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Microbiological Indicators of Frozen Fish and Sensitivity of Psychrotrophic Microflora to Antibiotics in the Absence and Presence of Residual Amounts of Antibacterial Drugs.

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ABSTRACT

Fish belongs to perishable food that requires appropriate temperature storage regimes to stop the flow of biochemical and microbiological processes. During storage of fish in the conditions of refrigerating chambers, cold-loving – psychrotrophic microflora dominates, which causes biochemical and organoleptic changes and affects sanitary and hygienic parameters. In the field of fish farming for the treatment and prevention of various diseases, antibacterial drugs are widely used. In frozen fish, which is imported into Ukraine, determination of the presence of residual amounts of antibacterial drugs is not carried out. The purpose of work was to investigate microbiological indicators of frozen fish imported into Ukraine, to identify the generic composition of psychrotrophic microflora, isolated from frozen fish in the absence and with the content of residual amounts of antibacterial drugs and to determine the sensitivity of the isolated microflora to antibiotics. It was found that frozen fish with the presence of antibacterial residues, on average about two orders is less contaminated with the microflora. Regardless of the presence or absence of antibiotics in frozen fish, the basis of its microflora are psychrotrophic microorganisms, which predominate amount of mesophilic bacteria in 1.3 – 1.6 times. It was found that, subject to the presence in the frozen fish of residual quantities of antibacterial drugs, a resistant psychrotrophic microflora is formed to detected antibiotics. As a result, a pathway for the transfer of resistant microorganisms and resistance genes from fish to consumers can take shape. Therefore, in order to prevent the consumption of fish with antibiotic residues, it is necessary to monitor their amount in fish throughout the chain from "production to sales in the trade network".

Keywords: frozen fish, psychrotrophic, mesophilic microflora, residual amounts of antibacterial drugs, antibiotic resistance.

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INTRODUCTION

Fish belongs to perishable food that requires appropriate temperature storage regimes to stop the flow of biochemical and microbiological processes [22, 35]. In the Ukrainian market sea fish are delivered mainly in a frozen form at a temperature – 12 - 18°C. When the technology of refrigeration chain is not observed, the fish quickly spoils and can be the source of food infections and toxicosis [13, 20, 27].

In the fish, in accordance with the requirements of normative and technical documents of Ukraine are regulated such microbiological indicators as the number of mesophilic aerobic and facultative anaerobic microorganisms up to 50 thousand CFU/g; bacteria of the group of intestinal sticks (coliform) are not allowed in 0.001 g of fish; Staphylococcus aureus in 0.01 g; pathogenic microorganisms, including genus Salmonella and Listeria monocytogenes are absent in 25.0 g and Vibrio parahaemolyticus is absentin 1.0 g. At the same time, in scientific publications [27, 37], scientists mainly focus on the dehiscence of frozen fish with mesophilic and coliform microorganisms. Studies [9, 17, 19] report that mesophilic microflora predominates in uncooked fish and seafood, as well as in fish from tropical and subtropical regions. For their storage under cold storage conditions, cold-loving – psychrotrophic microflora [14, 29] dominates, which, according to many scientists, causes biochemical and organoleptic changes in fish and affects sanitary and hygienic parameters [10, 30, 34]. So, scientists [6, 14, 29] indicate that due to non-observance of temperature storage regimes, the fish quickly spoils due to the development of gram-negative nonfermenting psychrotrophic microorganisms of the genera Pseudomonas spp., Acinetobacter spp., Moraxella spp., Flavobacterium. However, the study to determine the fertilization of frozen fish with psychrotrophic microflora does not provide normative and legal requirements.

In addition, antibiotics are widely used in the field of fish farming for the treatment and prevention of various diseases [12]. Uncontrolled use of antibacterial drugs leads to their accumulation in fish and seafood [1, 3]. The use of a large number of antibiotics can lead to the emergence of antibiotic resistant bacteria in aquatic environment, increased resistance to antibiotics in fish diseases, and the transfer of resistance determinants to pathogens that cause human illness [4, 11, 18, 25]. However, in frozen fish, which is imported into Ukraine, the determination of the presence of residual amounts of antibacterial drugs is not carried out.

Consequently, taking into account the abovementioned, conducting studies to determine the microbiological characteristics of frozen fish imported into Ukraine, in particular, the sterilization of its psychrotrophic microflora and residual amounts of antibacterial drugs is relevant.

The purpose of work was to investigate microbiological indicators of frozen fish imported into Ukraine, to identify the generic composition of psychrotrophic microflora isolated from frozen fish in the absence and with the content of residual amounts of antibacterial drugs and to determine the sensitivity of isolated microflora to antibiotics.

