a long time. Conjugative transfer of the plasmid from the control donor occurs in intestinal tract with a rather high frequency, while in the group with the killer donor transconjugants are absent. The reduced number of recipient cells in the second group also proves the effectiveness of the studied strain against pathogenic forms of *E. coli*. The use of conjugative mechanism in creating probiotic agent for poultry farming is in line with global trends. More research allows us to fully reveal the biotechnological potential of the genetically modified strain *E. coli* ŽP, including its effect on zootechnical indicators of birds. In particular, it seems important to study its effectiveness in treatment and

prevention of escherichiosis in animals and to evaluate the therapeutic and anti-epidemic potential.

REFERENCES

1. Fisinin V.I. *Materialy XIX Mezhdunarodnoi konferentsii «Mirovye i rossiiskie trendy razvitiya ptitsevodstva: realii i vyzovy budushchego»* [Proc. XIX Int. Conf. «World and Russian poultry development trends: realities and future challenges»]. Sergiev Posad, 2018: 9-48 (in Russ.).
2. Nefedova V.N., Maiorova S.V. *Ekonomika i Biznes: teoriya i praktika*, 2017, 8: 60-64 (in Russ.).
3. Mezentsev S.V., Telegin N.G. *BIO*, 2004, 10: 5-8 (in Russ.).
4. Miles T.D., McLaughlin W., Brown P.D. Antimicrobial resistance of *Escherichia coli* isolates from broiler chickens and humans. *BMC Veterinary Research*, 2006, 2(7): 1-9 (doi: 10.1186/1746-6148-2-7).
5. Koga V.L., Rodrigues G.R., Scandorieiro S., Vespero E.C., Oba A., de Brito B.G., de Brito K.C.T., Nakazato G., Kobayashi R.K.T. Evaluation of the antibiotic resistance and virulence of *Escherichia coli* strains isolated from chicken carcasses in 2007 and 2013 from Parana, Brazil. *Foodborne Pathogens and Disease*, 2015, 12(6): 479-485 (doi: 10.1089/fpd.2014.1888).
6. Mora A., Viso S., Lypez C., Alonso M.P., García-Garrote F., Dabhi G., Mamani R., Herrera A., Marzoa J., Blanco M., Blanco J.E., Moulin-Schouleur M., Schouler C., Blanco J. Poultry as reservoir for extraintestinal pathogenic *Escherichia coli* O45:K1:H7-B2-ST95 in humans. *Veterinary Microbiology*, 2013, 167(3-4): 506-612 (doi: 10.1016/j.vetmic.2013.08.007).
7. *Antimicrobial resistance: Global report on surveillance.* WHO Library Cataloguing-in-Publication Data, 2014.
8. *Proekt Rasporyazheniya Pravitel'stva Rossiiskoi Federatsii «Ob utverzhdenii Strategii preduprezhdeniya i preodoleniya ustoichivosti mikroorganizmov i vrednykh organizmov rastenii k lekarstvennym preparatam, khimicheskim i biologicheskim sredstvam na period do 2030 goda i dal'neishuyu perspektivu»* (podgotovlen Minzdravom Rossii 08.06.2017) [Draft Order of the Government of the Russian Federation «On approval of the Strategy for preventing and overcoming the resistance of microorganisms and pests of plants to drugs, chemical and biological agents for the period up to 2030 and beyond» (prepared by the Ministry of Health of Russia on 08.06.2017)] (in Russ.).
9. Jadhav K., Sharma K.S., Katoch S., Sharma V.K., Mane B.G. Probiotics in broiler poultry feeds: a review. *International Journal of Animal and Veterinary Science*, 2015, 2: 4-16.
10. Bidarkar V.K., Swain P.S., Ray S., Dominic G. Probiotics: potential alternative to antibiotics in ruminant feeding. *Trends in Veterinary and Animal Sciences*, 2014, 1: 1-4.
11. Parker R.B. Probiotics the other half of the antibiotics story. *Animal Nutrition and Health*, 1974, 29: 4-8.
12. Kavitha R.B., Desai J., Deepika Reddy A.R., Radhakrishna P.M. Effect of probiotics supplementation on the performance of broilers. *Indian Journal of Animal Nutrition*, 2007, 24(3): 142-146.
