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REVERSAL OF ENTEROCOCCI STRAINS ANTIBIOTIC SENSITIVITY DURING THEIR CULTIVATION IN HUMAN AND ANIMAL CELL CULTURES

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One of the crucial criteria for probiotics evaluation is their property of antibiotic resistance, which should be characteristic for the selection of the promising strain for production technology. But these properties are capable of significantly varying, for example, their loss may occur during technological passages, and acquired resistance (plasmid) may be present. Plasmid resistance appears due to the presence of R-plasmids and can occur during antibiotic therapy, chemotherapy, and radiation therapy.

This paper presents the results of determining the sensitivity of the enterococcus strain isolated from the probiotic "Linex" and enterococci isolated from newborn children before and after cultivation in cell cultures, which were used as a model. It was established that after the cultivation of a probiotic strain of Enterococcus faecium and the clinical strain of Enterococcus faecalis in human and animal cell cultures, there are changes in the strain's diameters of the growth inhibition zones around the disks with antibiotics, which may indicate a reversal of their sensitivity and resistance to antibiotics.

Key words: *enterococci, antibiotics, reversion, cell cultures.*

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РЕВЕРСІЯ ЧУТЛИВОСТІ ШТАМІВ ЕНТЕРОКОКІВ ДО АНТИБІОТИКІВ ПРИ ЇХ КУЛЬТИВУВАННІ В КУЛЬТУРАХ КЛІТИН ЛЮДИНИ І ТВАРИН

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Одним із критеріїв оцінки пробіотиків є властивість їх антибіотикорезистентності, що повинна бути характеристикою відбору перспективних для виробничої технології штамів. Але ці властивості здатні до значного варіювання, наприклад, може відбуватися їх втрата при технологічних пасажах, або може бути присутня так звана набута резистентність (плазмідна). Плазмідна резистентність зумовлена присутністю R-плазмід та може виникати при антибіотикотерапії, хіміотерапії, променевій терапії макроорганізму.

У роботі представлено результати визначення чутливості штаму ентерококу, виділеного з препарату-пробіотика «Лінекс», та ентерококів, ізольованих від новонароджених дітей, при їх культивуванні у культурах клітин, які слугували моделлю макроорганізму. Встановлено, що після культивування пробіотичного штаму ентерококів Enterococcus faecium та клінічного штаму Enterococcus faecalis у культурах клітин людини та тварин відбуваються зміни у розмірах зон затримки росту штамів навколо дисків з антибіотиками, що може вказувати на реверсію їх чутливості та резистентності до антибіотиків.

Ключові слова: *ентерококи, антибіотики, реверсія, культури клітин.*

To determine the natural sensitivity of enterococcal strains isolated from the probiotic preparation and enterococci isolated from newborn children, the method of determining the sensitivity of bacteria and fungi to antibiotics when cultivated in human and animal cells was used (1). Usually, diagnostic laboratories use the standard disk-diffusion method for determining the sensitivity of microorganisms to antibiotics. But it is known that when microbial cells interact with cells of a macroorganism, the adhesive properties of bacteria can change, which could lead to a change in the antimicrobial sensitivity of bacteria. The phenomenon of reversal of sensitivity to antibiotics was first discovered in lactic acid bacteria during their interaction with human lymphoblastoid cells.

The purpose of the research was the determination of the natural sensitivity of clinical strains of enterococci isolated from newborn children and the strain of enterococci isolated from the probiotic drug "Linex" during cultivation in cell cultures.

The objects of research were strains of enterococci of the *Enterococcus faecalis species*, isolated from the biotopes of newborn children (navel, stomach contents, intestinal contents) and the strain of enterococci — *Enterococcus faecium*, isolated from the probiotic drug "Linex". Enterococcal agar and Muller-Hinton agar were used to preserve the biological activity of the studied strains. The presence of changes in the sensitivity of the strains was studied after their cultivation in cell cultures: HEp-2 — human tumour cell line; BHK — Syrian hamster kidney cells; MDCK — dog kidney cells; RK-13 — rabbit kidney cells. A suspension of cells of the studied bacteria (at a concentration of 1.0×10^8 CFU/ml — 0.5 units according to the McFarland standard) was inoculated into tissue cultures. Susceptibility to antibiotics was studied before cultivation in cell cultures (initial) and after (final) using commercially produced discs (HiMedia, India; "Aspect", Ukraine).