MATERIALS AND METHODS

30 samples of frozen fish selected in supermarkets of Ukraine were investigated for the last two years. 135 cultures of psychrotrophic microorganisms were isolated and analyzed: 75 isolates from samples of frozen fish in the absence of antibacterial drugs in it, and 60 in the presence of residual amounts of antibiotics.

Studies to determine the residual amounts of drugs of antibacterial substances: sulfaguanidine, sulfacetamide, sulfapyridine, sulfadiazine, sulfamethoxazole, sulfathiazole, sulfamerazine, sulfamethizole, sulfabenzamide, sulfamethazine, sulfhinoxoline, sulfadoxine, sulfadimethoxine, penicillin G, cephalexin, ampicillin, penicillin V, amoxicillin, trimethoprim, nafcillin, oxacillin, josamycin, spiramycin, nalidixic acid, flumecin, oxalic acid, norfloxacin, ciprofloxacin, spectinomycin, denofloxacin, enrofloxacin, marbofloxacin, sarafloxacin, dyfloxacin, lincomycin, gentamicin, doxycycline, chlortetracycline, tetracycline, oxytetracycline, kanamycin, apramycin, streptomycin, dihydrostreptomycin, parommycin, sulfamoxole, sulfafenazole, sulfamethoxypyridazine, sulfamonometoxin, tiamulin in fish were carried out using methods: liquid chromatography using a liquid chromatograph with tandem quadrupole mass spectrometric detector Waters and microbiological (screening –method for determining the residual quantities of antibacterial drugs of the group macroleads and β-lactams, tetracycline group, sulfanilamides, group of quinolones and aminoglycosides).

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The number of mesophilic aerobic and facultative anaerobic microorganisms was determined at a temperature of 30ºC, incubation of crops for 72 hours on Nutrient Agar (Himedia, India), number of psychrotrophic microorganisms – at temperature 6.5°C incubation of sowing for 10 days on Nutrient Agar (Himedia, India). The isolation of staphylococci was carried out in the medium of Baird-Parker Agar (Himedia, India), bacteria of Enterobacteriaceae family – on Endo Agar (Himedia, India), titre of coliform bacteria– in the medium Kesler (Pharmaktiv, Ukraine), mushroom and yeast – onSabouraud Dextrose Agar (Himedia, India), enterococci – BileEsculinAzide Agar (Himedia, India), Salmonella - 3M Petrifilm Salmonella Express System (3M Petrifilm SALX), and Listeria –on 3M Petrifilm Environmental Listeria (EL) Plate. We also used plates for the biochemical identification of nonfermenting microorganisms "Neferm test-24" ("BioMerieux S.A.", France). Identification of isolated microorganisms was carried out on the basis of morphological, tincorial, culture and biochemical data in accordance with the 9th edition of the determinant of Bergy bacteria [36]. The determination of sensitivity of microorganisms, isolated from frozen fish, to antibiotics was carried out by the classical Kirby-Bauer disk diffusion method.

Statistical processing of results was carried out using methods of variation statistics using the program Statistica 6.0 (StatSoft Inc., USA). Nonparametric methods of research were used (Wilcoxon criteria, Mann-Whitney criteria). The arithmetic mean (x), the standard error of the average (SE) were determined. The difference between the comparable values (SE) was considered to be significant for $P < 0,05$.

RESULTS

Our previous studies found that frozen fish, imported to the Ukrainian market, in 10% of cases contain residues of antibacterial drugs of various pharmacological groups (nalidixic acid, sulfanilamides, antibiotics: aminoglycosides, tetracyclines, penicillins, fluoroquinolones, etc.). The quantitative determination of residual amounts of antibacterial drugs in fish meat by the chromatographic method revealed excess of maximum permissible amount in accordance with EU Regulation 37/2010 on the content of tetracycline and spectinomycin. It has also been established that there are residual quantities of antibiotics gentamicin, difloxacin and paromycin in the fish, which reached the limit of maximum allowable amount for these antibiotics at 100 µg/kg. Therefore, taking into account the given study, we have conducted the determination of contamination of microflora of frozen fish, imported into Ukraine, depending on the presence of residues of antibacterial drugs in it (Table 1).

As can be seen from the data in Table 1, that in the absence of residues of antibacterial drugs in frozen fish, the content of mesophilic and psychrotrophic microorganisms was one-two orders of magnitude larger than that of fish containing antibiotics. All samples of fish, in the absence of antibiotics, and with their content, were enclosed in a microbiological standard in the amount of mesophilic microorganisms up to 5×10^4 CFU/g of fish.

