EXTENDED HABILITATION REFERENCE

(Annotation of the materials under art. 65 (1) of the Regulations for the development of academic staff of PU "Paisii Hilendarski", including self-evaluation of overall contributions and input)

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Participant in the competition for the academic position "Professor" in the field of higher education **4. Natural Sciences, Mathematics and Informatics**, professional field **4.3 Biological sciences**, scientific specialty **Microbiology**,

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To participate in the competition for the academic position "Professor" in the scientific specialty Microbiology, I hereby present a total of 36 scientific works that have not been used in the procedures for obtaining the educational and scientific degree "Doctor" and for the academic position of "Assistant Professor" and "Assc. Professor". They are classified into the following groups (in accordance with the list of scientific papers):

- 18 publications in journals with impact factor or impact rang;
- 12 publications in scientific journals without impact factor;
- 3 publications in proceedings of conferences and congresses;
- 3 textbooks.

The contributions of the submitted materials for participation in the competition for the academic position "Professor" in the scientific specialty of Microbiology can be grouped in four areas:

I. Microbial enzymes;

I. Microbial pathogenesis (virulence factors);

III. Environmental microbiology;

IV. Contributions towards the field of education;

I. Contributions in the field of microbial enzymes:

- **determination of the activity of hydrolytic enzymes in Gram-positive and Gram-negative bacteria;**
- **optimization of culture conditions for enzyme production by the strain-producers;**

- **isolation and purification of enzymes;**
- **determination of enzymatic and molecular properties of purified proteins.**

Microorganisms produce various enzymes that enable them to assimilate a variety of substrates, colonize living space and this contributes to their ubiquity in the environment. Enzymes are biological catalysts that can conduct various reactions under physiological conditions in cells without a large expenditure of energy. Their use in industrial processes guarantees a reduced amount of toxic substances used, high selectivity of the reactions carried out, facilitated purification of the resulting products, reduction of technological steps, higher quality of the final product, and less environmental pollution. More and more production processes are replacing chemical-technological steps with biotechnological ones. Some of these areas are food production, animal feed, detergents and cleaners, textiles, pharmaceuticals, cosmetics, and fine chemicals.

Bacteria appear to be excellent producers of many extracellular, hydrolytic enzymes due to their ease of cultivation, short life cycles, and the ability to isolate and purify enzymes by conventional methods. Among the bacterial producers, species of the genus *Bacillus* are distinguished. They can synthesize more than 40 different enzymes that find application in various industrial processes. Microorganisms are generally preferred enzyme producers over plant and animal sources because microbial protein production is cheaper, easier to control, and more reliable.

The monograph "Phospholipases C produced by *Bacillus* species" (**Publication III. 1.**) presents personal results and generalizations on the production and purification of phospholipases C from *Bacillus cereus, B. thuringiensis,* and *B. sphaericus* species, and compares them with the state of the art in this field.

Phospholipases are a heterogeneous group of enzymes that hydrolyze acyl ester and phosphoester bonds of phospholipids and have diverse functions - catabolic (providing substrates for growth), structural (maintaining and patterning membranes), regulatory (forming bioactive lipid molecules involved in signal transduction). The phospholipases C hydrolyze the ester bond between diacylglycerol and the substituted phosphoric acid in the phospholipid molecule or that between N-acylsphingosine and choline-esterified phosphoric acid. Some bacterial phospholipases C are pathogenicity factors as they are an active component of bacterial toxins, but also contribute to tissue colonization, progression of infection, and suppression of the immune response. Identification of these enzymes is of great importance as the development of inhibitors for their action may generate potential vaccines and therapeutic agents that will reduce the impact of related diseases in animals and humans.

Phospholipases are major mediators of intracellular and intercellular signaling. Hydrolysis of phospholipids generates bioactive molecules such as diacylglycerol, phosphatidic, lysophosphatidic, and arachidonic acids, which are involved in many physiological and pathophysiological processes such as membrane transport, cell proliferation, signal transduction, and apoptotic cell damage.

The interaction of phospholipases with membrane phospholipids can also be used to study the phospholipid composition of membranes or completely simulate the action of eukaryotic phospholipase C on cellular metabolism. Since no eukaryotic phospholipase C has

been purified or cloned to date, species of the genus *Bacillus*, and in particular *B. cereus*, is the preferred producer and putative model for mammalian phospholipases C.

