MINISTRY OF HEALTH OF THE RUSSIAN FEDERATION

GENERAL MONOGRAPH (GM)

Microbiological purity **GM.1.2.4.0002.15**

Instead of State Pharmacopoeia (SP) XI, version 2, p. 193, changes, changes Nos. 2, 3, GM 42 -0016-04, SP XII, Part 1, GM 42-0067-07

This General Monograph applies to the methods of determination of the microbiological purity of non sterile pharmaceutical preparations (NPPs) including pharmaceutical preparations (PPs) that contain live microorganisms, as well as excipients and intermediates. Moreover, these techniques are used to determine the effectiveness of antimicrobial preservatives and the monitoring of industrial premises of pharmaceutical enterprises and individual laboratories.

NPPs (substances, various forms of preparations – tablets, capsules, granules, solutions, suspensions, syrups, ointments, suppositories, etc. and excipients) may be contaminated with microorganisms. The NPP is allowed to contain a limited number of microorganisms in the absence of certain species that threat to human health.

1. Recommendations for the quality of pharmaceutical preparations

Tables 1 and 2 show the quality requirements for the pharmaceutical preparations (preparations and substances), as well as excipients used in manufacturing pharmaceuticals.

Table 1: Microbiological purity of pharmaceutical preparations

Category	Preparations	Recommendations
1	Preparations, inter alia biological medicinal preparations, including immunobiological drugs which are required to be “Sterile”	Preparations should be sterile

2	• To apply locally, topically, vaginally • To be injected in the ear, nose • To apply for respiratory therapy • Transdermal patches Except for those preparations or immunobiological drugs which must be sterile	• The total number of aerobic bacteria and yeasts and moulds (in total) does not exceed 10^2 CFU/g or CFU/mL of the drug or CFU/patch (including the adhesive layer and backing) • Absence of *Pseudomonas aeruginosa* in 1 g or 1 mL of the drug or on 1 patch (including the adhesive layer and backing) • Absence of *Staphylococcus aureus* in 1g or 1 mL of the drug or on 1 patch (including the adhesive layer and backing) • Absence of Enterobacteriaceae resistant to bile, in 1 g or 1 mL of the preparations used in respiratory therapy • Absence of *Candida albicans* in 1 g or 1 mL of vaginal drugs
3	A. For oral or rectal use Except for those preparations or immunobiological drugs which must be sterile	• The total number of aerobic bacteria does not exceed 10^3 CFU/g or CFU/mL • The total count of yeasts and moulds does not exceed 10^2 CFU/g or CFU/mL • Absence of *Escherichia coli* in 1 g or 1 mL
	B. For oral use: made of raw materials from natural origin (animal, vegetable or mineral), which level of microbial contamination cannot be reduced during pretreating. Except for herbal medicinal preparations and immunobiological drugs containing live microorganisms that belong to categories 4 and 5	• The total number of aerobic bacteria does not exceed 10^4 CFU/g or CFU/mL • The total count of yeasts and moulds does not exceed 10^2 CFU/g or CFU/mL • The total number of Enterobacteriaceae resistant to bile does not exceed 10^2 CFU/g or CFU/mL • Absence of *Escherichia coli* in 1 g or 1 mL • Absence of *Salmonella spp.*in 10 g or 1 mL • Absence of *Staphylococcus aureus* in 1 g or 1 mL
4	Herbal medicinal preparations and raw material from vegetable nature A. Applied in the form of infusions and decoctions prepared using boiling water	• The total number of aerobic bacteria does not exceed 10^7 CFU/g • The total count of yeasts and moulds does not exceed 10^5 CFU/g • *Escherichia coli* does not exceed 10^2 CFU/g
	B. Prepared without boiling water	• The total number of aerobic bacteria does not exceed 10^5 CFU/g • The total count of yeasts and moulds does not exceed 10^4 CFU/g • The total number of Enterobacteriaceae resistant to bile does not exceed 10^3 CFU/g • Absence of *Escherichia coli* in 1 g • Absence of bacteria of the genus *Salmonella* in 25 g

5	Immunological drugs that contain live microorganisms	
5.1. Vaccines		
	A. For injections	The presence of microorganisms-contaminants is not allowed (to be detected in accordance with the “Sterility” GM)
	B. For the intradermal use and epidermal scarification (application)	• The total number of aerobic microorganisms, yeasts and moulds does not exceed 50 CFU/mL • Absence of Enterobacteriaceae in 1 mL • Absence of *Pseudomonas aeruginosa* in 1 mL • Absence of *Staphylococcus aureus* in 1 mL (to be detected in accordance with the pharmacopoeial monograph or regulatory documentation)
	C. For oral use (tablets)	• The total number of aerobic bacteria does not exceed 10^3 CFU/preparation item (g) • The total count of yeasts and moulds is less than 10 CFU/preparation item (g) • Absence of bacteria in the family *Enterobacteriaceae* in a preparation item (g) • Absence of *Pseudomonas aeruginosa* in a preparation item (g) • Absence of *Staphylococcus aureus* in a preparation item (g) (to be detected in accordance with the pharmacopoeial monograph or regulatory documentation)
5.2. Bacteriophages		
	A. Solutions for oral and rectal use	The presence of microorganisms-contaminants is not allowed (to be detected in accordance with the “Sterility” GM)
	B. For oral use (tablets), local and external application (ointment)	• The total number of aerobic bacteria does not exceed 10^2 CFU/g • Less than 10 yeasts and moulds in 1 g • Absence of bacteria in the family *Enterobacteriaceae* in 1 g • Absence of *Pseudomonas aeruginosa* in 1 g • Absence of *Staphylococcus aureus* в 1 г (to be detected in accordance with Sections 4, 5, 6 of the present General Monograph)
5.3. Probiotics		
	A. For oral, intravaginal use (lyophilisates, suspensions, powders)	• Absence of bacteria-contaminants in a preparation item/г (mL)

		• Absence of yeasts and moulds in a preparation item/г (mL) • For preparations containing E. coli: Absence of PFU/g (PFU/mL) of Bacteriophage
	B. For oral, intravaginal, rectal use (tablets, capsules, suppositories)	• The total number of aerobic bacteria does not exceed 10^2 CFU/preparation item (g) • The total count of yeasts and moulds is less than 10 CFU/preparation item (g) • Absence of Enterobacteriaceae in a preparation item (g) • Absence of *Pseudomonas aeruginosa* in a preparation item (g) • Absence of *Staphylococcus aureus* in a preparation item (g) • For preparations containing E. coli: Absence of PFU/g of Bacteriophage (for preparations with *E.coli* content of not less than 10^{10} CFU, not more than 10 PFU of Bacteriophage is allowed)
6	Immunobiological drugs that contain inactivated microorganisms, antigens, antibodies, proteins, peptides, glycoproteins, etc., in which micro-contaminants are allowed	
	1. For oral, intranasal use	• The total number of aerobic bacteria does not exceed 50 CFU/g or CFU/mL • Absence of Enterobacteriaceae in 1 g or 1 mL • Absence of *E.coli* in 1 g or 1 mL • Absence of bacteria of the genus *Salmonella* in 1 g or 1 mL • Absence of *Pseudomonas aeruginosa* in 1 g or 1 mL • Absence of *Staphylococcus aureus* in 1 g or 1 mL • Less than 10 yeasts and moulds in 1 g or 1 mL (to be detected in accordance with the pharmacopoeial monograph or regulatory documentation)
	2. For rectal administration	• The total number of aerobic bacteria does not exceed 10^2 CFU/g • Absence of bacteria in the family *Enterobacteriaceae* in 1 g • Absence of *Pseudomonas aeruginosa* in 1 g • Absence of *Staphylococcus aureus* in 1 g

		• Less than 10 yeasts and moulds in 1 g (to be detected in accordance with the pharmacopoeial monograph or regulatory documentation)

Table 2: Microbiological purity of substances and excipients for drugs production

Category	Substances and excipients	Recommended norms
1.2	Substances for production of	
	A. Sterile drugs that are not subjected to sterilization	Substances should be sterile
	B. Sterile drugs that are subjected to sterilization; Non sterile pharmaceutical preparations that belong to category 2	• The total number of aerobic bacteria, yeasts and moulds (in total) does not exceed 10^2 CFU/g or CFU/mL • Absence of Enterobacteriaceae resistant to bile in 1 g or 1 mL • Absence of *Pseudomonas aeruginosa* in 1 g or 1 mL • Absence of *Staphylococcus aureus* in 1 g or 1 mL
2.2	Non-natural substances for the production of non sterile pharmaceutical preparations	• The total number of aerobic bacteria does not exceed 10^3 CFU/g or CFU/mL • The total count of yeasts and moulds does not exceed 10^2 CFU/g or CFU/mL • Absence of *Escherichia coli* in 1 g or 1 mL
3.2	Natural substances (vegetable, animal, or mineral) for the production of non sterile pharmaceutical preparations	• The total number of aerobic bacteria does not exceed 10^4 CFU/g or CFU/mL • The total count of yeasts and moulds does not exceed 10^2 CFU/g or CFU/mL • Absence of *Escherichia coli* in 1 g or 1 mL • Absence of bacteria of the genus *Salmonella* in 25 g or 25 mL • Absence of *Pseudomonas aeruginosa* in 1 g or 1 mL • Absence of *Staphylococcus aureus* in 1 g or 1 mL • The total number of Enterobacteriaceae resistant to bile does not exceed 10^2 CFU/g or CFU/mL
4.2	Excipients (wheat flour, starch, talc, etc.)	• The total number of aerobic bacteria does not exceed 10^3 CFU/g or CFU/mL • The total count of yeasts and moulds does not exceed 10^2 CFU/g or CFU/mL • Absence of *Escherichia coli* in 1 g or 1 mL • Absence of bacteria of the genus *Salmonella* in 25 g or 25 mL • Absence of *Pseudomonas aeruginosa* in 1 g or 1 mL • Absence of *Staphylococcus aureus* in 1 g or

		1 mL • The total number of Enterobacteriaceae resistant to bile does not exceed 10^2 CFU/g or CFU/mL
5.2	Substances for the production of biological medicinal preparations, including immunobiological drugs	
	A. For the production of sterile drugs that are not subjected to sterilization	Substances should be sterile
	B. For the production of sterile drugs that are subjected to sterilization	• The total number of aerobic bacteria does not exceed 10^2 CFU/g or CFU/mL • Absence of Enterobacteriaceae in 1 g or 1 mL • Absence of *Pseudomonas aeruginosa* in 1 g or 1 mL • Absence of *Staphylococcus aureus* in 1 g or 1 mL • Less than 10 yeasts and moulds in 1 g or 1 mL
	C. For the production of immunobiological drugs that contain live microorganisms: – in which the presence of microorganisms-contaminants is not allowed (live vaccines (injectable dosage forms), Bacteriophages, solutions for oral and rectal use); – in which the presence of microorganisms-contaminants is limited (live vaccines for oral use (tablets); Bacteriophages (tablets))	The presence of microorganisms-contaminants is not allowed (to be detected in accordance with the "Sterility" GM)
	D. For the production of immunobiological drugs-probiotics that contain live microorganisms in which the presence of microorganisms-contaminants is allowed (tablets, capsules, suppositories)	• Absence of foreign aerobic bacteria in 200 mg • Less than 10 yeasts and moulds in 200 mg
	E. For the production of immunobiological drugs that contain inactivated microorganisms, antigens, antibodies, proteins, peptides, glycoproteins, etc.	Substances should be sterile

Notes to Tables 1 and 2

1. In the regulatory documents other standards may be listed as an exception, depending on the composition of the PP and the properties of their production process.

2. In the regulatory documents for the medicines for children, the more strict standards may be made, namely:

– 1 g (mL) of the medicines for infants (0-1 year) should not contain more than 50 aerobic bacteria, yeasts and moulds (in total) with the absence of Enterobacteriaceae resistant to bile, *Pseudomonas aeruginosa, Staphylococcus aureus;*

– 1 g (mL) of the medicines for children (older than 1 year) should not contain more than 500 aerobic microorganisms and 50 yeasts and moulds (in total) with the absence of Enterobacteriaceae resistant to bile, *Pseudomonas aeruginosa*, *Staphylococcus aureus.*

3. In the regulatory documents for the immunobiological drugs-probiotics for children, the more strict standards may be made, namely:

– for infants (3 months-1 year) for oral use (tablets, capsules, etc.), rectal use (suppositories): not more than 10 aerobic bacteria in 1 item/g; with the absence of Enterobacteriaceae in 1 item of the preparation, *Pseudomonas aeruginosa, Staphylococcus aureus*, yeasts and moulds;

– for children (older than 1 year) for oral use (tablets, capsules), rectal use (suppositories) not more than 50 aerobic bacteria in 1 g of the drug; with the absence of Enterobacteriaceae, *Pseudomonas aeruginosa, Staphylococcus aureus*, yeasts and moulds in 1 item of the drug.

4. If the test detected other pathogenic bacteria, in addition to the above, it is believed the quality of medicines, substances and excipients does not comply with the Microbiological purity characteristic.

The pharmaceutical preparations test for the microbiological purity should be carried out under aseptic conditions using the following methods and culture media.

The test includes the methods of preparation of various dosage forms, sampling for the analysis, the methods for quantitative determination of microorganisms viability, the detection and identification of individual species of bacteria which presence is unaccepted or limited in pharmaceutical preparations, as well as culture media, solutions and reagents needed for testing.

For the incubation of inoculations in culture media for bacteria, the standard temperature is 32.5±2.5 ºC, for fungi is 22.5±2.5 ºC.