Table 1: Contamination with microflora of frozen fish, depending on the presence of residual amounts of antibacterial drugs M±m, n=30

Samples of frozen fish	Number of mesophilic microorganisms, CFU/g of fish	Number of psychrotrophic microorganisms, CFU/g of fish	Number of S. aureus, CFU/g of fish	Titer of coliform bacteria
Fish without content of residual amounts of antibiotics	$3.8\pm2.1\times10^4$	$8.3\pm3.5\times10^{4*}$	78.5±4.2	0.1–0.001
Fish with the presence of residual amounts of antibiotics of tetracycline group	$7.1\pm3.5\times10^2$	$9.5\pm6.1\times10^{2*}$	$< 10^1$	< 1
Fish with the presence of antibiotics of penicillin group	$4.4\pm2.6\times10^3$	$7.1\pm5.3\times10^{3*}$	$< 10^1$	0.1
Fish with the presence of	$3.7\pm1.8\times10^3$	$6.1\pm2.9\times10^{3*}$	$< 10^1$	1

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antibiotics of aminoglycosides				
Fish with the presence of drugs of fluoroquinolone group	$8.7±4.1×10^2$	$9.2±5.7×10^2$	$< 10^1$	< 1

Note. $p < 0.05$ – concerning the mesophilic microflora

It was also found that the content of psychrotrophic microorganisms in frozen fish without antibiotics exceeded the amount of mesophilic microorganisms by 2.2 times ($p < 0.05$). In frozen fish with presence of residual amounts of antibiotics tetracycline, penicillin and aminoglycoside groups, the content of psychrotrophic microorganisms exceeded mesophilic microflora in 1.3 and 1.6 times ($p < 0.05$), respectively. The content of fish in the residues of drugs of fluoroquinolone group found the least amount of insemination to its microorganisms. The content of psychrotrophy does not exceed the amount of 10^3 CFU/g and the probable difference between the number of psychrotrophic and mesophilic microorganisms was not established.

Staphylococcus aureus was not isolated from the samples of frozen fish containing antibiotics, and in fish, in their absence, it was 78.4 ± 4.2 CFU/g, which also complies with regulatory requirements. Similar patterns were observed in regard to coliform bacteria. The titer of coliform bacteria in fish without antibiotics was in the range of 0.1 to 0.001, and their quantity in accordance with the standard is not allowed in 0.001 g of fish. Consequently, samples were found with an excessive number of coliform bacteria in fish in the absence of antibacterial drugs in it. At the same time, all samples containing antibiotics had the titer of coliform bacteria, which did not exceed 0.1 g.

In general, from the data obtained, it follows that frozen fish with the presence of residues of antibacterial drugs, on average two orders of magnitude is less contaminated with the microflora. Regardless of the presence or absence of antibiotics in frozen fish, the basis of its microflora are psychrotrophic microorganisms, which predominate amount of mesophilic bacteria in 1.3 – 1.6 times.

The next part of the work was to determine and compare the generic composition of the psychrotrophic microflora extracted from frozen fish in the absence and with the content of residues of antibacterial drugs. The results of research on the identification of psychrotrophic microflora of frozen fish in the absence of antibiotics in it are shown in Figure 1.

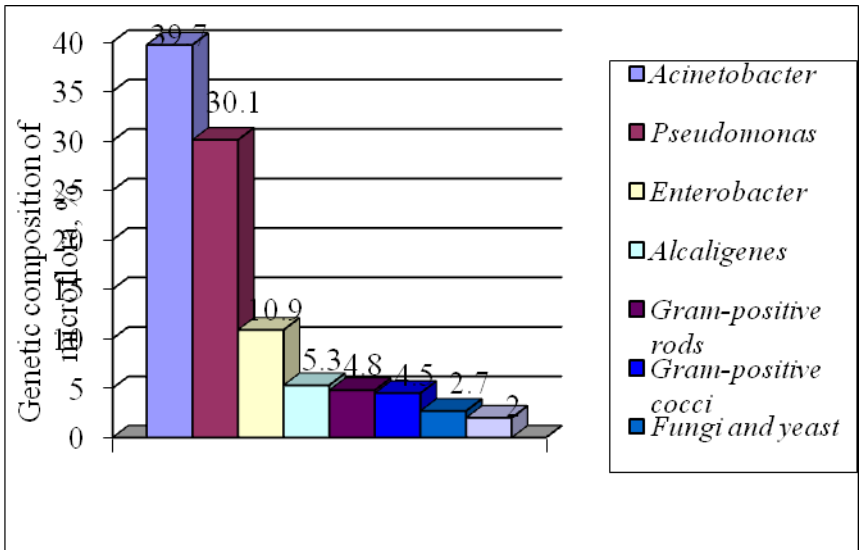

Fig 1: Genetic composition of psychrotrophic microflora of frozen fish in the absence of residual amounts of antibacterial drugs in it.