Research materials and methods. Microorganism strains: *Enterococcus faecium*, isolated from "Linex"; clinical strain *Enterococcus faecalis* isolated from newborn babies. Cell cultures: HEp-2; BHK; MDCK; RK-13. RPMI-1640 medium for growing cell cultures without the addition of antibiotics. Nutrient media for enterococci and determining antibiotic sensitivity: enterococcal agar, Muller-Hinton agar. Disks with antibiotics manufactured by Himedia, India and "Aspect", Ukraine, registered in Ukraine: aminoglycosides (gentamicin, amikacin); cephalosporins (ceftazidime, ceftriaxone, cefuroxime, cefepime); vancomycin, linezolid, amoxicillin; oxacillin, benzylpenicillin; macrolides — azithromycin; tigecycline, lincomycin, clindamycin, furazidin; fluoroquinolones (ciprofloxacin, levofloxacin). The disc-diffusion method was used for the study. Control was carried out with standard test cultures: *Escherichia coli* 25922, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853. Depending on the diameter of the growth inhibition zone of the tested bacteria around the discs with antibiotics, the studied strains were divided into three groups: sensitive — S; resistant — R, and intermediate — I.

Results and discussion. The investigated strain of *Enterococcus faecium* was pre-cultivated on enterococcal agar, the isolated colony was selected and screened on simple nutrient agar in a test tube. The culture grown after 24 hours of incubation in a thermostat at a temperature of +37 °C was used to study its sensitivity to antibiotics. A suspension of cells of the studied bacteria (at a concentration of 0.5 Mcfarland units) was inoculated on the Muller-Hinton medium, and discs with antibiotics were applied. Growth inhibition zones were measured after 18–24 hours. The obtained data are listed in Table 1.

Table 1. Susceptibility to Enterococcus faecium ("Linex")

Name of antibiotics	Growth inhibition in mm	
vancomycin	28	S
linezolid	28	S
ciprofloxacin	19	I
ceftazidime	0	R
cefuroxime	0	R
amoxicillin	0	R
benzylpenicillin	0	R
oxacillin	0	R
lincomycin	12	R
clindamycin	14	R
gentamicin	10	R
ceftriaxone	0	R
cefepime	0	R
amikacin	11	R
tigecycline	28	S
furazidin	20	S
azithromycin	15	R
levofloxacin	14	R

R — resistant; S — susceptible; I — intermediate

The investigated strain of *Enterococcus faecium* was resistant to 13 antibiotics: 3rd generation cephalosporins: ceftazidime, cefuroxime, ceftriaxone; 4th generation — cefepime; also to amoxicillin, benzylpenicillin, oxacillin, lincomycin, clindamycin, 2nd and 3rd generation aminoglycosides: gentamicin and amikacin; to macrolide — azithromycin. Sensitivity was found to vancomycin, linezolid, tigecycline and furazidin. *Enterococcus faecium* was moderately resistant to ciprofloxacin and resistant to levofloxacin.

Enterococcus faecium, isolated from enterococcal agar and previously grown on nutrient agar in a test tube, was used for introduction into cell cultures. Next, the *Enterococcus faecium* strain was inoculated in cultured monolayer cell lines for 24 hours. For this purpose, a 1 cm^3 suspension of microorganisms (at a concentration of 1.0×10^8 CFU/ml — 0.5 units according to the McFarland standard) was inoculated into cell cultures and cultivated in RPMI-1640 medium without the addition of serum and antibiotics in a thermostat at a temperature of ±37 °C.

As a control, a suspension of *Enterococcus faecium* was used (at a concentration of 0.5 units according to the McFarland standard).

Table 2. Sensitivity of Enterococcus faecium after passage through cell cultures

		vancomycin	linezolid	ciprofloxacin	ceftazidime	cefuroxime	amoxicillin
Zones of growth inhibition in mm							
1	HEp-2	30	34	25	0	0	0
2	BHK	28	30	22	0	0	0
3	MDCK	30	34	26	0	0	0
4	RK-13	30	36	25	0	0	0

Table 2 shows the zones of growth inhibition after passage through cell cultures.

E.faecium was re-isolated from the cell cultures after 24 hours of incubation and it was studied whether the indicators of sensitivity to antibacterial drugs had changed.

Table 3. Comparison of the sensitivity of E.faecium before and after passage through cell cultures

		vancomycin	linezolid	ciprofloxacin	ceftazidime	cefuroxime	amoxicillin
Zones of growth inhibition in mm of E.faecium before passage							
		28	28	19	0	0	0
Zones of growth inhibition in mm. E faecium after passage							
1	HEp-2	30	34	25	0	0	0
2	BHK	28	30	22	0	0	0
3	MDCK	30	34	26	0	0	0
4	RK-13	30	36	25	0	0	0

As can be seen from the table, the growth inhibition zone of *E.faecium* after passage through cell culture HEp-2 increased by 2 mm to vancomycin, by 4–6 mm to linezolid, by 5–6 mm to ciprofloxacin (from I to S); *E.faecium* remained stably resistant to ceftazidime, cefuroxime and amoxicillin. After passage through the culture of BHK cells, the parameters did not change, with the exception of sensitivity to ciprofloxacin, which increased by 2 mm, thus from moderately sensitive *E.faecium* became sensitive (from I to S).