The results presented in the monograph can be systematized according to the phospholipase activities of different *Bacillus* species.

*Bacillus cer*eus strains synthesize all three types of phospholipases type C - phosphatidylcholine-specific (PC-PLC), phosphatidylinositol-specific (PI-PLC), and sphingomyelinase C (SMase C). Ninety-three percent of the strains analyzed produce PC-PLC, 53.4% PI-PLC, and 81% SMase C. The highest activity is established for PC-PLC. The activity in the culture medium of *B. cereus* strain No 51 (16.8 U/ml) is increased by 54% by optimization of culture conditions. Phospholipase C is secreted into the culture medium at the end of the exponential growth phase - 10^{th} hour.

A highly efficient purification scheme for phospholipase C strain *B. cereus* No 51 was established. The purified enzyme has a specific activity of 319 U/mg with a 43% activity yield. The obtained phospholipase C has a molecular mass of 23-26 kDa as determined by gel filtration and SDS-PAGE electrophoresis, respectively. The enzyme hydrolyzes phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine in that order of activity. Does not show activity to phosphatidylinositol and sphingomyelin. Sodium deoxycholate and cetyltrimethylammonium bromide at a concentration of 0.1% stimulated the hydrolysis of phosphatidylcholine by 40% and 20%, respectively. Phospholipase activity is stimulated by Zn^{2+} and Ca^{2+} ions at a concentration of 5 mM, by 60% and 35%, respectively. The inactivated by EDTA enzyme is completely reactivated after the addition of Zn^{2+} ions, and partially by Mg^{2+} and Mn^{2+}, indicating that the enzyme belongs to the Zn-metalophospholipases.

The optimal pH value for the hydrolysis of phosphatidylcholine was 7.2. Phospholipase C from *B. cereus* strain No 51 exhibited high stability in the pH range 6.0-8.0. The temperature optimum for enzyme action is 35-37°C. Treatment at temperatures of 50 and 60°C, respectively, for 30 min resulted in a loss of about 50% of activity, but in the presence of high concentrations of urea, the enzyme retained activity at higher temperatures.

Bacillus cereus strains synthesize phosphatidylcholine-specific and phosphatidylinositol-specific phospholipase C, but there is no correlation in the production of the two enzymes. *Bacillus cereus* strain No 93 was selected as a producer of the PI-PLC, which is secreted into the medium at the end of the exponential growth phase (8^{th} hour). The enzyme was purified from the culture medium 1080-fold, with an activity yield of 32% by a four-step scheme including ultrafiltration, isopropanol precipitation, ion-exchange chromatography, and gel filtration.

The purified PI-PLC has a molecular mass of 27±1 kDa and has a high specificity for phosphatidylinositol. Enzyme hydrolysis is maximal at 35-40°C and pH 7.0-7.5. The enzyme does not contain and does not need metal ions for its activity. Hydrolysis of phosphatidylinositol is stimulated by anionic (Na-deoxycholate) and nonionic (Triton x 100) detergents. PI-PLC retained high activity in the pH range 6.0-8.0, and thermal stability analysis shows that temperatures above 60°C lead to a significant loss of activity.

Only *B. cereus* was found to produce sphingomyelinase C. There was a correlation between phospholipase and sphingomyelinase activity of the cultures. *Bacillus cereus* strain No 79 was selected as a producer of SMase C. It secreted the enzyme in the middle of the stationary growth phase (16^{th} hour). The maximum of sphingomyelinase production lags that of PC-PLC, which is a good prerequisite for the separation of the two activities. Sphingomyelinase C was purified 238-fold, with a yield of 31% by a combination of ultrafiltration and chromatography on DEAE-cellulose and Sephadex G-75, respectively.

The molecular mass of the enzyme determined by gel filtration is 24±1 kDa. Hydrolysis of sphingomyelin reaches a maximum at pH 6.5 - 7.0. Sphingomyelinase activity is stimulated by Mg^{2+} ions; the effect of Ca^{2+} ions depends on the physical state of the substrate. Enzyme activity is completely inhibited by 0.25 mM EDTA; the inactivated enzyme is reactivated by Mg^{2+} ions. Hydrolysis of sphingomyelin is stimulated by sodium deoxycholate and Triton x 100. Analysis of the hemolytic activity of phospholipase C against sheep erythrocytes showed Ca^{2+} and Mg^{2+} ions dependent adsorption and hemolysis.