From the data of Fig. 1 it is found that the largest part of psychrotrophic microflora is made up of bacteria of the genus Acinetobacter – 39.7±2.8%, the third part among identified microorganisms belongs to

the genus Pseudomonas, s 10.3±1.1% are bacteria of the genus Enterobacter. These identified three strains of bacteria constitute the main psychrotrophic microflora of frozen fish – 80%.

In the range of 4.5 to 5.3%, three groups of microorganisms were distinguished: the genus Alcaligenes, gram-positive rod forms and gram-positive cocci forms of bacteria. The smallest niche in the composition of the detected psychrotrophic microflora was microorganisms of the genus Aeromonas and fungal microflora from 2 to 2.7%.

When identifying the psychrotrophic microflora of frozen fish with the content of residual amounts of antibiotics (Fig. 2), it was established that the growth of bacteria of the genus Pseudomonas was 1.7 times ($p<0.05$), they accounted for half of isolated microflora. Also, decrease of 1.8 times ($p<0.05$) of bacteria of the genus Acinetobacter, compared to their content in frozen fish without antibiotics and 1.4 fold increase ($p<0.05$) in the number of bacteria of the genus Enterobacter was detected. However, in general, three species of bacteria occupy almost the same part of composition of psychrotrophic microflora as in the fish without the presence of antibiotic residues – 85±3%.

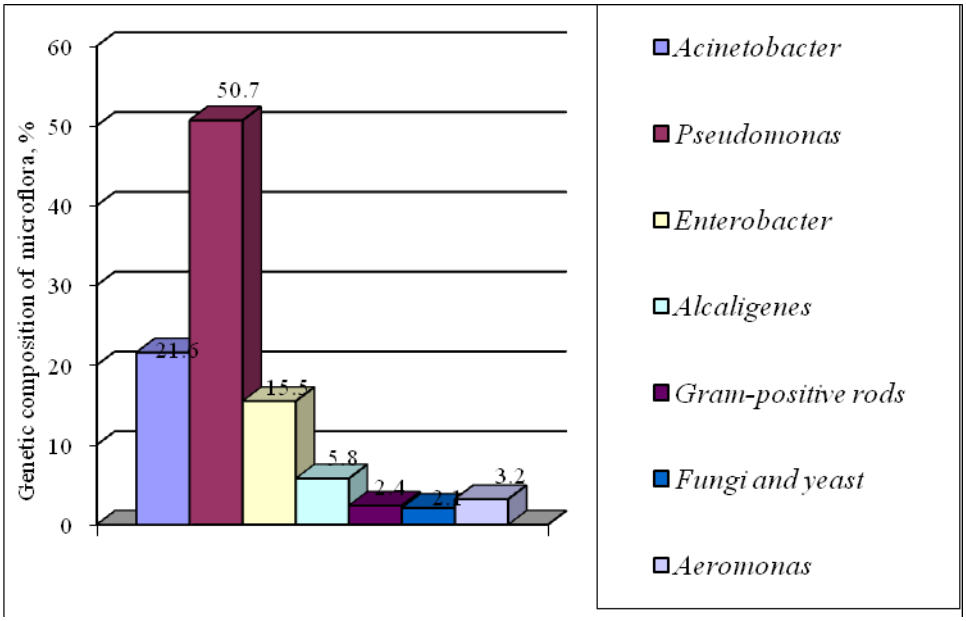

Fig 2: Genetic composition of psychrotrophic microflora of frozen fish in the presence of residual amounts of antibacterial drugs in it.

With regards to other representatives of identified microorganisms, the following may be noted. The bacteria of the genus Alcaligenes and the fungal microflora were allocated in the amount the same as from the fish without antibiotics, in the proportion of bacteria of the genus Aeromonas it was 1.5 times more ($p<0.05$), and on gram-positive rods 2.0 times ($p<0.05$) less. Also, in the presence of antibiotics in the psychrotrophic fish, no gram-positive coliform bacteria were identified, which is obviously due to its high sensitivity to the detected residual amounts of antibacterial drugs.

Thus, the obtained data indicate that in fish with the presence of residual quantities of antibiotics there is a change in the generic composition of psychrotrophic microflora due to the growth of bacteria of the genus Pseudomonas, Enterobacter and Aeromonas, which is probably due to the stability of these microorganisms to antibacterial drugs.

The next part of the work was to determine the sensitivity of psychrotrophic microflora to antibiotics and to compare the sensitivity of microorganisms, isolated from the meat of frozen fish in the absence of residual amounts and the presence of antibacterial agents in it.