2. Operation with test strains of microorganisms

To carry out the test (the determination of antimicrobial action of a PPs, quality of culture media, biochemical testing of identified microorganisms), one should use test strains of microorganisms, deposited in the official domestic and foreign collections, such as:

- State Collection of Pathogenic Microorganisms (GKPM), Russia;
- Russian Collection of Pathogenic Fungi, (RKPG);
- All-Russian Collection of Microorganisms (VKM) Russian Academy of Science, Russia;

- American Type Culture Collection (ATCC), USA;

- National Collection of Type Cultures (NCTC), UK;

- Collection of the Institut Pasteur (CIP), France.

Test strains of microorganisms used in the tests are given in Table 3.

Table 3: Test strains of microorganisms used in the tests

Name of microorganism	Test strain number
Bacillus subtilis	GKPM 010011, ATCC 6633
Bacillus cereus	GKPM 010014, ATCC 10702
Escherichia coli	GKPM 240533, ATCC 25922, ATCC 8739
Salmonella enterica subsp. *enterica* serovar *abony* (former name *Salmonella abony)*	GKPM 100329, IHE* 103/39, NCTC 6017, CIP 80.39
Pseudomonas aeruginosa	GKPM 190155, ATCC 9027
Staphylococcus aureus	GKPM 201108, ATCC 6538
Staphylococcus epidermidis	GKPM 202001, ATCC 14990 GKPM 202004, ATCC 12228
Candida albicans	GKPM 303903, GKPM 303901, RKPGY401/NCTC 885-653, ATCC 10231, NCPF 3179
Aspergillus brasiliensis *(*former name *Aspergillus niger*)	VKM F1119,ATCC 9642, ATCC 16404, NCPF2275 RKPGF106

*IHE – Institute of Hygiene and Epidemiology, Prague, Czech Republic

Besides the test strains of microorganisms listed in Table 3, it is possible to use other cultures which morphological, tinctorialm and biochemical properties are typical.

If necessary, the set of test microorganisms can be reduced or increased.

The test-microorganisms in the lyophilized form in ampules, test tubes on semisol-id agar should be stored at a 2-8 °C temperature. Cultures of microorganisms on the disk are stored at a temperature not higher than -20 °C.

Not more than 5 passages from a stock culture are allowed.

2.1. Activation of the lyophilized test strains of microorganisms

Lyophilized test strains of microorganisms in ampoules are prepared from official collections with the Certificate of Conformity (test strain passport).

The opening of the ampoules should be carried out under aseptic conditions according to the manufacturer's instructions.

To restore the viability of the culture not less than 2 isolate passages in culture medium are necessary corresponding to the biological properties of the test strain when incubating under the optimal temperature for the test strain. To get isolated colonies of test strain, one should carry out the isolate passage in an appropriate solid culture medium.

The culture incubation finished, one examines the morphology of grown colonies, microscopes Gram-stained smears, studies the biochemical properties using test-systems approved for use. The test-strain of a microorganism must have typical morphological, tinctorial, biochemical properties in accordance with the presented collection certificates.

After the confirmation of the test-strain properties, the culture is subcultured in the proper medium (first passage) and incubated under standard conditions.

To get *A. brasiliensis* conidia, the culture is incubated in Sabouraud glucose agar (or medium No. 2) for 5-7 days under standard conditions.

2.2. Activation of test-strains of microorganisms stored on disks

The disk is placed in liquid medium corresponding to the needs of the microorganism. After incubation under appropriate conditions, one performs the same operations and in the same sequence as when the activation of the lyophilized culture.

2.3. Storage of test-strains under deep freeze

The test-strains of microorganisms should be stored under deep freeze at a temperature of 70±5 °C below zero (cryosystem). The cryosystem consists of a set of sealed tubes containing ceramic beads embedded in specific cryo liquid and lead cryo block with cells. The operation with test-strains is carried out in full compliance with the recommendations of the cryosystem manufacturer.

3. Determination of antimicrobial activity

In order to avoid an incorrect assessment of the results, before testing for microbiological purity, it is necessary to determine the possibility of manifestation of the antimicrobial effect against certain types of microorganisms.

The method of determining the antimicrobial effect is based upon the comparison of the growth rate of the test-strains of microorganisms in the presence/absence of the test preparation.

3.1. Preparation of inoculum

24-hours broth cultures of bacteria grown on Casein soya bean digest broth or medium No. 8 and 24-48 hours *C. albicans* culture grown in Sabouraud liquid medium (Casein soya bean digest broth or medium No. 8) are diluted with sterile 0.9 % solution of sodium chloride at a 1:1,000 ratio (*B. cereus, C. albicans*) and a 1:100,000 ratio (*E. coli, S. abony, P. aeruginosa, S. aureus*) up to concentration of appr. 10^4 CFU/mL.

B.subtilis spore suspension is also diluted up to concentration of 10^4 CFU/mL.

A. brasiliensis culture from slope Sabouraud glucose agar or from medium No. 2 is washed with 0.9 % solution of sodium chloride with 0.05 % solution of Tween-80. The quantity of conidia in 1 mL of swab is determined using the Goryaev's chamber or agar dish method, and the swab is diluted to a concentration of 10^4 conidia in 1 mL.

3.2. Sample preparation for the determination of antimicrobial effect

To a PP one adds suitable diluent to get a 1:10 dilution. The diluent is generally phosphate buffer solution with sodium chloride and peptone (pH 7.0), neutralizing liquid or buffer solution that contains not more than 5% of Tween-80.

For dilution of preparations with known antimicrobial effect one uses neutralizing liquid. From 1:10 dilution one prepares serial dilutions of 1:50, 1:100, 1:500, 1:1,000, etc.

3.3. Methods for determination of antimicrobial effect

The presence of antimicrobial effect is tested using one of the methods as follows.

3.3.1. Determination of antimicrobial effect during testing the microbiological purity

Each dilution of the test preparation in the amount of 1 mL is placed in 6 Petri dishes of a 90 mm diameter, two of which are added with 0.2 mL of *B. cereus* (or *B .subtilis* spores) suspension, the other two dishes are added with 0.2 mL of working suspension of *C. albicans* culture, the last two dishes are added with 0.2 mL of *A. brasiliensis* conidia suspension. The dishes with bacteria are filled with 10-15 mL of melted and cooled up to a 42.5±2.5 °C temperature casein soya bean digest agar or medium No. 1, the dishes with fungi cultures are filled with the same amount or Sabouraud agar or medium No. 2.

By 1.0 mL of each dilution of the preparation is introduced in test tubes with 10 mL of liquid media: Mossel broth and casein soya bean digest broth (or similar – medium No. 3 and medium No. 8). By 1 mL of suspension of the *E. coli, S. abony, P. aeruginosa, S. aureus* test-strains (each test strain separately) is then introduced in a test tube with medium corresponding to the needs of a microorganism.

The control dishes and test tubes are added with the same amount of solvent instead of the drug dilutions.

The bacteria inoculations are incubated under standard conditions for 48 hours and fungi– for 5 days.

3.3.2. Replication method

The replication method is recommended for the determination of antimicrobial effect of non-water-soluble (suspensions, emulsions, etc.) or colored medicines.

In sterile Petri dishes one introduces by 1 mL of each dilution of the test specimen. In control dishes, one puts by 1 mL of diluent used for dilution. In the Petri dishes, both during the experiment and the control, one adds 10-15 mL of melted and cooled up to a 42.5±2.5 ºC temperature of casein soya bean digest agar or medium No. 1, in others – the same amount of Sabouraud agar or medium No. 2 and thoroughly mixes it. After agar solidification, the dishes are dried out in an oven or a laminar flow hood to remove the condensate from the surface of the medium on which one then applies the working suspension of each test strain of bacteria and fungi in the form of plaques with an inoculation loop, pipette or replicator. The in-

oculations are incubated under standard conditions for 48 hours and fungi cultures – for 5 days.

3.4. Recording and interpretation of results

The incubation time finished, one examine the inoculations and notes the appearance of the typical growth of test-microorganisms in the control dishes and test tubes (without specimen) and test ones (with various dilutions of the specimen). In cases that hinder recording the results (change in color or turbidity of the liquid medium as a result of interaction of the PP with culture medium), one makes subculturing in the agarized media.

If *E. coli, S. abony, P. aeruginosa, S. aureu*s growth is available in culture media, one makes a conclusion on the absence of antimicrobial effect of the study specimen.

The growth of test-microorganisms in test dishes and test tubes similar to control ones is marked with a "+" sign, the absence of the growth is marked as "-". If one observes reduction in the number of colonies on the dishes or the absence of growth of test-microorganisms, one makes a conclusion about its antimicrobial effect. The first serial dilution of the specimen, with no antimicrobial effect is used for inoculation in the proper medium.

3.5. Ways to eliminate the antimicrobial effect of a PP

To eliminate the antimicrobial effect of a PP, the methods are recommended, as follows:

- The increase in dilution of the specimen due to a larger volume of diluent or culture medium within the permissible norms of microbial contamination (instead of the standard phosphate buffer solution, one uses neutralizing liquid as as a diluent (see para. 9) of laboratory or industrial production);
- The use of specific inactivators (for example, the use of β-lactamase for certain β-lactam antibiotics and p-aminobenzoic acid (PABA) for sulfanilamides) that neutralize antimicrobial effect of the specimen, but not depresse the growth of microorganisms that contaminate the PPs;

- The use of non-specific inactivators for preparations with preservatives. After validation, the buffer solution and (or) culture medium may be added with Tween-80, soy or egg lecithin, etc.;
- For the preparations that are soluble in water or in isopropyl myristate (IPM), the method of membrane filtration is used, followed by washing the filters.

3.5.1. Inactivation of some antibiotics

To inactivate penicillins and cephalosporins, regardless of their dosage forms, in buffer solution used for dissolving, suspending or emulsifying the sample, as well as in culture media prior to their us one adds the sterile solution of β-lactamase in the amount specified in the regulations.

3.5.2. Inactivation of sulfanilamides

To inactivate sulfanilamides, irrespective of their dosage form, in a buffer solution used for dissolving, suspending or emulsifying the sample, as well as in culture media, if necessary, prior to sterilization, one adds PABA at 0.05 g/L of medium, if the antimicrobial effect cannot be eliminated by dilution.

3.5.3. Inactivation of preservatives found in PPs

To inactivate the preservatives found in some pharmaceutical preparations, in buffer solution where the sample is emulsified and in the culture media prior to sterilization, one adds nonspecific inactivators as follows: 3% Tween-80 and 0.3% lecithin (egg or soya bean) of medium volume. If the preparation has more than 2 preservatives of different chemical structure, the medium is added with 3% Tween-80, 0.3% lecithin, 0.1% L-histidine and 0.5% sodium thiosulphate simultaneously. The inactivators of antimicrobial effect of PPs are listed in Table 4.

Table 4: The inactivators of antimicrobial effect of PPs

Chemical compounds	Inactivants or method
Glutardialdehyde, mercurials	sodium hydrogen sulfite (sodium bisulfite)
Phenols, alcohols, aldehydes, sorbates	dilution

Aldehydes	Glycine
Quaternary ammonium compounds (QACs), bisbiguanides, parahydroxybenzoates (parabens)	Lecithin
QACs, iodinated compounds, parabens	Polysorbate, Tween-80
Mercurials	Thioglycollate
Mercurials , halogens, aldehydes	Thiosulphate
Ethylene diamine tetraacetate (EDTA)	Mg(II) or Ca(II) ions

If the method of membrane filtration cannot be used in the PP quality analysis, and all the above methods to eliminate the antimicrobial effect of a specific test strain of microorganism are ineffective, this kind of tests is not performed.

4. PPs sampling

From each test series of PPs, one recovers samples required in accordance with the category of the preparation, from a sufficient number of different packages (not less than 3–10).

For liquid- or solid based aerosols, one collects 10 containers, for transdermal patches – 10 patches.

In some cases (if high cost of the preparation and/or small volume of the series) the sample may be reduced in some cases up to 2-3 g (mL). The reduction of the number of samples with the name of the test method should be justified and approved in the established order by the applicable standards.

4.1. Solid dosage forms*:*

- 10.0 g of sample – to determine the total amount of bacteria and fungi in 1 g of the preparation, for the test of the absence of *P. aeruginosa, S. aureus* and *E. coli*;

- 25.0 g of sample – to determine the bacteria of the genus *Salmonella*;

- 10.0 g of sample – for quantitative determination of Enterobacteriaceae resistant to bile.

4.1.1. Tablets, dragees, granules, powder, etc.

10.0 g of the sample are dispersed (if necessary) and transferred in 100 ml of buffer solution. One then carries out quantitative and qualitative determination of microorganisms.

4.1.2. Capsules

10.0 g of sample are transferred in 100 mL of buffer solution that contains not more than 5 % Tween-80 and heated up to 40 ºC max. After suspending the capsules in the buffer solution, one carries out the quantitative and qualitative determination of microorganisms.

4.2. Semi-solids*:*

- 10.0 g of sample – to determine the total amount of bacteria and fungi, for the test for the absence of *P. aeruginosa, S. aureus, E. coli* in 1 g of the preparation;

- 10.0 g of sample – for the test for the absence in 1 g or the quantitative determination of Enterobacteriaceae resistant to bile.

4.2.1. Ointments, liniments, creams, suppositories, easily mixed with water

10.0 g of sample are placed in a sterile flask that contains 100 mL of buffer solution and sterile glass beads with a 5-6 mm diameter. The mixture is heated in water bath at a temperature not higher than 40 °C and is shaken vigorously until a homogeneous emulsion, which is used for quantitative and qualitative determination of microorganisms.

