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ANTIBIOTIC SENSITIVITY OF LACTOBACILLI ISOLATED FROM DIFFERENT SOURCES IN THE ODESA REGION

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Abstract. Recently, possible co-application of probiotics and antibiotics has been widely discussed. This form of combined therapy, because of its commonly recognised advantage, is widely used to prevent antibiotic-associated diarrhoea and induced dysbiosis. Due to the increased application of lactobacilli as probiotics, EFSA has developed a number of requirements concerning their safety and functionality. Every commercial probiotic should be able to obtain the Qualified Presumption of Safety status (QPS).Though QPS is a status attributed to species, individual species differ greatly in their genome content, including those belonging to the genus Lactobacillus. Infectious bacteria and strains that may possess genes responsible for virulence or antibiotic resistance should not be used lest pathogenic genes be passed on to other species. The human gastrointestinal tract, due to the immense amount of bacteria and the close contact between them, is a possible place for gene transfer. The main hazard is the transfer of antibiotic resistance determinants from commensal bacteria and the emergence of resistance to common microbial infections, which can prevent successful antibiotic treatment. A condition of using bacterial strains as probiotics is their safety, in particular, the absence of antibiotic resistance determinants. The purpose of the work was to determine the antibiotic sensitivity/resistance of lactobacilli isolated from different sources in the Odesa region. By the disc diffusion method, it has been determined how sensitive the 13 lactobacilli strains isolated from auto-fermented vegetables, raw meat, and newborn children's gastrointestinal tract are to 15 antibiotics with different mechanisms of action. It has been established that the sensitivity/resistance depended on the strain and specific antibiotic. An obvious feature of the strains under study was their higher sensitivity to antibiotics that inhibited the synthesis of protein and nucleic acids, as opposed to antibiotics that affected the synthesis of the cell wall and cytoplasmic membrane. The results of the comprehensive research have allowed selecting the Lactobacillus spp. strains O1, B4, 175, M2, and M3 as the most promising for the creation of probiotic preparations.

Key words: probiotics, lactobacilli, antibiotic resistance/sensitivity.

Introduction. Formulation of the problem

Probiotics are defined as "live microorganisms which when administered in an adequate amount confer health benefits to the host" (FAO/WHO, 2002) [1]. Alternatively, probiotics are defined as live microbial feed supplements that beneficially affect the host animal by improving its intestinal microbial balance [2]. Probiotics can be included in various types of food products, medicines, nutritional supplements, etc. Lactobacilli and bifidobacteria are most often used as probiotics. One of the most important criteria for the selection of bacterial strains to be used in the food industry is their safety, in particular, the absence of antibiotic resistance determinants. In Europe, according to the Qualified Presumption of Safety (QPS) approach established by the European Food Safety Authority (EFSA, 2008), the nature of any antibiotic resistance determinant present in a candidate microorganism should be determined prior to approval for the QPS status. So, antibiotic resistance *per se* is not a safety issue: it only becomes such when there is a risk of resistance transfer [3,4].

As components of products, supplements, biological preparations, probiotics directly enter the human and animal body that is why it is very important to determine the level of resistance to antibiotics. This is part of the safety assessment of microorganisms used as probiotics [5].

Analysis of recent research and publications

Resistance of microorganisms to antimicrobial drugs is a serious global risk for human health [6]. The increased number of resistant microbes largely results from the incorrect use of antimicrobial drugs in health care and agriculture [7,8]. Moreover, there is now an

opinion that resistance to antibiotics arises not only from incorrect or excessive use, but can also be one of the consequences of environmental pollution with harmful substances [9].

Lactic acid bacteria play an important role in the food industry as starters and probiotics. Antimicrobial resistance is becoming more common, and the risk of the spread of antibiotic resistance genes is increasing, that is why it is important to consider beneficial bacteria as a potential pool of transmissible antibiotic resistance genes. The more so as fermented food contains a large number of live bacteria and, therefore, is a favourable environment for the transfer of genes, including resistance genes [10]. This phenomenon is receiving more and more attention, since lactic acid bacteria as a potential reservoir of resistance genes can transmit them to pathogenic bacteria. This leads to the dissemination of antibiotic resistance among pathogens and makes it more difficult to treat infections caused by these bacteria. Importantly, many studies show that genes of this kind can be transferred in both directions. Although conjugation is the most common way of dissemination of antibiotic resistance genes, transformation and transduction may also play an important role in this process, even greater than previously thought [11].