In tabl. 2 is the study on the determination of sensitivity to antibiotics of microorganisms, isolated from frozen fish, in the absence of residues of antibacterial drugs in its meat.

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Table 2: Sensitivity of psychrotrophic microflora, isolated from frozen fish to antibiotics, in the absence of antibiotic residues in it, %, n = 75

Antimicrobial drugs, amount of active substance in the disk	Types of bacteria				
	Pseud-omonas spp.,n=15	Acineto-bacter spp., n=15	Entero-bacter spp.,n=15	S. aureus, n =15	Alcaligenes spp., n = 15
Benzylpenicillin, 10 units	0	86.7	60.0	86.7	86.7
Amoxicillin, 20 µg	0	86.7	73.3	93.3	933
Doxycycline, 10 µg	86.7	93.3	80.0	86.7	86.7
Tetracycline, 30 µg	80.0	86.7	80.0	80.0	93.3
Levofloxacin, 5 µg	93.3	100	86.7	93.3	100
Ciprofloxacin, 5 µg	93.3	100	93.3	93.3	100
Ceftriaxone, 30 µg	93.3	100	100	100	100
Ceftazidime, 30 µg	100	100	100	93.3	100
Gentamicin, 10 µg	93.3	100	86.7	83.3	93.3
Kanamycin, 30 µg	93.3	100	93.3	86.7	100
Furamag, 300 units	86.7	100	100	93.3	100

From data of table 2 it is evident that, basically, microorganisms, isolated from fish in the absence of residues of antibacterial drugs in it, were sensitive to antibiotics. So, the sensitivity of Pseudomonas spp. to the antibiotics of tetracycline group: tetracycline and doxycycline and nitrofuran drug - furamag ranged from 80.0 to 86.7%.

The drugs of fluoroquinolone group, levo- and ciprofloxacin, aminoglycosides (gentamicin and kanamycin), and cephalosporins of the III generation showed bactericidal effects on virtually all isolated cultures of Pseudomonas spp. – sensitivity ranged from 93.3 to 100%. Antibiotics of penicillin family: benzylpenicillin and amoxicillin, did not influence on the culture of Pseudomonas spp., which is associated with the natural resistance of these bacteria to this group of antibiotics

Bacteria of the species Acinetobacter spp. and Alcaligenes spp. practically in the same number were highly susceptible to antibiotics of different groups. At the same time, the effectiveness of most antibiotics, taken in the experiment, was 100%. Only the effectiveness of penicillin and tetracycline groups was slightly lower and ranged from 86.7 to 93.3%.

Bacteria of the species Enterobacter spp. were susceptible to antibiotics, but more stable than the species Acinetobacter spp. and Alcaligenes spp.Thus, only cephalosporins and nitrofuran drug - furamagshowed good antimicrobial activity – thesensitivity was 100%. Sensitivity of Enterobacter spp. to other antibiotics ranged from 80.0 to 93.3%, and to drugs of penicillin group from 60.0 to 73.3%.

Staphylococcus aureus, isolated allocated from the fish in the absence of residual amounts of antibacterial drugs, was highly sensitive in 86.7-100% of cases, almost to all antibiotics.

Consequently, the results of studies indicate that the most common types of psychrotrophic microflora, isolated from frozen fish in the absence of residual amounts of antibacterial drugs, were mostly highly susceptible to antibiotics. Despite the fact that among the investigated species Pseudomonas spp. proved to be resistant to drugs of pencillin group.

Table 3 shows the study of sensitivity to antibacterial drugs of psychrotrophic microorganisms isolated from frozen fish in the presence of residual amounts of antibiotics in the tetracycline group.

Table 3: Sensitivity of isolated bacteria from frozen fish to antibiotics, in the presence of residues of tetracycline in it, n = 60, %

Antimicrobial drugs, amount of active substance	Types of bacteria			
	Pseudomonas spp.,n=15	Acinetobacter spp., n=15	Enterobacter spp.,n=15	Alcaligenes spp., n = 15

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Benzylpenicillin, 10 units	0	40.0	33.3	40.0
Amoxicillin, 20 µg	0	46.7	46.7	53.3
Doxycycline, 10 µg	13.3	13.3	20.0	20.0
Tetracycline, 30 µg	66	13.3	13.3	6.6
Levofloxacin, 5 µg	73.3	73.3	73.3	73.3
Ciprofloxacin, 5 µg	73.3	80.0	73.3	86.7
Ceftriaxone, 30 µg	73.3	93.3	86.7	100
Ceftazidime, 30 µg	93.3	100	100	100
Gentamicin, 10 µg	73.3	80.0	86.7	86.7
Kanamycin, 30 µg	73.3	80.0	80.0	86.7
Furamag, 300 units	86.7	100	100	100