4.2.2. Ointments, liniments, creams, suppositories, hard water-miscible

10.0 g of sample are mixed with sterile Tween-80, which quantity should not exceed 1/2 of the sample volume (here 5 g). The mixture is heated in water bath or in an oven up to a temperature not higher than 40 °C (in exceptional cases up to 45 °C) and is gently stirred. And the heating time should not exceed 30 min. One than adds the required amount of pre-heated to an appropriate temperature sterile phosphate buffer solution with glass beads. The mixture is gently stirred to obtain homogeneous emulsion diluted at 1:10, which is used for qualitative and quantitative

determination of microorganisms. One can use other technical means, methods of homogenization observing aseptic rules and temperature control modes.

***4.3. Liquid dosage forms**:*

- 10.0 mL of sample are examined to determine the total amount of microorganisms and fungi in 1 mL of the preparation, for the test of the absence of *E. coli, P. aeruginosa, S. aureus*;

- 25.0 mL of sample – for the test of the absence of bacteria of the genus *Salmonella*;

- 10.0 mL of sample – for quantitative and qualitative determination of Enterobacteriaceae resistant to bile.

4.3.1. Solutions, suspensions, syrups, medicines

10.0 mL of sample are transferred in 90 mL of buffer solution, mixed, and one carries out the quantitative and qualitative determination of microorganisms.

4.3.2. Solutions in oils, emulsions

10.0 g of sample are placed in a sterile flask that contains 90 mL of buffer solution with Tween-80 which quantity should not exceed 5% and glass beads. The mixture is heated in water bath up to a temperature not higher than 40 °C and is shaken vigorously until a homogeneous emulsion, which is used for the quantitative and qualitative determination of microorganisms.

4.4. Aerosols

4.4.1. Alcohol-based aerosols and solids

3.0 g of sample are transferred (after propellant evaporation) in 30 mL of buffer solution, mixed and the quantitative and qualitative determination of microorganisms is carried out. Not less than 1.0 g of sample for respiratory therapy is used for the test on the absence of Enterobacteriaceae resistant to bile.

4.4.2. Oil-based aerosols

3.0 g of sample are transferred (after propellant evaporation) in a sterile flask that contains 30 mL of buffer solution with Tween-80 which quantity should not exceed 5% and glass beads. The mixture is heated in water bath up to a temperature

not higher than 40 °C and is shaken vigorously until a homogeneous emulsion, which is used for the quantitative and qualitative determination of microorganisms. Not less than 1.0 g of sample for respiratory therapy is used for the test on the absence of Enterobacteriaceae resistant to bile.

4.5. Transdermal patches

When sampling transdermal patches, one uses the sample that contains 10 units. Using special instruments, from each of 10 patches, one removes a ptotective film. If necessary, the patch is cut off with sterile scissors to smaller pieces which are then transferred in a 1,000 mL flask that contains 500 mL of sterile buffer solution and glass beads (conditional dilution at 1:50 rate). The flask is heated in water bath up to 40 ^{0}C max, is shaken vigorously during 30 min.

50 mL of the swab received are used for the quantitative and qualitative determination of microorganisms by means of membrane filtration and the test for the absence of *P. aeruginosa u S. aureus.*

If it is known the patch has antimicrobial effect, the diluent is added with an appropriate inactivator (Tween-80 and/or Lecithin).

If the swab from the transdermal patches cannot be used for the determination by means of membrane filtration, one uses the method of direct inoculation in culture media using the 1:50 dilution**.**

4.6. Herbal medicinal preparations

Herbal medicinal preparations (HMPs) include preparations produced from one kind or some kinds of raw material and sold in secondary (consumer) packages (packs, bags, briquettes, etc.).

From each series of the herbal medicinal preparation to be controlled, one recovers an integral sample from which one separates a sample to determine the microbiological purity – at least 5 unopened consumer packs of a total weigh not less than 50 g.

Prior to the test, the consumer packs are opened using sterile instruments, the samples are taken in equal quantities, mixed and transferred in a sterile flask.

For the quantitative determination of aerobic microorganisms and fungi, the sample of 10.0 g weigh (fruits, bark, roots and rhizomes, buds, etc.) or 2.0 g (grass, leaves, flowers and others with a large coefficient of water absorption) are transferred in a sterile flask. If the sample mass of 10.0 g, the flask is filled with 100 mL of 0.9 % sterile solution of sodium chloride. The flask with the sample tested is shaken on a shaker or a shaking apparatus for at least 15 min. The swab received is considered as a 1:10 dilution rate. To the sample of a 2.0 g mass, the flask is added with 200 mL of 0.9 % sterile solution of sodium chloride. The swab received is diluted at a 1:100 rate.

If the sample is hard wetted, the flask is added with surface active substance – sterile Tween-80 at 0.1 % of the solution volume.

From the HMPs received that correspond to 1:10 or 1:100 dilutions, one prepares serial tenfold dilutions in the same diluent. The quantitative determination of aerobic bacteria and fungi is carried out by an agar dish method as specified in Section 5.

The test for the absence of *E. coli, Salmonella* and Enterobacteriaceae resistant to bile is carried out in accordance with the methods specified in Section 6.

4. Methods of quantitative determination of aerobic microorganisms

Depending of the PP nature and its physical and chemical properties, one uses one of the variants of the agar dish method (deep, two layer, surface, modified deep), method of membrane filtration or a test tube method of the most probable numbers.

5.1. Agar dish methods

To culture microorganisms, one uses agarized culture media: casein soya bean digest agar or medium No. 1 that is dry for the control of microbial contamination – to grow bacteria, Sabouraud glucose agar or medium No. 2 that is dry for the control of control of microbial contamination – to grow yeasts and moulds.

For each sample dilution, one uses not less than 2 Petri dishes with specific medium.

5.1.1. Deep method

In a sterile Petri dish of a 90 mm diameter one adds 1 mL of a test sample prepared for the analysis. One then adds 15–20 mL of melted and cooled to a 42.5±2.5 C temperature of sterile agarized culture medium and mixes quickly by circular movements. If a bigger diameter of Petri dishes, the medium volume is increased to 20–25 mL. The agar solidified, the dishes are turned down and the inoculations are incubated.

5.1.2. Two-layer method

15-20 mL of the melted agarized sterile culture medium is added in a sterile Petri dish of a 90 mm diameter and is left for solidification. If a bigger diameter of Petri dishes, the medium volume is increased. The agar surface in the dish is dried out.

1 mL of the sample prepared for the analysis is added in a test tube with 4 mL of corresponding melted and cooled to a 42.5±2.5 C temperature culture medium. The test tube content is quickly mixed. The content is then poured on the surface of the solidified and dried out agar in the Petri dish, by distributing uniformly the upper layer of the medium with circular movements. After solidification, the dish is turned down and placed in an oven for uncubation.

5.1.3. Surface method

15-20 mL of melted and cooled to a 42.5±2.5 C temperature sterile culture media are added in each sterile Petri dish of a 90 mm diameter and are left for solidification. If a bigger diameter of Petri dishes, the medium volume is increased. The agar surface in the dish is dried out in an oven or a laminar flow hood.

0.1 mL of the sample prepared for the analysis is placed with a sterile pipette on agar and uniformly distributed with a spatula on the medium surface.

The dishes are turned down and placed in an oven for incubation.

5.1.4. Modified deep method

1.0 mL of the sample prepared for the analysis is added in a sterile Petri dish of a 90 mm diameter. One then adds 7-10 mL of melted and cooled to a 42.5±2.5 C temperature culture medium and mixes quickly by circular movements. After solidification, the dishes are turned down and placed in an oven for uncubation.

5.1.5. Recording and interpretation of the results received by agar dish methods

The inoculations are observed every day. The colonies are counted every 48–72 hours (preliminary result) and in 5 days (final result).

To receive reliable results, the dishes are selected where the number of bacteria does not exceed 250, and the fungi colonies – 50. If the results of the two subsequent dilutions revealed that the number of colonies on the dishes is within the limits above, the results are counted based on a poorer dilution.

If approximately more than 250 colonies of bacteria or more than 50 colonies of fungi grew on the dishes, one makes a number of subsequesnt dilutions of the sample by using an appropriate value for inoculation.

If on casein soya bean digest agar (or medium No. 1) one detected additionally fungi colonies, they are summeraized with the number of bacteria and the total number of aerobic microorganisms which is stipulated for each PP category is determined.

If in the culture medium there is no growth of microorganisms, the results are noted in the test log as follows: when inoculating the PP at a 1:10 dilution – "1 g (or 1 mL) of a pharmaceutical preparation contains less than 10 bacteria (or fungi)"; when inoculating the PP at a 1:100 dilution – "1 g (or 1 mL) of a pharmaceutical preparation contains less than 100 bacteria (or fungi)", etc.

The number of microorganisms *(N)* in 1 g or in 1 mL is counted by the formula as follows:

$$N = \frac{\sum c}{n} \cdot d \cdot 10,$$

where: *c* is the number of colonies on all Petri dishes;

n is the number of Petri dishes;

d is the sample dilution coefficient;

10 is the coefficient of recalculation of the passage in a dish in the amount of 0,1 mL.

*Example***.** When inoculating 1.0 mL of the sample, from the 10^{-2} dilution 168 and 215 colonies grew on two dishes:

$$N = \frac{168 + 215}{2} \cdot 1 \cdot 10^2 = 191.50 \cdot 10^2 = 1.9 \cdot 10^4.$$

The result received is rounded up to 2 meaning numbers – 19,000 and is written as $1.9 \cdot 10^4$ of colony-forming units (CFU).

If it is necessary to count the total number of microorganisms (bacteria and fungi in total) in 1 g or in 1 mL of a pharmaceutical preparation, one should add the number of aerobic bacteria and the number of fungi.

Notes.

Due to the fact the HMPs that represent pjarmaceutical preparations or their parts (leaves, flowers, grass, fruits, seeds, bark, roots and rhizomes, etc.) are nonhomogenous as for the number of aerobic bacteria and fungi, the limits of allowable microbial contamination of the herbal medicinal preparations are interpreted as follows:

- If the number of microorganisms in 1 g does not exceed 10^5 CFU, the maximum allowable concentration is $5 \cdot 10^5$ CFU/g;
- If the number of microorganisms in 1 g does not exceed 10^7 CFU, the maximum allowable concentration is $5 \cdot 10^7$ CFU/g, etc.

For other categories of pharmaceutical categories (except for HMPs), the limits of allowable microbial contamination are interpreted as follows:

- If the number of microorganisms in 1 g or in 1 mL does not exceed 10^2 CFU, the MAC is $2 \cdot 10^2$ CFU/g or CFU/mL;
- If the number of microorganisms in 1 g or in 1 mL does not exceed 10^3 CFU, the MAC is $2 \cdot 10^3$ CFU, etc.

The variants of dish agar method (deep, two-layer, and deep modified) may be used for the test of different pharmaceutical preparations regardless of the level of microbial contamination. It is preferable to use the surface agar method when testing the PP with a high level of microbial contamination. In order to reduce the period of receiving the results of qualitative determination of bacteria and fungi, which colonies have a tendance to crowding, a modified agar method of inoculation is used.

5.2. Method of membrane filtration

Method of membrane filtration is used for the quantitative and qualitative determination of microorganisms in PPs that have or do not have antimicrobial effect in

particular for solutions and water soluble PPs, as well as for fat-containing preparations that are soluble in water or in isopropyl myristate (IPM).

5.2.1. Conditions of the test performance

The construction of the membrane filtration should allow an easy removal of a filter, with its subsequent passage in culture media. One uses the membrane filters with pores diameter of 0.45 mcm max which are able to catch efficiently microorganisms; that fact is to be validated. The membrane material should be selected so the components of the test preparation do not affect on its efficiency. Filters of cellulose nitrat are used for water, oil, and diluted alcohol solutions (less than 30 %), of cellulose acetate – for alcohol solutions (more than 30 %), acids, alkali. Membrane filtration is carried out under aseptic conditions using vacuum.

5.2.2. Test procedure

As a rule, the sample is solved in buffer solution at a 1:10 ratio. Washing liquid is first introduced in a funnel of a filtering device (appr. 5 mL) to wet the filter. One adds a required amount of the preparation corresponding to 1 g of a test sample, and filters it immediately. If an antimicrobial effect of the PP, in order to wash the membrane, one uses 0.9 % solution of sodium chloride or liquids below (No. 1, No. 2, No. 3); for that not less than 3 portions of 100 mL of appropriate sterile washing liquid pass through the filter. If necessary, to the washing liquid, one can add suface active substances (f.ex. Tween-80) or inactivators with an antimicrobial effect. Not more than 500 mL of the washing liquid may be passed through one membrane.

If the method is validated, it is allowed to use less than 3 portions of washing liquid to wash the membranes.

In order to determine if the membranes are washed of the filtered preparation with an antimicrobial effect, after solution filtration, the last portion of the washing liquid is added with 1 mL of the suspension of the test-strain of microorganisms of the cultures that correspond to a category of test sample. The number of each microorganism entered separately should not exceed 100 CFU/mL.

The growth of the test-strains in the filters proves the absence of antimicrobial effect of the pharmaceutical preparation. If the antimicrobial effect stays, one uses specific or nonspecific inactivators or increases the volume of the washing liquid.

The swab from transdermal patches passes through each membrane filters by 50 mL (that corresponds to 1 patch).

The filtration finished, the membranes are subcultured in appropriate culture media poured in Petri dishes or vials with liquid culture media. The dishes with filters are turned down. The cultures in the dishes and in the vials are incubated under standard conditions.