This makes it very important task to define transmissible antibiotic resistance markers when assessing the safety of lactic acid bacteria, since genes determining resistance to some antimicrobial drugs (for example, chloramphenicol, erythromycin, streptomycin, tetracycline, and vancomycin) and located on mobile genetic elements (plasmids or transposons) have already been characterised in lactococci, lactobacilli, and enterococci isolated from food [12]. However, the transfer of antibiotic resistance genes from lactic acid bacteria to bacteria of the resident microbiota of the human gastrointestinal tract, and therefore to pathogenic bacteria, has not been fully studied.

It should be emphasised that lactic acid bacteria can have both natural and acquired resistance to antimicrobial drugs [12]. For instance, Shao *et al.* [13] demonstrated that two isolates of *L. plantarum* had aaadA and ant(6) genes associated with streptomycin resistance, and overexposure to this antibiotic sharply increased the minimum inhibitory concentration and increased cross-resistance to other antibiotics of this class. On the other hand, it was reported that 6% of strains isolated from some pharmaceutical and dairy products from Egypt had tetracycline [tet(M)] and/or erythromycin [erm(B)] resistance genes [14].A similar study reported on the frequent occurrence of lactobacilli resistant to vancomycin (58%), erythromycin (10.8%), tetracycline (4.3%), gentamicin (48%), and ciprofloxacin (26%) in Turkish fermented dairy products [15]. However, the studies conducted by Flores and Mayo [16] revealed that no transfer of tetracycline [tet(M)] and erythromycin [erm(B)] resistance genes from *S. thermophilus* to *L. delbrueckii* was detected during yogurt production and storage. Even though some publications confirm the transfer of resistance determinants, the two most common resistance genes in lactic acid bacteria are the tetracycline [tet(M)] and erythromycin [erm(B)] resistance genes. These are followed by CAT genes that encode chloramphenicol resistance [17]. The wide range of potential applications of lactic acid bacteria in industry and in human and animal health care calls for their detailed investigation. Antimicrobial resistance is considered an important safety concern during their evaluation and approval as probiotic cultures. On the other hand, the sensitivity of the strains to the antibiotics recommended by the European Food Safety Agency (EFSA) makes them safe for direct use in the food industry, animal husbandry, and agriculture [18].

The purpose of the work was to determine how sensitive/resistant to antibiotics are lactobacilli isolated from different sources in the Odesa region.

The objectives of the study were:

–to establish the sensitivity/resistance of the studied lactobacilli to antibiotics by the discdiffusion method;

–to find out the resistance levels of the studied lactobacilli;

–to select the *Lactobacillus* strains most promising for the creation of probiotic preparations.

Research materials and methods

Thirteen lactobacilli strains isolated from different ecological niches were used in experiments: from auto-fermenting vegetables (*Lactobacillus spp.*B1, B3, B4, B5, B6, O1), from raw meat (*Lactobacillus spp*. M1, M2, M3, M6), and from newborn children's digestive tract (*Lactobacillus spp.* 146, 275,175). All the studied *Lactobacillus* strains are stored in the museum of the Department of Microbiology, Virology, and Biotechnology of Odesa I.I. Mechnikov National University.

The sensitivity/resistance of lactobacilli to antibiotics was determined using a disc diffusion method [6,10]. Fifteen antibiotics having different mechanisms of action were used. Penicillins were represented by benzylpenicillin (PEN – 1 million IU/disc); cephalosporinsbycefazolin (CZ – 30 μg/disc), cefuroxime (CFX– 30 μg/disc), ceftazidime (CAZ – 10 μg/disc), and cefixime (CFM – 5 μg/disc); aminoglycosides bynetilmicin (NET – 30 μg/disc) andstreptomycin (STR – 300 μg/disc); tetracyclines bytetracycline (TET – 30 μg/disc); macrolides by erythromycin (ERY– 15 mg/disc); lincosamides byclindamycin (CLN – 10 μg/disc); phenicolsbychloramphenicol (CL – 30 μg/disc); glycopeptides by vancomycin (VAN – 30 μg/disc); fluoroquinolones byciprofloxacin(CIP – 5 μg/disc); polymyxins bypolymyxin B (PMB – 300 IU/disc);and rifampins byrifampicin (RIF – 5 μg/disc).