From data of table 3 it is evident that isolated psychrotrophic microorganisms from fish in the presence of residual amounts of tetracycline were significantly more resistant to antibacterial drugs. Thus, drugs of penicillin group generally did not act on cultures Pseudomonas spp., the sensitivity of other types of bacteria to penicillin ranged from 33.3 to 40.0%, and amoxicillin was sensitive to about 50% of the studied cultures. The effectiveness of antibiotics in the tetracycline group was very low; the number of sensitive cultures to tetracycline and doxycycline did not exceed 20.0%. Drugs of fluoroquinolone group levo- and ciprofloxacin showed a stable bactericidal effect on all isolated psychrotrophic bacteria, their sensitivity ranged from 73.3 to 86.7%. It should be noted that the anti-microbial activity in the antibiotics of cephalosporins is quite high: ceftriaxone and ceftazidime and nitrofuran drug – furamag. Number of susceptible strains of Pseudomonas spp. to the given doses ranged from 73.3 to 93.3%, sensitivity of all other isolated bacteria ranged from 86.7 to 100%.

The antimicrobial activity of aminoglycosides: gentamicin and kanamycin was also at a high level, the sensitivity of all isolated bacteria ranged from 73.3 to 86.7%.

Thus, studies have found that in the presence of residual quantities of antibiotics tetracycline in the frozen fish, microflora is found that is resistant to the drugs of this pharmacological group. In addition, in general, all microflora showed lower sensitivity to antibiotics of other groups.

Table 4 shows the results of studies of sensitivity of psychrotrophic microorganisms to antibacterial drugs, isolated from fish containing residual amounts of antibiotics of the penicillin group.

Table 4: Sensitivity of bacteria, isolated from frozen fish to antibiotics in the presence of residual amounts of penicillin in it, n = 60, %

Antimicrobial drugs, amount of active substance	Types of bacteria			
	Pseudomonas spp.,n=15	Acinetobacter spp., n=15	Enterobacter spp.,n=15	Alcaligenes spp., n = 15
Benzylpenicillin, 10 units	0	13.3	0	13.3
Amoxicillin, 20 µg	0	13.3	6.7	13.3
Doxycycline, 10 µg	66.7	80.0	73.3	80.0
Tetracycline, 30 µg	60.0	86.7	73.3	86.7
Levofloxacin, 5 µg	93.3	100	93.3	100
Ciprofloxacin, 5 µg	93.3	100	100	100
Ceftriaxone, 30 µg	93.3	93.3	86.7	100
Ceftazidime, 30 µg	100	100	86.7	100
Gentamicin, 10 µg	93.3	100	86.7	100
Kanamycin, 30 µg	100	100	86.7	93.3
Furamag, 300 units	93.3	100	93.3	100

From data of Table 4 it is evident that the isolated psychrotrophic microorganisms formed resistance to the drugs of penicillin group, since the sensitivity of bacteria did not exceed 13.3%. This is probably due to the presence of residues of antibiotic data in frozen fish. At the same time, drugs of other pharmacological

groups were rather highly active to isolated microorganisms. Thus, tetracyclines exhibited a stable bactericidal effect on all isolated psychrotrophic microorganisms taken in an experiment with a sensitivity of 60.0 to 86.7%.

The antimicrobial drugs of fluoroquinolone, cephalosporin and aminoglycoside group showed the highest efficiency – the sensitivity of bacteria was from 86.7 to 100%.

Results of studies of sensitivity to antibiotics of psychrotrophic microorganisms, isolated from fish with the content of residual amounts of antibiotics of aminoglycosides, are given in Table 5.

Table 5: Sensitivity of bacteria, isolated from frozen fish to antibiotics in the presence of residual gentamicin in it, n = 60, %

Antimicrobial drugs, amount of active substance	Types of bacteria			
	Pseudomonas spp.,n=15	Acinetobacter spp., n=15	Enterobacter spp.,n=15	Alcaligenes spp., n = 15
Benzylpenicillin, 10 units	0	53.3	20.0	60.0
Amoxicillin, 20 μg	0	66.7	46.7	46.7
Doxycycline, 10 μg	73.3	80.0	73.3	80.0
Tetracycline, 30 μg	66.7	73.3	66.7	80.0
Levofloxacin, 5 μg	80.0	86.7	80.0	86.7
Ciprofloxacin, 5 μg	86.7	93.3	86.7	93.3
Ceftriaxone, 30 μg	93.3	100	93.3	100
Ceftazidime, 30 μg	100	100	100	100
Gentamicin, 10 μg	6.6	13.3	6.7	20.0
Kanamycin, 30 μg	6.7	6.7	6.7	20.0
Furamag, 300 units	93.3	100	86.7	93.3

From data of Table 5 it is evident that the regularity is observed regarding the sensitivity of the isolated microflora from fish with the presence of residual amounts of antibacterial drugs. This pattern was characterized by the formation of stable microflora in fish meat to the antibiotic residues found in it. Thus, according to the data of Table 5, in meat of frozen fish, residual amounts of antibiotics of the aminoglycoside group were detected, as a result, isolated psychrotrophic microorganisms exhibited sensitivity to gentamicin and kanamycin to only 20.0% of cultures.