In MDCK and RK-13 cell cultures, an increase in the diameters of the growth inhibition zone of *E.faecium* was also noted by 2 mm to vancomycin, by 4–6–8 mm to linezolid, by 6–7 mm to ciprofloxacin (from I to S); to ceftazidime, cefuroxime and amoxicillin, *E.faecium* remained stably resistant as well.

The next stage of research was to determine the sensitivity of the clinical strain of *E. faecalis* before and after passage through cell cultures.

Analysis of the data provided in Table 4 showed that in the case of passaging through RK-13 cell culture, two zones of growth inhibition of the *E.faecalis* strain were formed. Colonies enterococci of the first zone have changed their sensitivity to 2nd and 3rd generation cephalosporins: ceftazidime, cefuroxime, and ceftriaxone for moderate resistance. *E.faecalis* zones of growth inhibition decreased by 8–9 mm to linezolid (that is, it turned from sensitive to moderately resistant); by 5–6 mm to ciprofloxacin (from S to I as well). In relation to the strain remained sensitive to vancomycin, amoxicillin, and tigecycline; to amikacin, it is still stably resistant.

Table 4. Comparison of the susceptivity of E.faecalis before and after passage through cell cultures

		vankomycin	linezolid	ciprofloxacin	ceftazidime	cefuroxime	ceftriaxone	amicacine	tigercycline	amoxicicline
		Befopre passage								
		19,90±0,10	29,86±0,14	21,68±0,32	26,80±0,33	26,80±0,33	26,23±1,18	10,01±1,31	24,45±1,64	35,70±0,30
		After passage								
1	HEp-2	20	32	22	22	30		9,80±0,22		
2	BHK	22	32	20	26	31		10,31±0,69		36
3	MDCK	20	30	20	18 (12)	30		9,65±0,35		40
4	RK-13	20,00±0,01	21,45±0,12 (10)	16,25±0,75	14,98±0,12 (8)	15,16±1,31 (8)	23,96±1,25 (7)	6,80±0,47	25,66±0,88	40,03±0,06

Table 5. Indicators of sensitivity of the clinical strain of E.faecalis to antibiotics before and after cultivation in RK-13 cell culture

Name of antibiotics	Before cultivation		After cultivation	
	Zone of growth inhibition (mm)	Sensitive or resistant	Zone of growth inhibition (mm)	Sensitive or resistant
Colonies of the first zone				
Amoxicillin	35.70±0.30	sensitive	40.03±0.06	sensitive
Amikacin*	10.01 ±1.31	resistant	6.80±0.47	resistant
Cefuroxime (II) °	17.36±0.64	sensitive	15.16±1.31	moderately resistant
Ceftriaxone (III) °	26.23±1.18	sensitive	23.96±1.25	moderately resistant
Ceftazidime (III) °	26.80±0.33	sensitive	14.98±0.12	moderately resistant
Vancomycin	19.90±0.10	sensitive	20.00±0.01	sensitive
Linezolid °	29.86±0.14	sensitive	21.45±0.12	moderately resistant
Ciprofloxacin °	21.68±0.32	sensitive	16.25±0.75	moderately resistant
Tigacil	24.45±1.64	sensitive	25.66±0.88	sensitive
Colonies of the second zone				
Cefuroxime (II) °*	17.36±0.64	sensitive	8.16±1.31	resistant
Ceftriaxone (III) °*	26.23±1.18	sensitive	6.96±1.25	resistant
Ceftazidime (III) *°	26.80±0.33	sensitive	7.98±0.12	resistant
Linezolid °*	29.86±0.14	sensitive	9.45±0.12	resistant

1. p<0.05. 2. * – antibiotics to which E.faecalis showed stability. 3. ° – antibiotics in relation to which reversion occurred

Colonies of the second zone due to reversion changed their sensitivity to resistance to drugs ceftazidime, cefuroxime, ceftriaxone and linezolid..

Colonies of the first zone of enterococci changed their sensitivity to 2nd and 3rd generation cephalosporins: ceftazidime, cefuroxime, ceftriaxone to moderate resistance. Zones of growth inhibition of *E. faecalis* decreased by 8–9 mm to linezolid (that is, they turned from sensitive to moderately resistant); by 5–6 mm to ciprofloxacin (from S to I as well). The strain remained sensitive to vancomycin, amoxicillin, and tigecycline; stably resistant to amikacin.

Colonies of the second zone due to reversion changed their sensitivity to resistance to the drugs ceftazidime, cefuroxime, ceftriaxone and linezolid.

Conclusions. After the cultivation of enterococcal strains in transplanted cultures of animal and human cells, their sensitivity to antibiotics was changed. This property has been found to be unstable: after passages, the sensitivity and resistance of the studied microorganisms are reversed. The same processes can occur in the human body and lead to ineffective treatment with antibiotics, the sensitivity to which is determined by traditional methods.

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