0.1 ml portions of suspensions of overnight broth cultures of lactobacilli with the concentration 10^7 CFU/ml were inoculated on the surface of MRS agar in Petri dishes. Discs were applied with sterile tweezers,

placing no more than 7 discs per dish. Incubation was carried out at 37°C for 24 hours. The results were recorded by measuring (accurate within 1 mm) the diameters of the growth inhibition zones around the discs with antibiotics, focusing on the zone of complete inhibition of visible growth. A microorganism strain was considered highly resistant at an inhibition zone diameter of 14 mm and smaller. If the diameter of the zone of inhibition ranged 15 to 25 mm, the strain was considered intermediate, if it was more than 25 mm, susceptible [19].

The studies were performed in triplicate. Statistical and graphical analysis of the results was performed using the Microsoft Office Excel 2016 software.

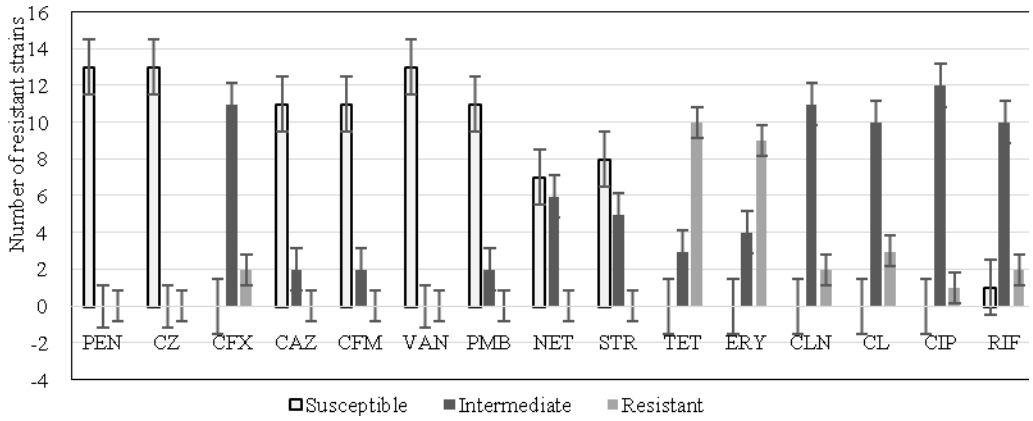
Results of the research and their discussion

Probiotic strains of microorganisms belonging to the species included in the EFSA QPS list (EFSA, 2012) are safe, and harmful effects of their use are very rare and often due to individual characteristics [20]. Undoubtedly, a complete safety analysis begins with the identification of the strain and determination of potential risks *in vitro* [21]. In the previous studies, we identified the isolated strains as representatives of the genus *Lactobacillus* and revealed their probiotic properties [22-25]. The sensitivity/resistance to antibiotics depended on the strain studied and on the antibiotic used. The relation to antibiotics, as well as many other bacterial properties, is often strain-specific, and the diameters of the zones of inhibition varied widely (Table 1).

A strain of the microorganism was classified as sensitive, intermediate, or resistant to each antibiotic considered (Fig. 1). It should be noted that sensitivity to antibiotics is not clearly dependent on the source of a strain.

Table 1 – Diameters of the zones of inhibition of the lactobacilli strains due to the action of antibiotics, mm (n=3)