Regarding the antimicrobial activity of drugs of other pharmacological groups, it was found that penicillins showed the least bactericidal activity, the sensitivity of the isolated bacteria was from 20.0 to 66.7%, except were Pseudomonas spp. that were resistant.

Sensitivity of isolated microorganisms to drugs of the tetracycline group ranged from 66.7 to 80.0%, and to fluoroquinolone was higher – from 80.0 to 93.3%. Cephalosporins showed the highest activity, so the sensitivity of isolated strains to ceftriaxone ranged from 93.3 to 100%, and all cultures were sensitive to ceftazidime.

The results of studies of sensitivity to antibiotics of psychrotrophic microorganisms isolated from fish with the content of residual amounts of preparations of fluoroquinolone group are given in Table 6.

Table 6: Sensitivity of bacteria, isolated from frozen fish to antibiotics in the presence of residual amounts of fluoroquinolone group, n = 60, %

Antimicrobial drugs, amount of active substance	Types of bacteria			
	Pseudomonas spp.,n=15	Acinetobacter spp., n=15	Enterobacter spp.,n=15	Alcaligenes spp., n = 15
Benzylpenicillin, 10 units	0	40.0	26.7	40.0
Amoxicillin, 20 μg	0	66.7	66.7	66.7
Doxycycline, 10 μg	66.7	73.3	73.3	86.7
Tetracycline, 30 μg	60.0	73.3	80.0	86.7

Levofloxacin, 5 µg	6.7	20.0	13.0	13.3
Ciprofloxacin, 5 µg	6.3	13.3	6.7	13.3
Ceftriaxone, 30 µg	80.0	93.3	86.7	66.7
Ceftazidime, 30 µg	86.7	93.3	86.7	100
Gentamicin, 10 µg	80.0	86.7	73.3	93.3
Kanamycin, 30 µg	86.7	80.0	73.3	93.3
Furamag, 300 units	93.3	100	86.7	93.3

As can be seen from Table 6, drugs of fluoroquinolone group: levo- and ciprofloxacin were not active to isolated microorganisms, since their sensitivity did not exceed 13.3%. Other pharmacological groups of antibiotics have shown a good bactericidal effect on microorganisms isolated from fish. Sensitivity of bacteria of the genus Acinetobacter, Enterobacter and Alcaligenes to penicillin ranged from 26.7 to 40.0%, and to amoxicillin – 66.7%. Stable high efficacy was shown by drugs of tetracycline series, the sensitivity of all isolated bacteria varied from 60.0 to 86.7%. Antibiotics of aminoglycosides and cephalosporins exhibited the highest bactericidal effect, the sensitivity of microorganisms ranged from 73.3 to 100%.

DISCUSSION

The safety and quality of the finished fish and seafood depends on the quality of the raw materials, conditions of its storage and processing technology [21]. Fresh fish quickly spoils, so after catching it is stored and sold in a cooled or frozen state. Faults of fish are associated with biochemical changes that arise as a result of active life of the microflora producing various enzymes [29]. The main microflora, which is active in the conditions of cold storage of fish, is psychrotrophic, it can develop at temperatures below +7°C, despite the higher optimum temperature for its development [14]. Therefore, various antibacterial drugs are widely used by manufacturers to stop the reproduction of microflora of fish in the technological chain "from catching to the consumer" [28]. Antibacterial drugs are used for feeding fish in aquaculture and are added to water during freezing [7]. Our studies found that frozen fish, imported into Ukraine, on average, contained about 10% of residual amounts of antibacterial drugs of various pharmacological groups. The chromatographic method of study revealed maximum permissible amount in accordance with EU Regulation 37/2010 on the content of tetracycline and spectromycin. Studies show the excess of maximum amount of antibiotics in products of animal origin on the content of tetracycline [2, 24,33]. The results of studies (Table 1) revealed that in the absence of antibiotic residues in frozen fish, the content of mesophilic and psychrotrophic microorganisms was one or two orders of magnitude higher compared to fish containing the residues of antibiotics tetracyclines, penicillins, aminoglycosides and fluoroquinolones. In addition, in the quantity of psychrotrophic microflora of frozen fish without antibiotics, the content of mesophilic content was 2.2 times higher ($p<0.05$), and in the presence of antibacterial drugs in 1.3-1.6 times ($p<0.05$). But after all microbiological tests, fish samples were put into the requirements of the national standard. The obtained results are consistent with the data, which indicate a significant fertilization of frozen fish with psychrotrophic microorganisms from 10^4 to 10^6 CFU/g. This may be due to contamination of water that is in contact with fish, unsatisfactory sanitary conditions during processing, lack of cooling or freezing.