Bacillus thuringiensis strains were found to secrete phosphatidylcholine-hydrolyzing and phosphatidylinositol-specific phospholipase C. Eighty-six percent of the cultures produce phosphatidylcholine-specific phospholipase C, which in strain *B. thuringiensis var. thuringiensis* 17 reach 19 U/ml at the end of the exponential growth phase (8^{th} hour). The activity remains relatively high for 6 hours, which facilitates the isolation of the enzyme. Optimization of the medium composition by the replacement of the nitrogen source, the addition of metal ions and sugars increase the initial activity by 58%. PC-PLC from *B. thuringiensis* strain 17 was purified by a combination of ultrafiltration, gel filtration, and FPLC (HiPrep DEAE) chromatography. A homogeneous enzyme preparation with a specific activity of 190 U/mg was obtained.

Phospholipase C from *B. thuringiensis var thuringiensis* 17 has a molecular mass of 25-30 kDa, determined by gel filtration and SDS-PAGE electrophoresis. Hydrolyzes phospholipids in the following order: phosphatidylcholine > phosphatidylethanolamine > phosphatidylserine. The enzyme has a maximum activity at pH 7.0 and temperature 35-37°C. Hydrolysis of phosphatidylcholine is stimulated by metal Zn^{2+} and Ca^{2+} ions at concentrations of 1 mM and 5 mM, respectively. After inactivation with EDTA, the enzyme activity is fully recovered by Zn^{2+} and partially by Mg^{2+} and Mn^{2+} ions. The phospholipase reaction is also stimulated by detergents such as sodium deoxycholate and Triton x 100 - 42 and 18%, respectively, at a concentration of 0.1%. The enzyme lost 72% of its activity after heat treatment at 80°C.

The gene encoding phospholipase C in *B. thuringiensis var. thuringiensis* strain 17 was isolated and sequenced. The highest degree of homology was found with the gene from *B. cereus* encoding PC-PLC.

Production of phosphatidylinositol-specific phospholipase C was detected in 69% of studied *B. thuringiensis* strains. The enzyme was secreted into the culture medium at the end of the exponential growth phase (10^{th} hour). Enriching the medium with glucose and yeast extract stimulated bacterial growth and enzyme production by up to 10%.

PI-PLC from *B. thuringiensis strain var. thuringiensis* 16H was purified 1051-fold, with a yield of 25%, by a scheme including ultrafiltration, isopropanol precipitation, cation-exchange chromatography, and gel filtration.

The enzyme from strain *B. thuringiensis var. thuringiensis* 16H has a molecular mass of 23±1 kDa. PI-PLC shows high specificity for phosphatidylinositol and does not hydrolyze other phospholipids. The Km for phosphatidylinositol was determined to be 1.4 x 10^{-3} M. The optimum pH value for enzyme action is 7.0 - 7.5, at 35-37°C optimal temperature. PI-PLC from *B. thuringiensis* 16H does not require divalent metal ions (Zn^{2+}, Ca^{2+}, Mg^{2+}), and its activity is inhibited by NaCl and KCl at concentrations higher than 0.1 M. EDTA, while o-phenanthroline at concentrations of 0.25 and 0.5 mM did not inhibit enzyme activity. The anionic detergent, sodium deoxycholate, and the nonionic detergent, Triton x 100, stimulate enzymatic hydrolysis at concentrations ranging from 0.2 to 0.8%, whereas cetyltrimethylammonium bromide has no effect. Purified PI-PLC from *B. thuringiensis* 16H exhibits ectoenzyme-releasing activity.

For the first time, the production of phosphatidylinositol-specific phospholipase C is demonstrated in *B. sphaericus* strains. The enzyme is secreted into the culture medium at the beginning of the stationary growth phase (12^{th} hour) and its activity is comparable to that of *B. thuringiensis* strains. The PI-PLC was partially purified (202-fold), with an activity yield of 26%, and is free of co-lipase activity. The approximate molecular weight determined by gel filtration is 34±1.5 kDa. The optimal pH value for the action of the enzyme, which exhibits high specificity for phosphatidylinositol is 7.2 - 7. 5.