13. Rozhdestvenskaya T.N., Yakovlev S.S., Kononenko E.V. *Zhivotnovodstvo*, 2012, 1(1): 54-56 (in Russ.).
14. Sushkova V.I., Ustyuzhaninova L.V. *Kormoproizvodstvo*, 2017, 3: 34-40 (in Russ.).
15. Higgins J.P., Higgins S.E., Vicente J.L., Wolfenden A.D., Tellez G., Hargis B.M. Temporal effects of lactic acid bacteria probiotic culture on *Salmonella* in neonatal broilers. *Poultry Science*, 2007, 86(8): 1662-1666 (doi: 10.1093/ps/86.8.1662).
16. Starovoitova S.A., Skrotskaya O.I. Probiotiki na osnove transgennykh mikroorganizmov. *Biotechnologia Acta*, 2013, 6(1): 34-45 (in Russ.).
17. Lebedeva I.A., Prokkoeva Zh.A., Shchepetkina S.V. *Materialy Mezhdunarodnoi nauchno-prakticheskoi konferentsii «Biotekhnologiya i obshchestvo v XXI veke»* [Proc. Int. Conf. «Biotechnology and society in the 21st century»]. Barnaul, 2015: 370-374 (in Russ.).
18. Crittenden R., Bird A.R., Gopal P., Henriksson A., Lee Y.K., Playne M.J. Probiotic research in Australia, New Zealand and the Asia-Pacific region. *Current Pharmaceutical Design*, 2005, 11(1): 37-53 (doi: 10.2174/1381612053382304).
19. Ushakova N.A., Nekrasov R.V., Pravdin V.G., Kravtsova L.Z., Bobrovskaya O.I., Pavlov D.S. *Fundamental'nye issledovaniya*, 2012, 1: 184-192 (in Russ.).
20. Livshits V.A., Chesnokova V.L., Aleshin V.V., Sokurenko E.V., Dalin M.V., Kravtsov E.G., Bykov V.A. *Shtamm bakterii Escherichia coli m17 fimh::kan/p colap, ispol'zuemyi dlya polucheniya probioticheskogo preparata. (RF) MPK^7 C 12 N 1/21, A 61 K 35/74. № 2144954. Zayavl. 15.01.98. Opubl. 27.01.2000* [The bacterial strain *Escherichia coli* m17 fimh::kan/p colap used to obtain the probiotic preparation. (RF) MPK^7 C 12 N 1/21, A 61 K 35/74. № 2144954. Appl. 15.01.98. Publ. 27.01.2000] (in Russ.).
21. Sokolova N.A., Khmel' I.A., Shegidevich E.A. *Veterinariya*, 2001, 11: 46-49 (in Russ.).

22. Budič M., Rijavec M., Petkovšek Ž., Žgur-Bertok D. *Escherichia coli* bacteriocins: antimicrobial efficacy and prevalence among isolates from patients with bacteraemia. *PLoS ONE*, 2011, 6(12): e28769 (doi: 10.1371/journal.pone.0028769).
23. Filutowicz M., Burgess R., Gamelli R.L., Heinemann J.A., Kurenbach B., Rakowski S.A., Shankar R. Bacterial conjugation-based antimicrobial agents. *Plasmid*, 2008, 60(1): 38-44 (doi: 10.1016/j.plasmid.2008.03.004).
24. Maslennikova I.L., Kuznetsova M.V., Toplak N., Nekrasova I.V., Žgur-Bertok D., Starčič Erjavec M. Estimation of the bacteriocin ColE7 conjugation-based «kill»—«anti-kill» antimicrobial system by real-time PCR, fluorescence staining and bioluminescence assays. *Letters in Applied Microbiology*, 2018, 67(1): 47-53 (doi: 10.1111/lam.12884).
25. Starčič Erjavec M., Petkovšek Z., Kuznetsova M.V., Maslennikova I.L., Žgur-Bertok D. Strain ŽP — the first bacterial conjugation-based «kill»—«anti-kill» antimicrobial system. *Plasmid*, 2015, 82: 28-34 (doi: 10.1016/j.plasmid.2015.10.001).
26. Versalovic J., Koeuth T., Lupski J.R. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research*, 1991, 19(24): 6823-6831 (doi: 10.1093/nar/19.24.6823).
27. Clermont O., Christenson J.K., Denamur E., Gordon D.M. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environmental Microbiology Reports*, 2013, 5(1): 58-61 (doi: 10.1111/1758-2229.12019).