Figure 1 shows that the composition of psychrotrophic microflora of frozen fish in the absence of residues of antibacterial drugs by 80% is represented by three genera of bacteria: Acinetobacter – 39.7±2.8%, Pseudomonas – 30.1±2.4 and Enterobacter – 10.3±1.1%. At the same time, in the presence of antibiotic residues in frozen fish (Fig. 2), it was found that the growth of bacteria of the genus Pseudomonas increased by 1.7 times ($p<0.05$) to 50.7±3.7% and decreased by 1.8 times ($p<0.05$) of bacteria of the genus Acinetobacter to 21.6±1.4%, compared with their content in frozen fish without antibiotics and increase of 1.4 times ($p<0.05$) of bacteria of the genus Enterobacter. The obtained results of the study coincide with the data [20], which report the contamination of frozen fish with bacteria of the genus Pseudomonas from 1.2×10^3 to 2.3×10^5 CFU/g. It is believed that this is due to the disease of the fish on the pseudomonosis. We have identified more bacteria of the genus Pseudomonas in frozen fish with residual amounts of antibiotics, probably also associated with feeding fish with antibiotics to prevent various infectious diseases.

Veterinary drugs, including antibiotics, in modern aquaculture are widely used for the prevention and treatment of various bacterial diseases of fish [28]. WHO has recognized that the use of antibacterial drugs for industrial livestock and aquaculture causes a global problem of formation of resistance to antibiotics [15, 16,

26]. In addition, it is indicated that food can serve as a source of formation and factor in the transmission of antibiotic-resistant microorganisms to humans [5, 23]. According to a recent report, more than 700000 people die from infections caused by antibiotic-resistant bacterial strains annually in the world [32].

When determining the sensitivity of psychrotrophic bacteria to antibiotics in microorganisms isolated from frozen fish meat in the absence of residual amounts of antibacterial drugs (Table 2), it was found that they were sensitive from 80.0 to 100% of cases. Exceptions were antibiotics of penicillin family that did not act on the culture of Pseudomonas spp. At the same time, in the presence of residual amounts of antibacterial drugs in fish, it was found the formation of resistance of microflora to antibiotics in it (Table 3-5). Thus, according to the data of Table 3, in the meat of frozen fish were found antibiotics of tetracycline group, while the sensitivity of microflora to antibiotics of this group did not exceed 20%. Despite the fairly high effectiveness of drugs of other pharmacological groups: fluoroquinolones, aminoglycosides and cephalosporins, sensitivity ranged from 73.3 to 100%, and the average efficacy of the penicillin group – the sensitivity of bacteria was up to 50%. The formation of microflora resistance in aquaculture to antibiotics was reported by researchers [2, 24, 33], which showed highly resistant strains to tetracycline, oxytetracycline, ampicillin and florfenicol. Other researchers [24] indicate that the percentage of resistant bacteria, isolated in aquaculture to the antibiotics of gentamicin, kanamycin, and enrofloxacin, was very low, from the total number of cultures examined. However, in general, the majority of researchers believe that residual amounts of antibacterial drugs in foods, including fish, are the source of formation in microorganisms of genes of multiresistance for antibiotics [7, 8, 31].

CONCLUSION

In general, the findings of the study indicate that it is impossible to maintain the quality and safety of fish without the use of cooling or freezing, which are aimed at inhibiting the development of microflora. However, in conditions of refrigeration of fish, the psychrotrophic microflora, which affects organoleptic parameters and reduces its safety and quality, becomes of paramount importance. Therefore, it is necessary to control the level of fish insemination by psychrotrophic microorganisms before beginning of freezing. Also, the results of the study found that, subject to the presence in the frozen fish of residual amounts of antibacterial drugs, is formed resistant to the detected antibiotics psychrotrophic microflora.

Consequently, a pathway for the transfer of resistant microorganisms and resistance genes from fish to consumers can be formed. Therefore, in order to prevent the flow of fish to consumers with antibiotic residues, it is necessary to monitor their amount in fish throughout the chain from “production to selling in trade network”.

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