Our studies and results confirm that *B. cereus* and *B. thuringiensis* species are among the best bacterial producers of phospholipase C. All three types of phospholipases, PC-PLC, PI-PLC, and SMase C, can be isolated from *Bacillus cereus* strains. The species *B. cereus* and *B. thuringiensis* have other advantages such as high enzyme activity, an early phase of production which shortens the cultivation time, relatively high resistance of enzymes to changes in the culture environment which provide a good prospect for their use as enzyme producers.

The results presented in the monograph are original and have not been published in periodicals or proceedings of scientific forums. The cited own publications are not submitted as peer-reviewed material in this competition.

Publication III. 2.16. presents detailed information on the optimization of culture conditions for the synthesis of phospholipase C in *B. thuringiensis strain* 17, commenting on the possibilities for the application of the enzyme in bioremediation processes. Although lipases and phospholipases are hydrolytic enzymes commonly synthesized by microorganisms, they have been successfully applied in industrial biosynthetic processes. Their ability to catalyze transesterification reactions can be used in the biosynthesis of fatty acid alkyl esters (known as biodiesels), which are an important source of renewable fuels. Another application of phospholipase enzymes with a high ecological effect is the possibility of degrading waste from various industries. The *B. thuringiensis* 17 strain has a high initial activity and has potential for industrial application, which is improved by detailing the culture conditions for phospholipase C production.

Species of the genus *Bacillus* have been known to produce a significant range of enzymes besides Phospholipase C. The production of alkaline phosphatase by *Bacillus cereus* and enzyme activity was also studied in **Publication III. 2.2**. The enzyme is produced after cultivation on modified nutrient media with low phosphate content. The highest activity was established during the middle stationary phase of growth. At this moment the activity was 0.31 U/mg for AP1 and 1.4 U/mg for AP II. The extracellular (AP I) and membrane-bound (AP II) alkaline phosphatases were purified 282-fold and 70-fold, respectively by a combination of chromatographic methods. The optimal pH for both enzymes is 9.5. The addition of Ca^{2+} ions completely reactivated enzymes, that were treated with EDTA. Temperatures of 80°C inhibit irreversibly the enzyme activity. The molecular weight determined by gel-filtration is 43±1 kDa for AP I and 44 ± 1 kDa – for AP II. Despite some differences in their activity, both enzymes are characterized by similar enzymatic properties, suggesting that part of the enzyme is released in the nutrient medium, which in terms provide inorganic phosphate for *Bacillus cereus*.

Alkaline phosphatases have been identified in a wide variety of organisms, including bacteria (*Escherichia coli, Bacillus species, Mycobacterium smegmatis, Thermotoga maritime, Haloarcula marismortui*) and humans. Although the actual purpose of the enzyme is still not fully understood, the simple hypothesis, that it is a means for the bacteria to generate free phosphate groups for uptake and use.

The activity of alkaline phosphatase was also analyzed in strains isolated from soil and identified as *Escherichia coli*, based on biochemical, morphological, and cultural characteristics (**Publication III. 3.2**). All of the strains were found to produce membrane-bound alkaline phosphatase and β-galactosidase. A significant phosphatase activity (between 10 *и* 20 U/ml) was found for three of them. In all cases, the highest activity was established at the stationary phase of growth. The correlation between growth and enzyme activity was discussed. The *E. coli* enzyme is the first bacterial alkaline phosphatase discovered and characterized. This enzyme has a wide application in the identification of secreted and integral proteins as an export protein; for the analysis of membrane topology and in the detection of insertion-tolerant sites in membrane proteins, which determines the interest in isolating strains with high phosphatase activity and the possibility of using them as efficient enzyme producers.

Bacillus species are among the most promising producers of proteolytic enzymes (alkaline and neutral proteases), which have high activity, a wide pH spectrum of action, temperature stability, and resistance to organic solvents. These enzymes are used in the production of bioactive peptides, organic synthetic reactions, detergents, also in the leather and food industries. One hundred sixty-six bacterial strains from genus *Bacillus* were tested for protease production (**Publication II. 2.10**.). Ninety percent of the studied strains demonstrated protease activity on nutrient gelatin and milk agar. A strain *Bacillus thuringiensis* was selected as the most promising for enzyme production based on its initial enzyme activity of 9.2 U/ml. The nutrient medium composition and cultivating conditions were optimized aiming for better yields. The highest protease activity of 15 U/ml was achieved in the following conditions: inoculation of the medium with 5% inoculum (6.0 McF), followed by 16 hours of cultivation in a liquid medium containing 0.5% glucose,

0.55% Bacto Peptone, 50mM phosphate buffer and 0.2% magnesium ions. The produced enzymes were partially purified 5.6-fold by ultrafiltration and size-exclusion chromatography on Sephadex G-75 and had a specific activity of 17.7 U/mg. The approximate molecular weights between 45 and 66 kDa were determined by SDS-PAGE.