Antibiotic	*Lactobacillus sp.*strains												
	175	275	146	O1	B1	B3	B4	B5	B6	M1	M2	M3	M6
Inhibitors of cell wall synthesis													
PEN	0.00	11.33 ±1.73	11.33 ±0.65	0.00	0.00	12.33 ±0.65	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CZ	0.00	0.00	0.00	0.00	11.33 ±1.31	0.00	11.67 ±0.65	0.00	0.00	14.33 ±1.31	0.00	0.00	0.00
CFX	18.67 ±1.31	20.33 ±1.73	27.67 ±1.73	21.00 ±1.13	25.33 ±0.65	23.33 ±1.73	22.67 ±1.73	26.67 ±0.65	25.67 ±0.65	23.33 ±1.73	20.00 ±1.13	19.33 ±0.65	22.33 ±0.65
CAZ	0.00	0.00	0.00	0.00	0.00	0.00	0.00	16.33 ±0.65	19.00 ±1.13	14.00 ±1.13	0.00	0.00	14.33 ±0.65
CFM	0.00	0.00	0.00	0.00	0.00	0.00	0.00	15.33 ±0.65	22.33 ±1.73	0.00	0.00	0.00	0.00
VAN	0.00	0.00	0.00	0.00	0.00	0.00	12.33 ±0.65	14.67 ±1.31	0.00	0.00	0.00	0.00	14.00 ±1.13
Inhibitors of CPM functions													
PMB	0.00	17.67 ±1.73	0.00	0.00	0.00	0.00	0.00	21.33 ±1.73	0.00	0.00	0.00	0.00	0.00
Inhibitors of protein synthesis													
NET	14.67 ±0.65	16.67 ±0.65	18.67 ±1.31	12.33 ±0.65	13.67 ±0.65	12.67 ±1.31	16.00 ±1.13	12.33 ±0.65	16.00 ±1.13	21.00 ±1.13	11.67 ±0.65	13.00 ±1.13	16.00 ±1.13
STR	0.00	13.33 ±0.65	15.33 ±0.65	19.67 ±1.73	0.00	0.00	19.33 ±1.73	20.33 ±1.31	15.67 ±1.31	0.00	0.00	0.00	13.67 ±1.73
TET	23.33 ±1.73	28.00 ±1.13	30.67 ±1.73	27.00 ±1.13	31.67 ±0.65	28.33 ±0.65	30.33 ±0.65	24.00 ±1.13	31.00 ±1.13	28.33 ±0.65	26.00 ±1.13	24.67 ±0.65	30.00 ±1.13
ERY	19.33 ±0.65	30.00 ±1.13	26.00 ±1.13	22.33 ±0.65	30.67 ±1.31	27.67 ±1.31	31.00 ±1.13	31.00 ±1.13	20.33 ±0.65	32.67 ±0.65	20.00 ±1.13	26.67 ±0.65	30.67 ±0.65
CLN	23.67 ±1.31	22.33 ±1.73	29.33 ±1.73	24.33 ±1.73	19.33 ±0.65	17.00 ±1.13	21.33 ±0.65	17.67 ±1.31	22.00 ±1.13	25.67 ±1.31	21.33 ±0.65	25.67 ±0.65	31.67 ±0.65
CL	16.67 ±1.73	23.00 ±1.13	21.67 ±1.73	20.00 ±1.13	27.67 ±0.65	23.33 ±1.73	25.00 ±1.13	22.67 ±1.31	25.00 ±1.13	22.67 ±0.65	26.00 ±1.13	23.67 ±1.31	27.33 ±1.73
Inhibitors of nucleic acid synthesis													
CIP	17.33 ±1.73	21.33 ±1.31	24.00 ±1.13	20.67 ±0.65	22.67 ±0.65	22.33 ±1.31	21.00 ±1.13	19.33 ±1.73	21.00 ±1.13	25.67 ±1.31	19.33 ±1.73	21.00 ±1.13	26.33 ±1.73
RIF	27.00 ±1.13	20.33 ±1.73	29.00 ±1.13	13.33 ±1.73	24.67 ±1.31	22.00 ±1.13	22.67 ±1.31	19.67 ±0.65	24.67 ±1.31	19.33 ±1.73	20.33 ±1.73	18.33 ±0.65	23.67 ±1.31



PEN – benzylpenicillin; CZ– cefazolin; CFX– cefuroxime; CAZ– ceftazidime; CFM– cefixime; VAN–vancomycin; PMB– polymyxin; NET–netilmicin; STR– streptomycin; TET–tetracycline; ERY– erythromycin; CLN–clindamycin; CL–chloramphenicol; CIP – ciprofloxacin; RIF – rifampicin.