5.2.3. Recording and interpretation of the results

The colonies are counted every 48–72 hours (preliminary result) and in 5 days (final result). The dishes are selected where the number of bacteria on the filters does not exceed 100, and the fungi colonies – 50, and the number of microorganisms per 1.0 г (1.0 mL) of the sample or per 1 patch is countered. If the filter contains a lot of microorganisms, it is required to make a number of subsequent dilutions of the sample and select the appropriate one.

The results on liquid culture medium are taken into account in accordance with Section 6.

5.2.4. Liquids to wash the filters

To wash the filters, one can use any sterile liquid that does not suppress the growth of microorganisms:

- 0.9 % solution of sodium chloride, pH 7.0±0.2 (after sterilization);
- Liquid No. 1: 1 g of fermented peptone is dissolved in 1,000 mL of purified water, filtered and centrifuged for clarification, poured in vessels and sterilized; pH 7.0±0.2;
- Liquid No. 2: 1 mL of Tween-80 is added to 1,000 mL of Liquid No. 1, poured in vials and sterilized. pH value after sterilization is 6.9±0.2. Liquid No. 2 is used if the preparation contains oil;

- Liquid No. 3: 5 g of meat peptone, 3 g of meat extract and 10 g of Tween-80 are dissolved in 1,000 mL of purified water, poured in vials and sterilized; pH after sterilization is 6.9±0.2.

5.3. Method of the most probable numbers (MPN)

The MPN method is used for the test of PPs with a low level of microbial contamination, as well as in the cases, if the use of other methods is impossible. The MPN method is less sensible and precise in comparison with a dish agar method or a method of memebrane filtration, and it is used only to determine the total number of bacteria as the results received when determining the total number of fungi especially moulds are deemed to be non-reliable.

5.3.1. Test procedure

The test sample is prepared as solution, suspension or emulsion at 1:10, 1:100, 1:1,000 dilution rates using an appropriate solvent. Liquid culture medium is poured in 12 sterile tubes of 9 mL volume each. Test tubes are put on a rack: 4 raws with 3 test tubes in each.

The 1st raw of test tubes is added with 1 mL of the test sample at a 1:10 dilution rate, the 2nd – with 1 mL at a 1:100 dilution rate, the 3rd – with 1 mL at a 1:1,000 dilution rate. Test tubes of the 4th raw are filled with 1 mL of a diluent which is used for dissolving, suspending and emulgating the sample. The inoculations are incubated under standard conditions during 3 days maximum.

5.3.2. Recording and interpretation of the results

The total number of test tubes in the 1st, 2nd, and 3rd raws are noted, in which the growth of microorganisms is visible. The medium in test tubes of the 4th raw (control over diluent) should stay sterile. The received three-figure number corresponds to the most probable number of vitable microorganisms in 1.0 g or in 1.0 mL of a pharmaceutical preparation (Table 5).

Table 5: The most probable number of microorganisms

Number of test tubes in each raw in which the growth is observed			The most probable number of microorganisms in 1 g (mL) of the preparation
Volume of the preparation in a test tube, in g (mL)			
0.1	**0.01**	**0.001**	
0	0	0	less than 3
0	0	1	3
0	1	0	3
0	1	1	6.1
0	2	0	6.2
0	3	0	9.4
1	0	0	3.6
1	0	1	7.2
1	0	2	11
1	1	0	7.4
1	1	1	11
1	2	0	11
1	2	1	15
1	3	0	16
2	0	0	9.2
2	0	1	14
2	0	2	20
2	1	0	15
2	1	1	20
2	1	2	27
2	2	0	21
2	2	1	28
2	2	2	35
2	3	0	29
2	3	1	36
3	0	0	23
3	0	1	38
3	0	2	64
3	1	0	43
3	1	1	75
3	1	2	120
3	1	3	160
3	2	0	93
3	2	1	150
3	2	2	210
3	2	3	290
3	3	0	240
3	3	1	460
3	3	2	1100
3	3	3	more than 1,100

Example. In the 1st raw, the growth of microorganisms is noted in 3 test tubes, in the 2nd – in 2 test tubes, in the 3rd – in 1 test tube. The number received "321" according to Table 5 corresponds to the number "150".

So the most probable number of bacteria in 1 g or 1 mL of the test sample is 150. If the results cannot be determined precisely due to the nature of the test preparation (medium turbidity, change in its color, etc.), the culture is subcultured in the appropriate liquid or agaraised medium in order to be sure in the presence of the growth of microorganisms.

6. Determination of separate types of microorganisms

The test includes the use of selective and diagnostic culture media.

6.1. Enterobacteriaceae resistant to bile

6.1.1 Test for the absence of Enterobacteriaceae resistant to bile (qualitative method)

To restore the vitability of microorganisms, one uses the pre-incubation of the sample in liquid culture medium. For that purpose, 10.0 g or 10.0 mL of the test sample are transferred in 100 mL of casein soya bean digest broth (or medium No. 8), mixed and incubated at a 22.5±2.5 ºC temperature for 2 hours, but not more than for 5 hours. The incubation finished, one remixes the content of the vial in which the vitability of microorganisms (homogenate A) was restored, and transfers 10 mL (the quantity that corresponds to 1 g or 1 mL of the sample) in 100 mL of the enrichment medium (Mossel broth). The inoculations are incubated for 24–48 hours under standard conditions. If the growth presence, the culture is subcultured with an inoculation loop in the Mossel agar or medium No. 4 which is incubated for 18–24 hours.

If in Mossel agar typical colonies of Enterobacteriaceae (Table 7) are detected which morphological and tinctorial properties represent non–spore-forming gram-negative bacilli not containing cytochrome oxidasey enzyme (see para.8.1), one considers the test sample is contaminated with Enterobacteriaceae resistant to bile.

6.1.2. Quantitative determination of Enterobacteriaceae resistant to bile

For inoculation, one uses 3 test tubes with 9 mL of Mossel broth in each. Homogenate A in the volume of 1 mL (corresponds to 0.1 g or 0.1 mL of the sample) is placed in the 1st tube, mixed thourougly, and 1 mL (corresponds to 0.01 g or 0.01 mL of the sample) is transferred in the 2nd test tube, remixed and 1 mL (corresponds to 0.001 g or 0.001 mL of the sample) is transferred in the 3rd test tube by changing a pipette after each stage. The inoculations are incubated for 24-48 hours.

In order to confirm the absence of Enterobacteriaceae resistant to bile, one subcultures the cultures with ainoculation loop from each test tube with a visible growth in Mossel agar (medium No. 4) and Petri dishes are incubated for 18–24 hours. One carries out a microscopique examination of the colonies detected in the dense medium. The detection of non–spore-forming gram-negative bacilli proves the presence of Enterobacteriaceae resistant to bile in a PP. The most probable number of Enterobacteriaceae resistant to bile in 1 g or 1 mL of the sample is determined by Table 6.

Table 6: Interpretation of results

Volume of the test sample			The most probable number of bacteria in 1 g (mL) of the sample
0.1 g (mL)	0.01 g (mL)	0.001 g (mL)	
1 mL of homogenate A	1 mL of homogenate A at a 1:10 dilution rate	1 mL of homogenate A at a 1:100 dilution rate	
+	+	+	more than 10^3
+	+	-	From 10^2 to 10^3
+	-	-	From 10^1 to 10^2
-	-	-	Less than 10^1

Notes: + presence of the growth; – absence of the growth

6.2. Escherichia coli bacteria

6.2.1. Test for the absence of E. coli bacteria (qualitative method)

10 g of the test sample dissolved or diluted with sterile phosphate buffer solution at a 1:10 dilution rate are transferred at the amount of 10 mL (corresponds to 1 g or 1 mL of the tested PP) in 100 mL of casein soya bean digest broth (or medium No. 8), mixed and incubated for 18–24 hours. If the growth presence, 1 mL of the vial content is transferred in 100 mL of MacConkey broth (or medium No. 3) and incubated for 24–48 hours at a 43±1 ºC temperature.

If the growth presence in the broth, one carries out subcultures the culture in MacConkey agar or medium No. 4 usin a bacteriological loop. The inoculations are incubated for 18–48 hours (MacConkey agar) or for 18–24 hours (medium No. 4). If after incubation, in the dense culture media one reveales colonies that are typical for *E. coli* (see Table 7), they are microscoped. If the small gram-negative bacilli

are found in smears, some typical colonies are subcultured in test tubes on a slope casein soya bean digest agar or medium No. 1 and incubated for 18–24 hours to accumulate pure culture of microorganism.

To identify the bacteria found, one uses biochemical tests for cytochrome oxidasey enzyme (see para.8.1), indole (see para.8.2) and the possibility to remove sodium citrate. For that purpose, from test tubes with pure culture, one carries out subculture in Simmons's agar (or medium No. 14) and casein soya bean digest broth (or medium No. 15). In 18–24 hours of incubation, one notes the growth of bacteria or their absence in Simmons's agar (or medium No. 14). The removal of citrate is specified by the deviation of the medium pH to an alkali side (change in color of the medium from green to blue). The presence of indole is determined by the appearance of a red ring on the surface of the casein soya bean digest broth (or medium No. 15) when adding Kovac's reagent.

If during the test, one finds typical gram negative bacilli not containing cytochrome oxidasey enzyme that do not remove sodium citrate and form indole, one considers the PP is contaminated with *E. coli* bacteria.

6.2.2. Quantitative determination of E. coli bacteria

Quantitative determination of *E. coli* bacteria is carried out the same way as the quantitative determination of Enterobacteriaceae resistant to bile (see para. 6.1.2) by subculturing their from homogenate A in test tubes with MacConkey broth (or medium No. 3). If the growth presence in test tubes (see Table 7), from each test tube, one subcultures the culture with an inoculation loop in MacConkey agar or medium No. 4. The inoculations are incubated under standard conditions for 24–48 hours (MacConkey agar) or for 18–24 hours (medium No. 4).

If in media above typical colonies of bacteria (see Table 7) are detected which morphological and tinctorial properties represent the gram-negative bacilli that do not contain cytochrome oxidasey enzyme, do not remove sodium citrate and form indole, one considers the PP is contaminated with *E. coli* bacteria. The most probable number of *E. coli* cells in 1 g or in 1 mL of the test sample is determined by Table 6.

6.3. Test for the absence of bacteria of the genus Salmonella

10 (25) g or 10 (25) mL of the test sample are transferred in 100 (225) mL of casein soya bean digest broth (or medium No. 8), mixed and incubated for 18–24 hours. Mixing finished, 0.1 mL is transferred in 10 mL of an accumulative broth for bacteria of the genus *Salmonella* – Rappoport-Vassiliadis medium – and incubated under standard conditions for 18–24 hours. Incubation finished, one subcultures the culture with an inoculation loop in one of two dense diagnostic media: Xylose lysine deoxycholate agar or Bismuth sulfite agar (medium No. 5) which are then incubated for 48 hours.

If in the media above one finds colonies typical for bacteria of the genus *Salmonella* (see Table 7), one fulfills microscopique examination. If in the smears one finds gram-negative bacilli, typical colonies are subcultred in the slope triple sugar–iron–agar (or medium No. 13), by placing a large amount of the culture with an inoculation loop first on a slope part of agar, and then by a prick in the column, not touching the test tube bottom. In 24 hours of incubation under standard conditions, one notes the change in color from red to yellow at the bottom of the culture medium (glucose fermentation). In the slope part of agar, the medium color does not change (the absence of sucrose and lactose fermentation). The blackening of the medium proves the formation of hydrogen sulfide, a typical sign of the most bacteria of the genus *Salmonella*. At the same time, one determines the presence of cytochrome oxidasey enzyme (see para.8.1), as well as other biochemical and serological tests, if additional confirmation is required.

If in the sample one finds bacteria which are typical by their cultural, morphological, and tinctorial properties (see Table 7), not containing cytochrome oxidasey enzyme, not fermenting sucrose and lactose and generating hydrogen sulfide, one considers the pharmaceutical preparation is contaminated with bacteria of the genus *Salmonella*.

6.4. Test for the absence of Pseudomonas aeruginosa bacteria

The test sample solved or diluted by sterile buffer solution at a 1:10 dilution rate is transferred at the volume of 10 mL (corresponds to 1 g or 1 mL) in 100 mL of liquid culture medium (casein soya bean digest broth or medium No. 8), mixed and и incubated under standard conditions for 24-48 hours. Incubation finished, if the presence of the growth, one subcultures the culture with an inoculation loop in the selective culture medium to identify Pseudomonas aeruginosa (Cetrimide agar or Cetylpyridinium chloride (CPC) agar – medium No. 16). The inoculations are in-

cubated under standard conditions for 24-48 hours. The separate colonies of microorganisms which tinctorial and morphological properties are gram-negative bacilli are subcultured in agar to identify blue-green pigment of pyocyanin (or medium No. 9). The inoculations are incubated for 24–48 hours.

To confirm if the selected bacteria belong to *P. Aeruginosa*, one determines the presence of cytochrome oxidasey enzyme (see para.8.1) and the possibility of the selected microorganisms to grow in the casein soya bean digest broth (or medium No. 8) at a 42±1 ºC temperature for 18–24 hours.

When testing the qualtity of of transdermal patches, 10 patches are placed in 500 mL of phosphate buffer solution and shaken carefully for at least 15 min.

The liquid received at the amount of 50 mL is passed through a sterile filter made of cellulose nitrate with a pore diameter of 0.45 mcm which is then transferred in 100 mL of casein soya bean digest broth (or medium No. 8). The inoculations are incubated for 24–48 hours. The incubation finished, if the presence of the growth, one subcultures the colonies with an inoculation loop in selective media – Cetrimide Agar or CPC agar. The further identification is held as specified above.