28. Guglielmetti E., Korhonen J.M., Heikkinen J., Morelli L., von Wright A. Transfer of plasmid-mediated resistance to tetracycline in pathogenic bacteria from fish and aquaculture environments. *FEMS Microbiology Letters*, 2009, 293(1): 28-34 (doi: 10.1111/j.1574-6968.2009.01512.x).
29. Merritt J.H., Kadouri D.E., O'Toole G.A. Growing and analyzing static biofilm. *Current Protocols in Microbiology*, 2005, 00(1): 1B.1.1-1B.1.18 (doi: 10.1002/9780471729259.mc01b01s00).
30. *GOST 34088-2017. Rukovodstvo po soderzhaniyu i ukhodu za laboratornymi zhivotnymi. Pravila soderzhaniya i ukhoda za sel'skokhozyaistvennymi zhivotnymi* [GOST 34088-2017. Guidelines for the maintenance and care of laboratory animals. Rules for keeping and caring for farm animals]. Moscow, 2018 (in Russ.).
31. Gillor O., Kirkup B.C., Riley M.A. Colicins and microcins: the next generation of antimicrobials. *Advances in Applied Microbiology*, 2004, 54: 129-146 (doi: 10.1016/S0065-2164(04)54005-4).
32. Karimi Torshizi M.A., Rahimi S.H., Mojgani N., Esmaeilkhanian S., Grimes J.L. Screening of indigenous strains of lactic acid bacteria for development of a probiotic for poultry. *Asian-Australasian Journal of Animal Science*, 2008, 21(10): 1495-1500 (doi: 10.5713/ajas.2008.80081).
33. Ogunbanwo S.T., Sanni A.I., Onilude A.A. Influence of bacteriocin in the control of *Escherichia coli* infection of broiler chickens in Nigeria. *World Journal of Microbiology and Biotechnology*, 2004, 20(1): 51-56 (doi: 10.1023/B:WIBI.0000013311.43842.74).
34. Tarakanov B.V. *Shtamm bakterii Escherichia coli, ispol'zuemyi dlya proizvodstva probiotika mikrotsikola V5/98. (RF) MPK C 12 N 1/20, A 61 K 35/74, C 12 R 1/19. № 2268297. Zayavl. 29.12.03. Opubl. 20.01.06. Byull. № 02* [The bacterial strain *Escherichia coli* used to produce the probiotic microcycol B5/98. (RF) MPK C 12 N 1/20, A 61 K 35/74, C 12 R 1/19. № 2268297. Appl. 29.12.03. Publ. 20.01.06. Bull. № 02] (in Russ.).
35. Tarakanov B.V., Aleshin V.V., Nikolicheva T.A., Polyakova L.L., Yakovleva A.A., Nikulin V.N., Palagina T.E. *Problemy biologii produktivnykh zhivotnykh*, 2008, 1: 20-36 (in Russ.).
36. Stahl C.H., Callaway T.R., Lincoln L.M., Lonergan S.M., Genovese K.J. Inhibitory activities of colicins against *Escherichia coli* strains responsible for postweaning diarrhea and edema disease in swine. *Antimicrobial Agents and Chemotherapy,* 2004, 48(8): 3119-3121 (doi: 10.1128/AAC.48.8.3119-3121.2004).
37. Cutler S.A., Lonergan S.M., Cornick N., Johnson A.K., Stahl C.H. Dietary inclusion of colicin E1 is effective in preventing postweaning diarrhea caused by F18-positive *Escherichia coli* in pigs. *Antimicrobial Agents and Chemotherapy*, 2007, 51(11): 3830-3835 (doi: 10.1128/AAC.00360-07).
38. Sonnenborn U. *Escherichia coli* strain Nissle 1917 — from bench to bedside and back: history of a special *Escherichia coli* strain with probiotic properties. *FEMS Microbiology Letters*, 2016, 363(19): fnw212 (doi: 10.1093/femsle/fnw212).
39. Chua K.J., Kwok W.C., Aggarwal N., Sun T., Chang M.W. Designer probiotics for the prevention and treatment of human diseases. *Current Opinion in Chemical Biology*, 2017, 40: 8-16 (doi: 10.1016/j.cbpa.2017.04.011).
40. Álvarez B., Fernández L.A. Sustainable therapies by engineered bacteria. *Microbiology and Biotechnology*, 2017, 10(5): 1057-1061 (doi: 10.1111/1751-7915.12778).