Fig. 1. Resistance levels of the lactobacilliunder study

These results make it clear that lactobacilli are more sensitive to antibiotics inhibiting the synthesis of protein and nucleic acids than to those interfering with the synthesis of the cell wall and cytoplasmic membrane. Inhibitors of the synthesis of the cell wall were represented by penicillins, cephalosporins, and glycopeptides. Almost all lactobacilli proved highly resistant to the antibiotics of these groups (with the exception of cefuroxime).Moreover, in most cases, active growth of lactobacilli was observed. According to these publications [11,14,26], lactobacilli are usually more sensitive to penicillin and more resistant to cephalosporins.

Many gram-positive bacteria, including bacteria of the genus *Lactobacillus*, are sensitive to vancomycin *in vivo* because its action is associated with inhibition of cell wall synthesis and changing of the permeability of membranes and RNA synthesis. The vancomycin-resistant phenotype of some lactobacilli perhaps is the best to characterise the internal resistance of lactic acid bacteria [27]. Vancomycin contacts precursors of peptidoglycan from the cell wall of the cytoplasmic membrane and binds to the D-alanine/D-alanine end of the pentapeptide, preventing polymerisation of the peptidoglycan precursors. In some species of lactic acid bacteria, the terminal D-alanine end is replaced by D-lactate or D-serine in the muramylpentapeptide. This prevents binding of vancomycin, and, therefore, the cell wall becomes resistant to the antibiotic. Chromosomal mutations, which were also found in lactobacilli, lead to the occurrence of phenotypes resistant to antibiotics of various groups. However, the risk of resistance transfer through chromosomal mutations is low [20].

Eleven *Lactobacillus* strains were highly resistant to polymyxin, an inhibitor of cytoplasmic membrane functions, two strains were considered intermediate. Other researchers, too, report about high resistance of lactobacilli, in particular, *Lactobacillus casei*, to polymyxin and vancomycin [4,13,27].

On the other hand, lactobacilli are usually sensitive to low concentrations of many protein synthesis inhibitors, such as chloramphenicol, macrolides, lincosamides, and tetracyclines, and their resistance to aminoglycosides is often noted [28]. This is confirmed by the results obtained (Table 1, Fig. 1). The studied strains were sensitive to tetracycline, erythromycin, clindamycin, and chloramphenicol, and their effects are associated with inhibition of the protein synthesis. The levels of resistance to these antibiotics are rated as "intermediate" and "susceptible," depending on the strain. Most strains were highly resistant to the aminoglycosides netilmicin and streptomycin, the resistance to the latter being greater.

Nucleic acid synthesis inhibitors in our experiment were represented by fluoroquinolones (ciprofloxacin) and rifampins (rifampicin). Pawar *et al.* (2020) and Dobreva *et al.* (2020) in their publications reported their findings on lactobacilli resistance to quinolones (its mechanisms are related to internal factors such as the cell wall structure, efflux features, and permeability) and to rifampicin (resistance to it may be the result of mutations) [4].In our investigation, the lactobacilli strains, on the contrary, were sensitive to antibiotics of these classes. In the case of rifampicin this can be explained by the absence of mutations, and the mechanism of sensitivity to ciprofloxacin requires more detailed studies.

Of interest are the reports about the finding of genes responsible for atypical antibiotic resistance among lactobacilli. Chloramphenicol resistance genes (*cat*; chloramphenicol acetyltransferase) were identified in *L. acidophilus*, *L. delbrueckii subsp. bulgaricus*, and *L. johnsonii*, as well as in *L. reuteri* and *L. plantarum*. Besides, erythromycin resistance genes responsible for the phenotype of resistance to macrolide, lincosamide, and streptogramin (MLS) were detected and identified in some lactobacilli species. However, the most common lactobacilli resistance determinants found are tetracycline resistance genes,

which are sometimes found in combination. To date, at least 11 different tetracycline resistance genes have been identified in lactobacilli, including genes encoding ribosomal protective proteins and efflux pumps. Genes of resistance to aminoglycosides and β-lactams were found far less often in lactobacilli [4,28].