If in the sample one detects bacteria typical for Pseudomonadaceae by their morphological and tinctorial properties (see Table 7) that form blue-green pigment of pyocyanin, contain cytochrome oxidasey enzyme and grow at a 42±1 ºC temperature, one considers the PP is contaminated with *P. aeruginosa* bacteria.

6.5. Test for the absence of Staphylococcus aureus bacteria

The test sample solved or diluted with sterile buffer solution at a 1:10 dilution rate is transferred at the amount of 10 mL (which corresponds to 1 g or 1 mL of the sample) in 100 mL of casein soya bean digest broth or medium No. 8, mixed and incubated for 24–48 hours. If the presence of the growth, the colonies are subcultured with a inoculation loop in a Mannit-Kochsalz-Agar (or medium No. 10) and incubated under standard conditions for 24-48 hours.

The appearance of typical golden-yellow colonies (see Table 7) after the incubation finishes, surrounded by yellow spots at the section with Mannit-Kochsalz-Agar proves the growth of *S. aureus* that ferments Mannit-Kochsalz-Agar. One

carries out microscopique examination of typical colonies. If the in the smears one finds gram-positive cocci arranged in clusters, one subcultures the colonies in casein soya bean digest agar (or medium No. 1), then incubates under standard conditions for 18–24 hours. To identify the bacteria, one carries out the test for the presence of coagulase (see para.8.3).

When testing the microbiological purity of transdermal patches, 10 patches are placed in 500 mL of phosphate buffer solution and gently shaken for at least 15 min.

The liquid received at the volume of 50 mL is passed through a sterile membrane filter made of cellulose nitrate with a pore diameter of 0.45 mcm which is then transferred in 100 mL of casein soya bean digest broth (or medium No. 8) and incubated for 24–48 hours. Incubation finished, if the presence of the growth, one subcultures cultures with a loop in Mannit-Kochsalz-Agar (or medium No. 10) to identify *S. aureus*. The inoculations are incubated for 48 hours.

If in the sample one detects bacteria (see Table 7) typical on cultural, morphological, and tinctorial properties that contain coagulase, remove mannite, one considers the PP is contaminated with *S.aureus*.

6.6. Test for the absence of Candida albicans fungi

The test sample solved or diluted by sterile buffer solution at a 1:10 dilution rate is transferred at the amount of 10 mL (that corresponds to 1 g or 1 mL of the sample) in 100 mL of Sabouraud broth, mixed and incubated for 3–5 days at a 32.5±2.5 °C temperature. If the presence of the growth, the colonies are subcultured with an inoculation loop in Sabouraud glucose agar (or medium No. 2) and incubated for 24–48 hours at the same temperature.

The growth of white round, convex, smooth and shiny colonies may indicate the presence of *Candida albicans* that is proved during the next identification, one of which stage is microscopique examination (Gram staining) that detects 4–8 mcm gram-positive budding oval or round yeast cells. To identify them, it is possible to use chromogenic medium intended for differentiating *C. albicans* and other species of fungi of genus *Candida*.

If in the sample one finds yeast-like fungi typical by morphological and tinctorial properties (see Table 7) identified as *C. albicans*, one considers the PP is contaminated with the fungi above.

6.7. Cultural, morphological and tinctorial properties of microorganisms

The specific cultural, morphological and tinctorial properties of some microorganisms that are possible contaminants of PPs are given in Table 7.

Table 7: Cultural, morphological and tinctorial properties of microorganisms-contaminants.

Culture media	Colony morphology	Gram staining
Escherichia coli		
MacConkey Broth	Discoloration of medium, turbidity, gas generation	Gram-negative bacilli, with no spores
Medium No. 3	Change in medium color, gas generation	
MacConkey agar	Brick-red colonies, may be surrounded by spots of the precipitated bile	
Medium No. 4	Crimson or pink colonies with a metallic shine, surrounded by spots of crimson color	
Mossel Agar	Red colonies surrounded by red spots of precipitation	
Salmonella spp.		
Rappaport-Vassiliadis Broth	Medium turbidity with the color retention or absence of visible growth	Gram-negative bacilli, with no spores
Xylose lysine deoxycholate agar	Red colonies with/without black center	
Bismuth sulfite agar (or medium No. 5)	Black colonies with anthracite shine, medium under colonies is painted black	
Mossel Agar	Red colonies surrounded by red spots of precipitation	
Pseudomonas aeruginosa		
Casein soya bean digest broth (Medium No. 8)	Turbidity, surface growth in the form of a film	Gram-negative bacilli, with no spores
Cetrimide Agar	Greenish colonies, green under UV light	
Medium No. 16 (CPC agar)	Greenish colonies, green under UV light	
Agar for detection of Pyocyanin, Medium No. 9	Blue-greenish colonies, blue-green under UV light	
Staphylococcus aureus		
Casein soya bean digest broth (Medium No. 8)	Unform turbidity	Gram-positive cocci arranged in clusters
Mannit-Kochsalz-Agar (or Medium No. 10)	Golden-yellow colonies surrounded by white spots	

Staphylococcus epidermidis		
Mannit-Kochsalz-Agar (or Medium No. 10)	White colonies, absence of yellow spots around colonies	Gram-positive cocci arranged in clusters
Candida albicans		
Sabouraud broth	Near-bottom growth	4–8 mcm gram-positive budding oval or round yeast cells
Sabouraud agar (Medium No. 2)	White, round, convex, smooth and shiny colonies	

7. Test repeating

If necessary, when detecting the contamination of a PP, one repeats the test part which results do not meet the requirements of regulatory documents. The analysis is performed at a twice amount of the preparation samples.

8. Biochemical tests for identification of microorganisms

8.1. Test for the presence of the cytochrome oxidasey enzyme (oxidase test)

Reagent: 1% solution of N,N-Dimethyl-1,3-phenylenediamine dihydrochloride. The solution is kept at 2-8 °C temperature in vials of neutral absorbent glass for an established validated shelf life. The solution should be colorless.

A strip of filter paper is wetted with a reagent. With a platinum loop or a glass rod, one applies a 24-hour pure culture of the test bacteria grown on casein soya bean digest agar (or medium No. 1). Dark red color appearing during 1 min proves a positive oxidase reaction. A positive control is a test-microorganism of *P. aeruginosa*, a negative one is a test-microorganism of *E. coli* (staining is absent).

8.2. Test for indole presence

Kovac's reagent:

- Amylic or isoamil alcohol - 75 mL
- *para*-Dimethylaminobenzaldehyde - 5 g
- Hydrochloric acid, concentrated - 20 mL

The appropriate quantity of *para*-Dimethylaminobenzaldehyde is dissolved in amylic or isoamil alcohol when heating in water bath at a 52.5±2.5 ºC temperature, cooled and hydrochloric acid is added drop by drop. The solution is kept in a place protected against sunlight at a 2–8 ºC temperature. The reagent should be yellow. If improper storage, the reagent color becomes brown, and the reagent is unusable.

In a test tube with casein soya nean digest broth (or medium No. 15), in which the daily studied culture grew, one adds 0.5 mL of Kovac's reagent and slightly shakens it. In 3-5 minutes in the presence of indole, one observes the appearance of a red ring on the surface of the medium in a test tube. A positive control is a test-microorganism of *E. coli*, a negative one is a test-strain of *S. abony* (staining is absent).

8.3. Test for the presence of a coagulase ferment (coagulase plazma reaction)

Dry citrate rabbit plasma is diluted according to the attached instruction with 0.9% sterile solution of sodium chloride and is poured by 0.5 mL in sterile test tubes. In a test tube with reduced rabbit plasma, one adds 1 inoculation loop of a daily pure culture of identified bacteria grown on casein soya bean digest agar (or medium No. 1). The 2nd test tube is not inoculated (negative control). A positive control is a test-strain of *S. aureus*, a negative one is test-strain of *S. epidermidis*. All test tubes are incubated under standard conditions. The coagulation plasma reaction is noted every hour for 4–6 hours by slightly tilting the test tube without shaking.

If the absence of a positive coagulation plasma reaction, the time of incubation is prolonged to 24 hours to receive final results. The test for the presence of coagulase is considered positive if a plasma bunch is found.

9. Culture media and solutions

To test the qulity of a PP for the microbiological purity, one uses culture media of domestic or foreign production.

In the preparation of culture media in the laboratory, one should strictly adhere to the above recipe, and when using commercial dry culture media – the manufacturing plant's instruction. Indicators and dyes included in the culture media are added as solutions of certain concentration. A required pH value of the culture medium is set at a 22.5±2.5 ºC temperature.

If no other directions in the regulatory documentation, the media are sterilized in an autoclave at a 121 °C temperature for 15 minutes, provided for the sterilization process is validated.

Phospahe buffer solution with sodium chloride and peptone (pH 7.0):

- Potassium Phosphate Monobasic 3.6 g
- Disodium hydrogen phosphate 7.2 g
- Sodium chloride 4.3 g
- Peptone (meat and casein) 1.0 g
- Purified water 1,000.0 mL

Neutralizing fluid

- Tween-80 30.0 g
- Lecithin (egg or soya bean) 3.0 g
- Histidine hydrochloride 1.0 g
- Peptone (meat and casein) 1.0 g
- Sodium chloride 4.3 g
- Potassium Phosphate monobasic 3.6 g
- Disodium hydrogen phosphate 7.2 g
- Purified water 1,000.0 mL

  pH after sterilization 7.6±0.2

Semiliquid agar to store test-microorganisms

- Pancreatic digest of casein 8.0 g
- Sodium chloride 5.0 g
- Microbiological agar 5.0 g
- Purified water 1,000.0 mL

  pH after sterilization 7.0±0.2

Casein soya bean digest agar

- Pancreatic digest of casein 15.0 g
- Papaic digest of soya bean meal 5.0 g
- Sodium chloride 5.0 g
- Microbiological agar 15.0 g
- Purified water 1,000.0 mL

  pH after sterilization 7.3±0.2

An alternative medium of domestic production to grow aerobic bacteria is medium No. 1 to control over microbial contamination, dry; meat-and-peptone agar (MPA); agarized culture media based on fish hydrolysate (FH).

Sabouraud broth

• Meat peptone	5.0 g
• Casein peptone	5.0 g
• Glucose monohydrate	20.0 g
• Purified water	1,000.0 mL
pH after sterilization	5.6±0.2

Sabouraud 4% Glucose Agar

• Peptone (meat or casein)	10.0 g
• Glucose monohydrate	40.0 g
• Biological agar	15.0 g
• Purified water	1,000.0 mL
pH after sterilization	5.6±0.2

An alternative medium of domestic production to grow yeasts and moulds is medium No. 2 (Sabouraud glucose agar) to control over microbial contamination, dried, of different manufacturers.
To increase the selectivity of the medium, in order to prevent the growth of bacteria, prior to sterilization, one adds 50 mg of chloramphenicol (laevomycetin) in 1 liter of the medium, or before filling in Petri dishes, in a melted medium, one adds 0.1 g of benzylpenicillin sodium salt and 0.1 g of tetracycline in 1 L of the medium as sterile solutions.

Enterobacteria Enrichment Broth – Mossel

• Pancreatic digest of gelatin	10.0 g
• Glucose monohydrate	5.0 g
• Dry ox gall	20.0 g
• Potassium Phosphate Monobasic	2.0 g
• Disodium hydrogen phosphate	8.0 g
• Brilliant green	0.015 g
• Purified water	1,000.0 mL
pH	7.2 ± 0.2

The medium is heated to 100 C for 30 minutes followed by fast cooling down.
An alternative medium of domestic production to grow aerobic bacteria is medium No. 3 to control over microbial contamination, dried; of different manufacturers.

Mossel Agar (Crystal violet, Neutral Red, Bile Agar with Glucose)

• Yeast extract	3.0 g
• Pancreatic digest of casein	7.0 g
• Bile salts	1.5 g
• Lactose monohydrate	10.0 g

- Sodium chloride 5.0 g
- Glucose monohydrate 10.0 g
- Microbiological agar 15.0 g
- Neutral Red 0.03 g
- Crystal violet 0.002 g
- Purified water 1,000.0 mL

pH 7.4±0.2

To be boiled. The medium is not autoclaved.
An alternative medium of domestic production to identify Enterobacteriaceae is medium No. 4 (Endo) to control over microbial contamination, dried; of different manufacturers.

MacConkey Broth

- Pancreatic digest of gelatin 20.0 g
- Lactose monohydrate 10.0 g
- Dry ox gall 5.0 g
- Bromocresol purple 0.01 g
- Purified water 1,000.0 mL

pH after sterilization 7.3 ±0.2

An alternative medium of domestic production to enrich Enterobacteriaceae is medium No. 3 to control over microbial contamination, dried, of different manufacturers.

MacConkey Agar

- Pancreatic digest of gelatin 17.0 g
- Peptone (meat and casein) 3.0 g
- Lactose monohydrate 10.0 g
- Sodium chloride 5.0 g
- Bile salts 1.5 g
- Microbiological agar 13.5 g
- Neutral Red 0.03 g
- Crystal violet 0.001 g
- Purified water 1,000.0 mL

pH after sterilization 7.1±0.2

Prior to sterilization is boiled for 1 min, by constantly shaking.
An alternative medium of domestic production to identify Enterobacteriaceae is medium No. 4 (Endo) to control over microbial contamination, dried, of different manufacturers.