Strains of the genus *Bacillus* were tested for extracellular amylolytic activity (**Publication III. 3. 9.**), Positive results were established for 31% of them, including 61% of the *B. cereus*, 31% *B. thuringiensis*, and 3% *B. sphaericus*. Activity ranged from 0. 9 to 2. 8 U/ml and was highest in *B. cereus* strain No 10. The enzyme secretion into the culture medium begins in the exponential phase (8^{th} hour/1 U/ml) but reaches a maximum in the late stationary phase, 36^{th} hour - 3.14 U/ml. The prolonged enzyme production during the growth cycle and the presence of three plateaus of activity suggest the synthesis of different amylase enzymes that are released into the culture medium during *B. cereus* growth. Secretion of the enzyme into the medium is not associated with cell lysis due to spore breakage. Enrichment of the medium with 0.1% ribose or glucose, respectively, yeast extract, and Ca^{2+} ions stimulated amylase activity.

Amylases are one of the most important industrial enzymes accounting for more than 25% of the global enzyme market. The amylases produced by *Bacillus* are widely used in various industries - in food, to produce glucose and glucose-fructose corn syrups, saccharification of starch, preparation of various alcoholic beverages, hydrolysis of starch in the dough to dextrins, di- and monosaccharides, which are digested by yeast; in the textile industry - for sizing without damaging textile fibers; are incorporated into detergents. The diverse applications of amylolytic enzymes increase the need to discover new producers and enzymes with specific properties.

Species of the genus Pseudomonas are active producers of extracellular enzymes. Members of this genus produce various enzymes, some of them unique, that enables them to digest a variety of substrates - carbohydrates, lipids (organic acids), proteins (amino acids), aromatic and aliphatic hydrocarbons. The productive metabolism of compounds defined as xenobiotics by these bacteria makes them indispensable in biotransformation and bioremediation processes. In **Publication III. 3.5**. *Pseudomonas* strains were examined for the production of lipolytic enzymes. Bacteria synthesize extracellular lipase and phospholipase type C. The majority of the strains of *Pseudomonas sp*. are producers of lipase and phospholipase C. Phospholipase C activity reaches a maximum in the initial stationary phase – 12^{th} hour, while the maximum lipase secretion is found in the late stationary phase. The production of both enzymes is positively influenced by the addition of extra carbon sources at a concentration of 0.5% for a soybean-casein medium that is suitable for the cultivation of species Pseudomonas.

The relatively high activity (for Gram-negative bacteria) has attracted particular attention to specific classes of enzymes of the genus *Pseudomonas*. Enzymes of *P. aeruginosa*, *P. cepacia*, and *P. fluorescens* are obtained in industrial conditions and are used in organic synthesis, including catalysis of reactions in aqueous solutions.

II. Contributions in the field of microbial pathogenesis:

- **identification of microorganisms associated with infections of the urinary tract (*Enterobacteriaceae, Enterococcus, Candida*);**
- **identification of virulence factors and correlation between them;**
- **elucidation of mechanisms of microbial pathogenesis.**

In **Publication III.1.** the importance of phospholipases C as virulence factors in some bacteria - *Clostridium perfringens, Staphylococcus aureus, Pseudomonas aeruginosa, Listeria monocytogenes, B. cereus*, etc. is commented. Several bacterial sphingomyelinases and phospholipases are essential for virulence of extracellular, facultative, or obligate intracellular pathogens, as these enzymes contribute to phagosomal escape or phagosomal maturation avoidance, favoring tissue colonization, infection establishment and progression, or immune response evasion. Of particular importance in microbial pathogenesis is the ability of sphingomyelinase C to induces hemolysis of erythrocytes from different species, which is directly correlated with the sphingomyelin content of their membranes. PI-PLC contributes to the "survival" and spread of bacteria during the infectious process, but in addition, its action results in the formation of signaling molecules (second messengers), such as protein kinase C and inositol triphosphate, which activate cascades of enzymatic reactions critical in a number of physiological and pathophysiological processes.