It is important to note that many genetic determinants of resistance are sometimes found in potentially mobile elements, such as transposons and plasmids, which can spread antibiotic resistance genes mainly through conjugation mechanisms. Localisation of these genes in the genome, their nucleotide sequence, and analysis of the flanking regions surrounding antibiotic resistance genes may be important for understanding the process of acquisition of these determinants, as well as their source or origin [20]. Some of these genes were found to be transferred *in vitro* between lactobacilli strains, as well as from lactobacilli to various gram-positive bacteria, including foodborne pathogens like *Staphylococcus* [14]. On the other hand, lactobacilli can get antibiotic resistance determinants from other gram-positive bacteria [10]. *In vitro* studies were also conducted in experimental animal models regarding the potential risks associated with lactobacilli carrying mobile determinants of antibiotic resistance [9]. In general, the results obtained confirm the hypothesis of a reservoir of resistance genes among intestinal bacteria and their role in the dissemination of antibiotic resistance. Currently, it is generally accepted that the possibility of transfer is related to the genetic basis of the resistance mechanism, that is, to whether the resistance is natural, or acquired as a result of chromosomal mutation(s), or acquired as a result of horizontal gene transfer [1,4,9].

Conclusion

Thus, the research conducted is part of the assessment of the safety of lactobacilli strains isolated from various sources in the Odesa region. EFSA requires that every strain intended for human/animal consumption should be tested for antibiotic resistance [11].It has been demonstrated that the investigated *Lactobacillus spp*. strains B1, B3, B4, B5, B6, O1, M1, M2, M3, M6, 146, 275, 175 showed variable sensitivity/resistance to antibiotics. Antibiotic resistance in lactic acid bacteria, in particular lactobacilli, can result from the often-uncontrolled use of antibiotics in everyday life. Detection of antibiotic resistance is one of the main criteria for selection of strains as possible probiotics. Based on the data obtained and the results of previous studies [10,19,22-25], the *Lactobacillus spp.* Strains O1, B4, 175, M2, and M3 were selected as the most promising for the creation of probiotic preparations.

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ВІДНОШЕННЯ ДО АНТИБІОТИКІВ ЛАКТОБАЦИЛ, ВИДІЛЕНИХ ІЗ РІЗНИХ ДЖЕРЕЛ В ОДЕСЬКОМУ РЕГІОНІ

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Анотація. Останнім часом широко обговорюється можливе спільне застосування пробіотиків і антибіотиків. Перевага такої форми комбінованої терапії визнана і широко використовується для профілактики антибіотикоасоційованої діареї та індукованого дисбактеріозу. У зв'язку зі збільшенням застосування лактобактерій як пробіотиків, EFSA розробило ряд вимог щодо їх безпеки та функціональності. Кожен комерційний пробіотик повинен мати можливість отримати статус кваліфікованої презумпції безпеки (QPS). Незважаючи на те, що QPS є статусом, який приписується виду, вміст геному значно відрізняється між видами, включно у представників роду *Lactobacillus*. Бактерії – збудники інфекційних захворювань та штами, які можуть володіти генами вірулентності або стійкості до антибіотиків, не повинні використовуватися черезризик передачі генів патогенності іншим видам. Шлунково-кишковий тракт людини, завдяки величезній кількості бактерій і тісному контакту між ними, є можливим місцем для передачі генів. Основною небезпекою є передача детермінант резистентності до антибіотиків від бактерій-коменсалів і поява антибіотикорезистентності, що перешкоджає успішному лікуванню антибіотиками.Однією із умов використання штамів бактерій як пробіотиків є їх безпечність, у тому числі відсутністьдетермінант резистентності до антибіотиків. Метою роботи було визначити чутливість/резистентність до антибіотиків лактобацил, виділених із різних джерел Одеського регіону. Відношення 13 штамів лактобацил, виділених із автоферментованих овочів, м'ясної сировини і шлунково-кишкового тракту новонароджених дітей до 15 антибіотиків з різним механізмом дії визначали диско-дифузійним методом.Виявлено, що чутливість/резистентність залежала від штаму і конкретного антибіотику. Очевидною ознакою досліджених штамів була вища чутливість до антибіотиків, що пригнічують синтез білка і нуклеїнових кислот, на противагу антибіотикам, що впливають на синтез клітинної стінки і цитоплазматичної мембрани. За результатами комплексних досліджень було відібрано штами штами*Lactobacillus*spp. О1, Б4, 175, М2 та М3 як найбільш перспективні для створення пробіотичних препаратів.

Ключові слова: пробіотики, лактобацили, антибіотикорезистентність/чутливість.