Cumulative environment for bacteria of the genus Salmonella (Rappaport-Vassiliadis Broth)

- Soya Peptone 4.5 g
- Hexaqua magnesium chloride 29.0 g
- Sodium chloride 7.2 g
- Potassium phosphate dibasic 0.18 g
- Potassium phosphate monobasic 1.26 g
- Benzal green 0.036 g
- Purified water 1,000.0 mL

pH after sterilization 5.2±0.2

The medium is autoclaved for 15 min. at a 115 ºC temperature.

Xylose, Lisine, Deoxycholate Agar

- Xylose 3.5 g
- L– Lisine 5.0 g
- Lactose monohydrate 7.5 g
- Sucrose 7.5 g
- Sodium chloride 5.0 g
- Yeast extract 3.0 g
- Phenol red 0.08 g
- Microbiological agar 13.5 g
- Sodium desoxycholate 2.5 g
- Sodium thiosulphate 6.8 g
- Ammonium ferric citrate 0.8 g
- Purified water 1,000.0 mL

pH 7.4±0.2

To be boiled. Cooled down to a 50 °C temperature and poured in Petri dishes. The medium is not autoclaved.

Bismuth sulfite agar

- Meat extract 5.0 g
- Meat peptone 10.0 g
- Glucose monohydrate 5.0 g
- Disodium hydrogen phosphate 4.0 g
- Ferrous sulphate 0.3 g
- Brilliant green 0.025 g
- Bismuth sulfite 8.0 g
- Microbiological agar 15.0 g
- Purified water 1,000.0 mL

pH 7.6±0.2

The medium is not autoclaved. The medium prepared is cloudy, of green color.

An alternative medium of domestic production to identify salmonella is medium No. 5 to control over microbial contamination, dried, of different manufacturers.

Casein soya bean digest broth

- Pancreatic digest of casein 17.0 g
- Papaic digest of soya bean meal 3.0 g
- Sodium chloride 5.0 g
- Potassium phosphate dibasic 2.5 g
- Glucose monohydrate 2.5 g
- Purified water 1,000.0 mL

pH after sterilization 7.3±0.2

An alternative medium of domestic production to grow bacteria is medium No. 8 to control over microbial contamination, dried, of different manufacturers.

Cetrimide Agar

- Pancreatic digest of gelatin 20.0 g
- Magnesium muriate 1.4 g
- Potassium sulphate dibasic 10.0 g
- Cetrimide (Cetylpyridinium bromide) 0.3 g
- Microbiological agar 13.6 g
- Glycerine 10.0 mL
- Purified water 1,000.0 mL

pH after sterilization 7.2±0.2

An alternative medium of domestic preparationion to identify P. aeruginosa is CPC (medium No. 16), i.e. agar to identify P. aeruginosa, dried.

CPC agar (medium No. 16)

- Dried fermentation peptone 20.0 g
- Potassium sulphate 7.6 g
- Magnesium sulphate heptahydrate 2.4 g
- Caustic ash 1.0 g
- Phenozan acid 0.2 g
- CPC (N -cetyl pyridinium chloride in aqueous solution) 0.3 g
- Microbiological agar 8.0 g
- Purified water 1,000.0 mL

pH 7.2±0.2

The medium is not autoclaved.

Pseudomonas agar medium for detection of pyocyanin

- Pancreatic digest of gelatin 20.0 g

- Ahydrous magnesium muriate 1.4 g
- Ahydrous potassium sulphate 10.0 g
- Microbiological agar 15.0 g
- Glycerine 10.0 mL
- Purified water 1,000.0 mL

pH after sterilization 7.2 ± 0,2

All the components except for glycerine are dissolved in water, heated, mixed, and boiled for 1 min. One then adds glycerine and sterilizes them.
An alternative medium of domestic production to identify P. aeruginosa is medium No. 9 to control over microbial contamination, dried, of different manufacturers.

Mannit-Kochsalz-Agar

- Dried fermentation peptone 10.0 g
- D-Mannit 10.0 g
- Sodium chloride 75.0 g
- Microbiological agar 15.0 g
- Phenol red 0.025 g
- Purified water 1,000.0 mL

pH after sterilization 74±0.2

An alternative medium of domestic production to identify S. aureus is medium No. 10 to control over microbial contamination, dried, of different manufacturers.

Triple sugar–iron–agar

- Meat extract 3.0 g
- Yeast extract 3.0 g
- Peptone (casein or meat) 20.0 g
- Sodium chloride 5.0 g
- Lactose monohydrate 10.0 g
- Sucrose 10.0 g
- Glucose monohydrate 1.0 g
- Ammonium ferric citrate 0.3 g
- Sodium thiosulphate 0.3 g
- Phenol red 0.025 g
- Microbiological agar 12.0 g
- Purified water 1,000.0 mL

pH after sterilization 7.4±0.2

The medium is poured in test tubes by filling 1/3 of their volume.Sterilization finished, the medium is left to be solidified so a column and a slope form over it.
An alternative medium of domestic production to identify Salmonella is medium No. 13 to control over microbial contamination, dried, of different manufacturers.

Simmons citrate agar

• Sodium chloride	5.0 g
• Ammonium dihydrogen phosphate	0.2 g
• Dipotassium phosphate	1.0 g
• Sodium citrate	1.0 g
• Bromthymol blue	3.0 g
• Microbiological agar	0.08 g
• Purified water	20.0 g
	1,000.0 mL
pH after sterilization	7.2±0.2

An alternative medium of domestic production to identify *E. coli* is medium No. 14 to control over microbial contamination, dried, of different manufacturers.

Culture glucose agar

• Tryptic digest of casein with 150 mg % amino nitrogen	150 mL
• Extract of fodder yeast	5.0 g
• Microbiological agar	20.0 g
• Glucose	5.0 g
• Sodium chloride	5.0 g
• Purified water	до 1,000.0 mL
pH after sterilization	7.2±0.2

To be sterilized at a 121 °C temperature for 15 min.

Meat-and-peptone agar (MPA) with 0.5 % glucose

• Infusion broth	1,000.0 mL
• Sodium chloride	5.0 g
• Dried fermentation peptone	10.0 g
• Microbiological agar	20.0 g
• Glucose	5.0 g
pH after sterilization	7.4±0.1

To be sterilized at a 121 °C temperature for 15 min.

Culture agar with 9 % sodium chloride (salt agar)

• Infusion broth	1,000.0 mL
• Sodium chloride	5.0 g
• Dried fermentation peptone	10.0 g
• Microbiological agar	20.0 g
	5.0 g

- Glucose

  pH after sterilization 7.4±0.1

Culture agar with 9 % sodium chloride may be prepared based on MPA with addition of 9 % sodium chloride.

Trypsin hydrolysate of meat with amino nitrogen 0.60+0.05 %

- Meat (beef) 1,000.0 g
- Potable water 2,000.0 mL
- Cattle pancreas 130.0 g
- Chloroform 1,000.0 mL
- Sodium bicarbonate 13.0 g

  pH after sterilization 7.4±0.1

To be sterilized at a 121 ^{0}C temperature for 15 min.

Blood agar with addition of defibrinated blood

- 2 % meat peptone agar 950.0 mL
- Defibrinated blood 50.0 mL

  pH after sterilization 7.4±0.1

In the melted and cooled to a 45 °C temperature of MPA, one adds defibrinated blood (human, sheep, rabbit), stirs until smooth and pours into Petri dishes.

Preparation of defibrinated blood. Blood is aseptically collected into a sterile vessel, at the bottom of which there are glass beads, plugged and shaken for 20-25 min before deposition of fibrin; liquid, not coagulated portion of blood is then added in the right amount to the MPA cooled to a 45+2 °C temperature.

Urea medium

- Digest of casein, fermented *E. coli* M-17 (amino nitrogen content (6.6±0.6 g/L[a)]) 80.0 mL
- Sodium chloride 5.0 g
- Microbiological agar 10.0 g
- Urea 10.0 g
- Lactose 10.0 g
- Glucose 1.0 g
- Andrade's combined indicator with addition of 0.35 % bromthymol blue)[b)] 40.0 mL
- Purified water up to 1,000.0 mL

  pH after sterilization 7.1±0.1

To be sterilized at 110 °C for 15 min.

[a] Preparation of fermented digest of casein. Micobial suspension is prepared by suspending 24-hours culture of *E. coli* M-17 grown in MPA in 0.9 % solution of sodium chloride. The microbial suspension is brought to 10 IU of turbidity on CO. To 1 L of digest of casein, one adds 3–5 mL of microbial suspension, incubates it for 24 hours at a 37+1 °C temperature and sterilizes it.

[b] Preparation of Andrade's combined indicator. To 400 mL of distilled water, one adds 1 g of fuchsinic acid and 64 mL of 1 M sodium hydroxide solution, maintains 1 day at a 37+1 °C temperature and 2 days at room temperature. One then plugs and stores it in a dark glass bottle at room temperature in a dark place. When preparing Andrade's combined indicator, to 100 mL of Andrade's combined indicator one adds 350 mg of bromthymol blue.

Preuss urea medium

• Trypsin hydrolysate of meat with amino nitrogen 0.60+0.05 % [a]	1,000.0 mL
• Microbiological agar	15.0 g
• Glucose	5.0 g
• Urea (50% aqueous solution)	20.0 mL
• Bromthymol blue	12.0 mL
• pH after sterilization	7.0±0.1

Gauze medium No. 2, agarized

• Hottinger's broth (700mg % amino nitrogen)	30,0 mL
• Dried peptone	5.0 g
• Sodium chloride	5.0 g
• Glucose	10.0 g
• Microbiological agar	30.0 g
• Purified water	up to 1,000.0 mL
pH after sterilization	7.2±0.2

Hottinger's broth

• Hottinger hydrolysate	24.0 g
• Sodium chloride	5.0 g
• Purified water	up to 1,000.0 mL
pH after sterilization	7.2±0.2

Egg yolk salt agar

• Meat peptone agar	850.0 mL
• Sodium chloride	90.0 г
• Egg yok suspension (1 egg yolk per 200 mL of 0.9 % solution pf sodium chloride)	150.0 mL
• Purified water	up to 1,000.0 mL

pH after sterilization	7.2±0.2

It is possible to change the composition of the culture media and to replace materials of animal origin with the components of industrial production provide for the confirmation of the quality and validation of their application for tests for the “Microbiological purity” characteristic.

10. Quality assessment of culture media

For each series of commercial media (dried and ready-to-use), and for each batch of the medium, made in the laboratory, one determines the growth, selective and diagnostic properties.

Basic biological criteria of quality of the culture media are their growth and selective properties, defined using microorganisms and certified culture media. Ready-to-use media with the manufacturer’s certificate are used as certified as well as the previously certified ones in the laboratory of a high quality medium.

Growth properties of the culture medium are the ability of a culture medium to provide an effective and typical growth of appropriate teststrains of microorganisms.

Selective properties are the ability of the culture medium to inhibit the growth of appropriate microorganisms from a microbial association.

Test-microorganisms, strains-assiociants and incubation conditions for the determination of growth and selective properties of culture media are shown in Table 8.

10.1. Growth properties of culture media

10.1.1. Preparation of working suspension of test-microorganisms

Cultures of bacteria and fungi *C. albicans* are washed from from the surface of slope agar by sterile 0.9 % solution of sodium chloride. One prepares standard suspensions of each test-strain corresponding to 10 IE by a standard on turbidity. For *B. subtilis*, *B. cereus* and *C. albicans* it is 10^7 CFU/mL concentration, for *E. coli*, *S. abony*, *P. aeruginosa*, *S. aureus* is 10^9 CFU/mL. Standardized suspension by the method of subsequent decimal dilutions is brought by sterile 0.9% solution of sodium chloride to a concentration of 10^3 CFU/mL. To determine the actual concen-

tration of working suspensions of bacteria and *C. albicans*, the cultures are plated by a surface method from the concentration of 10^3 CFU/mL by 0.1 mL per Petri dish with an appropriate certified agarized medium.

To wash conidia *A. brasiliensis* from Sabouraud glucose agar, one uses sterile 0.9% sodium chloride solution containing 0.05% Tween-80. The number of conidia in 1 mL of suspension is determined by a Goryaev's chamber or by inoculation of an appropriate dilution in the certified Sabouraud glucose agar or medium No. 2. For inoculation, on prepares working suspension of *A. brasiliensis* with conidia concentration of about $0.5 \cdot 10^3$ in 1 mL, which is inoculated by a surface method by 0.1 mL in dishes with Sabouraud glucose agar (or medium No. 2).

The prepared working suspensions of test-microorganisms are used to determine the growth properties of culture media. The number of the cells of test strains to be added into liquid or agarized culture media should not exceed 10^2 CFU.

10.1.2. Test of agarized media

The test and certified agarized media are poured into Petri dishes with a 90 mm diameter by 15-20 mL, with agar drying out after solidification. By 0.1 mL of the working suspension of test microorganism with a 10^3 CFU/mL concentration is inoculated by a surface method in Petri dishes with the test and certified media in duplicate.

In agarized media after incubation, one counts colonies of test-strains of microorganisms and determines the coefficient of germination K_g according to a formula:

$$K_g = \frac{N}{N_o},$$

where: N is an arithmetic mean of colonies in the Petri dish with the test medium;

N_o is an arithmetic mean of colonies in the Petri dish with the certified medium.

10.1.3. Test of liquid media

Liquid test and certified culture media are poured in sterile test tubes of 15×150 mm by 10 mL. By 0.1 mL of the working suspension of the test-strain of microorganism with a 10^3 CFU/mL concentration are inoculated in test tubes with test and

standard media (by 3 test tubes for each type of medium). To be incubated at an appropriate temperature for a minimal time required for that test. The growth of microorganisms is determined visually.

10.1.4. Requirements to growth properties of culture media

The test agarized medium is considered to be applicable if the coefficient of germination is not less than 0.7 in comparison with certified culture medium.

The test agarized medium is condidered to be applicable if in the test and certified media one visually notes the same growth of a test-strain.

10.2. Determination of selective properties of culture media

10.2.1. Test procedure

To determine the selective properties of the culture media, the test and certified media are contaminated with strains-associants, each individually, with the inoculation dose 2 orders higher than the dose of the test-strain.

Inoculation in the agarized media is held by a surface method. In 2 dishes with each medium (test and standard), one adds by 0.1 mL of the working suspension of the strain-associant with a 10^5 CFU/mL concentration.

In order to inoculate in liquid culture media, in 2 test tubes of each medium, one adds 0.1 mL of the working suspension with a concentration of 10^5 CFU/mL of a strain-assiociant. In all inoculated culture media (in Petri dishes and in tubes), after the longest incubation period for the test at the appropriate temperature, one notes the absence of the strain-associant growth.

10.2.2. Requirements for the selective properties of culture media

The test selective medium is considered to be applicable if during the inoculation of strains-assocoants, one notes the total absence of their growth.

10.3. Determination of diagnostic properties of culture media

10.3.1. Test procedure

The diagnostic properties are tested for the culture media as Mossel agar (or medium No. 4), MacConkey agar, Xylose, Lysine, Deoxycholate agar (or medium No. 5), Cetrimide agar (or CPC agar), agar for detection of pyocyanin (or medium No.

9), Mannit-Kochsalz agar (or medium No. 10), triple sugar–iron–agar (or medium No. 13), Simmons citrate agar (or medium No. 14).

To confirm the diagnostic properties of the culture medium, with an inoculation loop one cultures broth culture of test-microorganisms (each separately) in two Petri dishes or in two test tubes with test medium. After incubation under standard conditions, one determines the characteristics of the test-strains of a certain type of microorganisms: the appearance of colonies, color, presence of a pigment, a halo around the colonies, the color change of the medium, etc. (see Table 7).

To confirm the selective properties of the diagnostic culture medium, one cultures broth culture of test-microorganisms (each separately) in test medium. After incubation under standard conditions, the growth of strains-associants should be absent.

10.3.2. Requirements for diagnostic properties of culture media

The test medium is considered to be applicable, if morphological and diagnostic properties of test-microorganisms correspond to the description given in Table 8, at the same time, there is no growth of strains-associants.

10.4. Sterility of culture media

Not less than 5 % of containers (bottles, test tubes) from each batch of prepared culture media are to be controlled on sterility while maintaining them at an appropriate temperature for 48-72 hours. Upon detection of microbial growth for at least in one of the containers under test, the batch of culture medium is to be destroyed.

10.5. Storage of culture media

Dried culture media should be stored tightly packed, in a dark, dry place at a temperature of 2-30 °C. After opening the package, on the bottle one needs to write the date, and the bottle is then stored at room temperature until the expiration date. Prepared from dry mixes, and poured into vials, culture media are stored for 1 month at room temperature or 3 months at a 2-8 °C temperature. Shelf life of the media poured in Petri dishes is 7 days at a 2-8 °C temperature. The exception to this rule applies to medium No. 4, poured in Petri dishes, the shelf life of which does not exceed 3 days when stored protected against light.

Table 8. Test-microorganisms and incubation conditions for the determination of growth and selective properties of culture media

Culture media	Note	Test-strains of microorganisms	Incubation conditions
1	2	3	4
• Casein soya bean agar • Medium No. 1 to grow bacteria	Identification of aerobic microorganisms	*Bacillus subtilis* ATCC 6633 or *Bacillus cereus* ATCC 10702; *Escherichia coli* ATCC 8739 or ATCC 25922; *Staphylococcus aureus* ATCC 6538	72 hours 32.5±2.5 ºC
• Sabouraud broth	Identification of yeasts	*Candida albicahs* PKPGY/NCTC 885-653 or ATCC 10231	5 days 22.5±2.5 ºC
• Sabouraud glucose agar • Medium No. 2 to grow fungi	Identification of yeasts and moulds	*Candida albicahs* PKPGY/NCTC 885-653 or ATCC 10231; *Aspergillus brasiliensis* ATCC 9642, ATCC 16404 VKMF1119 or A. niger PKPGF106	5 days 22.5±2.5 ºC
• Mossel broth • MacConkey Broth • Medium No. 3	Enterobacteriaceae Enrichment	*Escherichia coli* ATCC 8739 or ATCC 25922; *Salmonella enterica* subsp. *enterica* serovar *abony* IHE 103/39 *or* NCTC 6017 Strains-associants: *Staphylococcus aureus* ATCC 6538	24–48 hours 32.5±2.5 ºC
• MacConkey agar • Mossel Agar • Medium No. 4 to identify Enterobacteriaceae	Identification of Enterobacteriaceae	*Escherichia coli* ATCC 8739 or ATCC 25922; *Salmonella enterica* subsp.*enterica* serovar *abony* IHE 103/39 *or* NCTC 6017 Strains-associants for the determination of selective properties: *Staphylococcus aureus* ATCC 6538	24–48 hours 32.5±2.5 ºC
• Xylose lysine deoxycholate agar • Bismuth Sulfite agar • Medium No. 5 to identify bacteria of the genus *Salmonella*	Identification of bacteria of the genus *Salmonella*	*Salmonella enterica ssp. enterica serovar abony* IHE 103/39 *or* NCTC 6017 Strains-associants for the determination of selective properties: *Escherichia coli* ATCC 8739 or ATCC 25922; *Staphylococcus aureus* ATCC 6538 or *Bacillus cereus* ATCC 10702	24–48 hours 32.5±2.5 ºC
• Casein soya bean broth • Medium No. 8 to grow bacteria	Accumulation of aerobic bacteria	*Bacillus cereus* ATCC 10702 or *Bacillus subtilis* ATCC 6633; *Escherichia coli* ATCC 8739 or ATCC 25922; *Staphylococcus aureus* ATCC 6538; *Pseudomonas aeruginosa* ATCC 9027	24 hours 32.5±2.5 ºC
• Agar for detection of Pyocyanin *P. aeruginosa*	Identification of *P. aeruginosa*	*Pseudomonas aeruginosa* ATCC 9027	24–48 hours 32.5±2.5 ºC

• Medium No. 9 to identify*P. aeruginosa*			
• Cetrimide Agar • CPC agar to identify *P. aeruginosa*	Identification of *P. aeruginosa*	*Pseudomonas aeruginosa* ATCC 9027 Strains-associants for the determination of selective properties: *Escherichia coli* ATCC 8739 or ATCC 25922; *Staphylococcus aureus* ATCC 6538	24–48 hours 32.5±2.5 ºC
• Mannit-Kochsalz-Agar • Medium No. 10 to identify *S. aureus*	Identification of *S. aureus*	*Staphylococcus aureus* ATCC 6538; *Staphylococcus epidermidis* ATCC 14990 or ATCC 1228 Strain-associant for the determination of selective properties: *Pseudomonas aeruginosa* ATCC 9027	48 hours 32.5±2.5 ºC
• Rappaport-Vassiliadis Broth	Enrichment of bacteria of the genus *Salmonella*	*Salmonella enterica* subsp. *enterica* serovar *abony* IHE 103/39 *or NCTC 6017*	24 hours 42.5±2.5 ºC
• Triple sugar-iron-agar • Medium No. 13 to identify bacteria of the genus *Salmonella*	Identification of bacteria of the genus *Salmonella*	*Salmonella enterica* subsp. *enterica* serovar *abony* IHE 103/39 *or NCTC 6017; Escherichia coli* ATCC 8739 or ATCC 25922	24 hours 32.5±2.5 ºC
• Simmons Citrate Agar • Medium No. 14 to identify *E. coli*	Identification of *E. coli*	*Escherichia coli* ATCC 8739 or ATCC 25922; *Salmonella enterica* subsp. *enterica* serovar *abony* IHE 103/39 *or NCTC 6017*	24 hours 32.5±2.5 ºC

11. Particularities of the test of the immunobiological drugs (IBD) that contain live microorganisms

Probiotics of medical use

11.1. Sampling

The tests are carried out under aseptic conditions.

From each series of PP, one samples 10 units (vials, capsules, tablets, suppositories)/g of the preparation, from not less than 5 different packages, if increasing series, plus 1 vial (capsule, tablet, suppository, etc.) from each thousand of units.

Two units of the test preparation (vials, capsules, tablets, suppositories, etc.) are integrated in 1 sample, if no other instructions in the regulatory documentation.

Suspensions: the content of two vials is combined in 1 sample, mixed 8-10 times with a pipette and the original dilution is got.

Lyophilisates for solution or suspensions for oral and topical use: the content of each vial is diluted by sterile 0.9 % sodium chloride solution, based on 1 mL per 1 dose. The suspension received is integrated from 2 vials, mixed and original dilution is got.

Tablets: each sample (2 tablets) is preliminarily aseptically ground in a mortar to a homogeneous state (or in a test tube to the state of powder) and then fractionally 20 mL is added of sterile 0.9% sodium chloride solution, mixed and a 1:10 dilution is got.

Powders: each sample (the content of two sachets) is placed in a в flask to which 20 ml of sterile 0.9% sodium chloride solution is added, mixed and a 1:10 dilution is got.

Capsules: each sample (2 capsules) is placed in a test tube to which 20 mL of sterile 0.9% sodium chloride solution preliminarily heated to a 39±1 °C temperature is added, and mixed.

Suppositories: each sample (2 suppositories) is placed in a test tube to which 20 mL of sterile 0.9% sodium chloride solution preliminarily heated to a 39±1 °C temperature is added and mixed.

The test tubes with capsules or suppositories are placed in water bath at a 39±1 °C temperature. In 10-30 minutes, the content is mixed homogeneously to give dilution of 1:10.

Test conditions for microbiological purity of probiotics are presented in Tables 9-10.

11. 2. Analysis methods

11.2.1. Direct inoculation method

From the suspension received of the test sample of an appropriate dilution, one cultures by 1 mL in Petri dishes or in large (d = 20 mm, h = 200 mm) test tubes (flasks) with slope columns of culture medium.

In Petri dishes, suspension is distributed on the surface of the culture medium without the use of a spatula (with caution circular movements). The dishes are incubated for 30-40 minutes on the plane of the table, without turning, until complete absorbtion of suspensions by agar, and then are turned upside down and incubated in an oven.

In the media that are sloped in test tubes (vials), the inoculation is distributed by spinning and incubated in an oven for 2 days at first in a horizontal position, and during the remaining period – vertically.

The suspension is inoculated in test tubes with slope urea agar by means of a Pasteur pipette as a prick to the bottom of the test tube, and the rest of the inoculation material when removing the pipette, is rubbed over the surface of the slope portion of the column of the medium and is incubated in an oven.

The sample inoculation in which the presence of foreign microorganisms and fungi is not allowed or not more than 50 CFU/U of preparation is permitted, is held from the original test sample dilution. The inoculation of preparation samples, in which the total number of aerobic microorganisms is allowed at a 10^2 CFU/U concentration of the preparation, is made from a 1:10 dilution (10^{-1}) of the test sample.

The preparations that include live coli bacteria: in Petri dishes with Endo agar, one inoculates by 0.5 mL of suspension of original dilution of the test sample. The material is distributed on the surface of the medium, with a Drigalski spatula, and then

with the same spatula, one inoculates the culture in a new Petri dish with Endo agar to receive isolated colonies.

When testing preparations, in which contamination is not allowed, before inoculation in medium, from each sample, one smears and then, depending on the presence of probiotic bacteria there, there is Gram or Ziehl–Neelsen staining and microscope examination. The smear is examined in 10 fields of view. The microslide should contain only bacteria that are caracteristic for the test sample of probiotic.

11.2.2. Method of the determination of the contamination degree with aerobic microorganisms

To determine the degree of contamination with aerobic bacteria, one prepares additional dilution, for which 1 mL of microbial suspension from original dilution or from 1:10 dilution (depending on the pharmaceutical form and the MAC of the microbial contamination norms) is transferred in the test tubes together with 9 mL of 0.9 % sodium chloride solution and mixed for 8–10 times, by receiving the dilution as follows, i.e. 10^{-1} or 10^{-2} respectively. One then inoculates cultures in Petri dishes by 1 mL of 10^{-1} and 10^{-2} dilution (or from original dilution and 10^{-1}) in 2 dishes for each dilution.

The inoculation of the preparations in which the total number of aerobic microorganisms is allowed at a 10^{2} CFU/U concentration of the preparation, is held from 10^{-1} and 10^{-2} dilution. The inoculation of the preparations in which the total number of aerobic microorganisms is allowed at a 50 CFU/U concentration max of the preparation, is held from original dilution of 10^{-1}.

11.2.3. Streak culture method

A Petri dish with agarized culture medium divided in 4-5 sectors. The inoculation from the original dilution of a test sample is begun in the 1st sector, carefully rubbing the suspension in agar with an inoculation loop. Then with the same inoculation loop, one keeps on inculating in the 2nd and subsequesnt sectors. In the last secors, isolated colonies should grow.

11.2.4. Method of determination of the presence of phage in coli-containing preparations

By 1.0 mL of original initial dilution of the test sample are plated on Petri dishes with MPA. The inoculation material is distributed over the entire surface of the medium, shaking the Petri dish to obtain a solid lawn. The rest of suspension is removed with a sterile Pasteur pipette. Closed dishes with inoculations are kept on the table, without turning, for 30-40 min (until complete absorption of the suspension in agar), after which they are turned upside down and incubated in an oven at a 20±2 °C temperature for 19±1 hours.

Incubation finished, the dishes are observed for the presence of phagolysis spots. If against the growth of *E. coli* on the surface of a Petri dish, one detects phagolysis spots of any size and form, one carries out a repeated inoculation on a double number of samples.

11.3. Preparation of culture media used for the determination of microbial contamination of preparations-probiotics

Ready culture media are melted in water bath, cooled to a temperature of 45±1 °C, poured by 25 mL in Petri dishes with a diameter of 90 mm, installed on a flat surface. Blood agar is poured with a 1.5-2 mm layer.

The medium solidified, the closed Petri dishes are placed in an oven upside down, at a 37±1 °C temperature for 48 hours for drying and controlling the culture medium sterility.

Dishes with Endo medium are dried out in a laminar flow hood (under laminar flow) with open lids for 45-50 min.

Culture media in wide test tubes (d=20 mm, h=200 mm) or in vials is melted and the column of culture medium is sloped.

11.4. Recording of results received

In 72 hours after the start of incubation of the inoculations and finally in 5-8 days, one counts the number of colonies of bacteria (permitted aerobic bacteria flora) in 2 dishes (of each dilution), finds the average value (for colonies grown on 2 dishes of one dilution) and multiplying it by the dilution ratio, one calculates the number of bacteria in 1 tablet (capsule, suppository, etc.) or in 1 g.

If when inoculating the sample from 10^{-1} and 10^{-2} dilutions, there is no growth, the result is noted as follows: “In 1 capsule (tablet, suppository, etc.) there is less than 10 CFU of bacteria”.

If in the inoculations there is the growth of conditionally pathogenic bacteria (Enterobacteriaceae, Proteus, hemolyzing bacteria, etc.) and fungi, one believes the quality of the preparation does not meet the requirements in terms of “Microbiological purity”.

If the number of aerobic microorganisms exceeds the permissible limit of the CFU number in 1 tablet (capsule, suppository, etc.), the control is repeated on a double number of samples.

Table 9: Conditions of the test for the microbiological purity of probiotics in which the presence of microorganisms-contaminants is not allowed (Suspensions and lyophilisates to prepare solutions and or суспензий for oral use and topical application)

Generic name	Culture media	Dishes, test tubes	Incubation conditions	Microorganisms taken into account	Results recording
Bifidum-containing preparations	MPA or FH-agar	Tubes (or Petri dishes)	37±1 ºC 8 days	Aerobic microorganisms	There should not be a growth
	Culture glucose agar or MPA with 0,5 % glucose	Tubes (or Petri dishes)	37±1 ºC 8 days	Aerobic microorganisms	
	Sabouraud agar	Tubes (or Petri dishes)	22±2 ºC 8 days	Yeasts and moulds	
Lactam-containing preparations (Lactobacilli – micro-aerophiles)	MPA	Petri dishes (or tubes)	37±1 ºC 8 days	Aerobic microorganisms	There should not be a growth
	Sabouraud agar	Tubes (or Petri dishes)	22±2 ºC 8 days	Yeasts and moulds	
Lactam-containing preparations (Lactobacilli – Facultative aerobes*)*	Culture agar with 9 % sodium chloride	Tubes (or Petri dishes)	37±1 ºC 8 days	Aerobic microorganisms	There should not be a growth
	Sabouraud agar with antibiotics	Tubes (or Petri dishes)	22±2 ºC 8 days	Yeasts and moulds	
	Endo agar	Petri dishes	37±1 ºC 24–48 h	Bacteria in the family of *Enterobacteriaceae*	
	MPA	Tubes (or Petri dishes)	37±1 ºC 8 days	Aerobic microorganisms	There should not be a growth of bacteria-contaminants (The growth of lactobacilli in the form of small grayish translucent colonies, forming a continuous lawn)

Coli-containing preparations	Culture agar with 9 % sodium chloride	Tubes (or Petri dishes)	37±1 ºC 8 days	Aerobic microorganisms	There should not be a growth
	Sabouraud agar with antibiotics	Test tubes (or Petri dishes)	22±2 ºC 8 days	Yeasts and moulds	
	Endo agar	Petri dishes	37±1 ºC 19±1 hour	Lactose negative bacteria in the family of *Enterobacteriaceae*	There should not be a growth of lactose negative colonies
	MPA (control on the absence of phagolysis)	Petri dishes	20±2 ºC 19±1 hours	To control over the phage contamination	There should not be phagolysis spots (for the preparations with *E. coli* content not less than 10^{10} it is allowed 10 BFE max)
Preparations that contain bacteria of the genus Bacillus (spore probiotics)	Sabouraud agar	Petri dishes	22±2 ºC 8 days	Yeasts and moulds	There should not be a growth of bacteria-contaminants (is possible the growth of bacteria of the genus *Bacillus* – smooth, white or yellowish-pink shaded colony)
	Endo agar	Petri dishes	37±1 ºC 24-48 hours	Bacteria in the family of *Enterobacteriaceae*	There should not be a growth of bacteria-contaminants (is possible the growth of bacteria of the genus *Bacillus* – small, colorless or slightly painted from pale-pink to reddish colonies)
	Gauze medium No. 2	Petri dishes	37±1 ºC 8 days	Aerobic microorganisms	There should not be a growth of bacteria-contaminants (is possible the growth of bacteria of the genus *Bacillus* – rough with scalloped edges pinkish-beige and smooth, white colonies)
	Blood agar	Petri dishes	37±1 ºC 24–48 h	Aerobic microorganisms	There should not be a growth of bacteria-contaminants (is possible the growth of bacteria of the genus *Bacillus* – with scalloped edges, rough grayish-pink and sweet beige-brown colonies, without hemolysis)

	Urea medium with Andrade's indicator or Preuss	Test tubes	37±1 ºC 72 hours	Bacteria of the genus *Proteus*	There should not be change in color of the medium (is possible the growth of bacteria of the genus *Bacillus* – small colorless colonies, medium redness is possible)
	Egg yolk-salt agar (or Medium No. 10)	Petri dishes	37±1 ºC 72 h	Staphylococci	There should not be a growth of bacteria-contaminants (is possible the groqth of bacteria of the genus *Bacillus* – round, white, well removing colonies)

Note. When suspicious colonies appear, microscope examination is to be carried out.

Table 10: Conditions of the test for the microbiological purity of probiotics in which the presence of foreign microorganisms and fungi is allowed (suppositories, tablets, capsules)

Generic name/Dosage form	Culture media	Dishes, tubes	Incubation conditions	Microorganisms taken into account	Results recording
Bifidum-containing preparations	MPA	Petri dishes	37±1 ºC 8 days	Aerobic bacteria	To count the number of bacterial colonies of permissible bacteria-contaminants
	Endo medium	Petri dishes	37±1 ºC 24–48 hours	Bacteria in the family Enterobacteriaceae	There should not be a growth
	Blood agar	Petri dishes	37±1 ºC 24–48 hours	Hemolyzing microorganisms	There should not be colonies surrounded by a hemolytic plaque
	Urea medium with Andrade's indicator or Preuss	Test tubes	37±1 ºC 24–48 hours	Bacteria of the genus *Proteus*	There should not be change in color of the medium
	Sabouraud agar	Petri dishes	22±2 ºC 8 days	Yeasts and moulds	There should not be a growth
	Medium No. 9	Petri dishes	37±1 ºC 24–48 hours	*Pseudomonas aeruginosa*	
	Medium No. 10	Petri dishes	37±1 ºC 24–48 hours	*Staphylococcus aureus*	
Lactam-containing preparations (Lactobacilli – microaerophiles)	MPA or Medium No. 1	Petri dishes	37±1 ºC 8 days	Aerobic microorganisms	To count the number of bacterial colonies of permissible bacteria-contaminants
	Endo medium	Petri dishes	37±1 ºC 24–48 hours	Bacteria in the family Enterobacteriaceae	There should not be a growth
	Blood agar	Petri dishes	37±1 ºC 24–48 hours	Hemolyzing microorganisms	There should be no colonies surrounded by a hemolytic plaque
	Urea medium with Andrade's indicator or Preuss	Test tubes	37±1 ºC 24–48 hours	Bacteria of the genus *Proteus*	There should not be change in color of the medium
	Sabouraud agar or Medium No. 2	Petri dishes	22±2 ºC 8 days	Yeasts and moulds	There should not be a growth

	Medium No. 9	Petri dishes	37±1 ºC 24–48 hours	*Pseudomonas aeruginosa*	
	Medium No. 10	Petri dishes	37±1 ºC 24–48 hours	*Staphylococcus aureus*	
Lactam-containing preparations (Lactobacilli – Facultative aerobes*)*	Culture agar with 9 % sodium chloride	Petri dishes	37±1 ºC 8 days	Aerobic microorganisms	To count the number of bacterial colonies of permissible bacteria-contaminants
	Endo medium	Petri dishes	37±1 ºC 24–48 hours	Bacteria in the family of *Enterobacteriaceae*	There should not be a growth
	Blood agar	Petri dishes	37±1 ºC 24–48 hours	Hemolyzing microorganisms	There should be no colonies surrounded by a hemolytic plaque
	Urea medium with Andrade's indicator or Preuss	Test tubes	37±1 ºC 24–48 hours	Bacteria of the genus P*roteus*	There should not be change in color of the medium
	Sabouraud agar with antibiotics	Petri dishes	37±1 ºC 8 days	Yeasts and moulds	There should not be a growth

12. Detemination of the water microbiological purity

12.1. Water for injections (in bulk)

The total number of aerobic microorganisms (bacteria and fungi) does not exceed 10 CFU in 100 mL. The presence of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* in 100 mL is not allowed.

For analysis of water microbiological purity for injection, one takes a sample in a volume of not less than 1,000.0 mL.

The test is carried out aseptically by the method of membrane filtration. For inoculation, one uses nitrocellulose membrane filters with pore diameter that does not exceed 0.45 mcm and an outer diameter of 47 mm. To wet a filter, on applies a sterile 0.9 % sodium chloride solution (not less than 5 mL).

To determine the total number of aerobic microorganisms, one filters 100 mL of water for injection in duplicate. The filtration finished, each filter is transferred to Petri dishes on the surface of agarized medium R2A with the composition as follows:

• Digest of casein	0.5 g
• Yeast extract	0.5 g
• Proteose-peptone	0.5 g
• Glucose	0.5 g
• Soluble starch	0.5 g
• Dipotassium phosphate	0.3 g
• Magnesium sulphate	0.024 g
• Sodium pyruvate	0.3 g
• Microbiological agar	15.0 g
• Purified water	1,000.0 mL
pH after sterilization	7.2±0.2

The inoculations are incubated in an oven at a 32.5±2.5 ºC temperature for 5 days. One then counts the colonies in 48–72 hours (preliminary results), in 5 days (final results) and determined an arithmetic average number of aerobic microorganisms (bacteria and fungi in total) in 100 mL of water.

To determine the total amount of aerobic microorganisms it is allowed to use casein soya bean digest agar or medium No. 1 to identify bacteria, Sabouraud agar or medium No. 2 to grow fungi.

To identify *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, one filters 100 mL of water in duplicate. According to the results received, to identify each microorganism, it is allowed to filter 200 mL of purified water through one filter.

Filtration finished, 2 filters are transferred in Petri dishes on the surface of agarized medium Endo (medium No. 4). The inoculations are incubated at a 32.5±2.5 ºC temperature for 24 hours, microscoped – crimson-red colonies with/without a metallic shine surrounded by crimson spots of precipitation, non-mucoid. Upon detection in smears of gram-negative bacilli, individual colonies are placed on a sloped in test tubes casein soya bean digest agar (medium No. 1) and are incubated for 18–24 hours. Incubation finished, one carries out identification according to para. 8*. If in the sample one finds non–spore-forming gram-negative bacilli not containing cytochrome oxidasey enzyme, not removing sodium citrate and forming indole, one considers the water is contaminated with *E. coli*.

The next two membrane filters are transferred in Petri dishes on the surface of agarized medium No. 9. The inoculations are incubated at a 32.5±2.5 ºC temperature for 24-48 hours. If presence in the filters placed in medium No. 9 colonies of bacteria that evolve a blue-green pigment of pyocyanin in agar, one carries out microscopic identification in accordance with para 8*. If in the sample one finds non–spore-forming gram-negative bacilli that evolve a blue-green pigment of pyocyanin containing cytochrome oxidasey enzyme and growing at a 42±1 ºC temperature, one considers the water is contaminated with *P. aeruginosa*.

To identify *S. aureus,* 2 membrane filters are transferred in Petri dishes on the surface of culture medium – Mannit-Kochsalz-Agar or medium No. 10 – and are incubated for 24–48 hours. Golden-yellow colonies surrounded by yellow spots are placed to casein soya bean digest agar or medium No. 1. One carries out microscopy and identification in accordance with para.8*. If in the sample, one finds Gram-

positive cocci arranged in clusters, fermenting Mannit-Kochsalz-Agar (medium No. 10), containing coagulase enzyme, one considers the water sample is contaminated with *S. aureus*.

Note.

* - To identify, one can use other methods (test-systems, automatic analysators, etc.).

12.2. Purified water

The total number of aerobic microorganismsns (bacteria and fungi) does not exceed 100 CFU/mL. The presence of *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* in 100 mL is not allowed.

To analize the microbiological purity of purified water, one takes a sample in the volume of not less than 1,000 mL.

To determine the total number of aerobic microorganisms, one filters the volumes of purified water as follows: 1 mL, 10 mL and 100 mL (in duplicate). For the test, it is allowed to use one volume of water, selected in accordance with the results received. The analysis then is carried out in accordance with para.12.1.