Contributions in the field of microbial pathogenesis are mainly related to the identification of bacteria isolated from outpatients with urogenital tract infections, the determination of virulence factors, the establishment of their drug resistance, which is necessary both for the application of appropriate therapy and for the discovery of new approaches to the treatment of infections. Particular attention is paid to the ability of bacteria to form biofilms as one of the key strategies for "survival" in host conditions and the factors that can modulate it.

Urinary tract infections (UTIs) are the most commonly diagnosed infections in humans and represent a serious health and economic problem for society. The treatment of these diseases is related to the correct identification of the causative agents, identification of virulence determinants, and their resistance to antimicrobial agents. Establishing drug resistance of microorganisms is essential in selecting appropriate therapy, but it is also imperative because of the ability of microorganisms to acquire resistance through different mechanisms. The increasing resistance of microorganisms is a global environmental problem and requires the search for alternative approaches to treating the infections caused.

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A study of 318 strains of *Enterobacteriaceae* associated with urogenital tract infections confirmed that *Escherichia coli* is the most common etiologic agent (64.8%),

followed by *Klebsiella spp*. (17%) and *Proteus mirabilis* (10.37%) (**Publication III. 3.10**.). Antibiotic susceptibility tests showed high resistance to ampicillin (49%), mecillinam (71%), doxycycline (41%) and sensitivity to cephalosporins (cefuroxime 84.6%; cefoxitin 83.7%; cefotaxime 91.5%; cefepime 87.7%) and fluoroquinolones (ciprofloxacin 85%, norfloxacin 79%, levofloxacin 83%). Significant resistance to nitrofurantoin was found (24%). Amongst the tested strains, 8.5% produced extended-spectrum β-lactamases (ESBLs). Eighty-four percent of the strains were resistant to the bactericidal activity of normal human serum. The results indicate that complement resistance is probably one of the obligate virulence factors for the majority of *Enterobacteriaceae* associated with urogenital infections. The results indicate that complement resistance is probably one of the obligatory virulence factors for the majority of Enterobacteriaceae associated with urogenital infections.

Publication III. 3.7. presents the results from virulence determinant analysis amongst 20 *Escherichia coli* strains isolated from urine samples of outpatients with urinary tract infections asymptomatic bacteriuria and pregnant women. The presence and distribution of virulence determinants as adhesins, motility, hemolysins, serum resistance, and biofilm-forming were investigated phenotypically and with multiplex PCR in correlation. We found that strains poses different combinations of virulence capabilities and the structure statistically most often found is the type 1 pili.

The results of a susceptibility study of 28 clinically isolated *E. coli* strains associated with urinary tract infections to β-lactams, aminoglycoside antibiotics, and sulfonamides are presented in **Publication III. 2.1.** The lowest susceptibility was found to β-lactam antibiotics (ampicillin and ampicillin/sulbactam) and relatively high to aminoglycoside antibiotics (gentamicin and amikacin). The highest sensitivity was recorded to nitrofurantoin and cefotaxime/clavulanic acid. Approximately half of the strains studied were multidrug-resistant, and six of them synthesized broad-spectrum β-lactamases (ESBLs). The plasmid profile of the isolates is analyzed and their involvement in drug resistance is discussed.

The biofilm-forming capacity of 50 strains *E. coli* was associated with different urinary tract infections was investigated for the elucidation of mechanisms of microbial pathogenesis (**Publication III. 2.3.**). Biofilm production was detected in 36% of the isolates, and 24% produced α-hemolysins. No significant correlation was established between biofilm-formation and α-hemolysis. A relatively low presence (16%) of type 1 fimbriae was found, while 23 (46%) of the strains showed MRHA activity that could be mediated by P fimbriae, X, FIC, and DR fimbriae. Type 1 fimbriae, which facilitate adhesion to host epithelial cells, are important in the initial stages of biofilm-formation, and it is possible that after successful colonization and transition to commensalism, strains may have "lost" some virulence factors. The studied isolates showed relatively high resistance against the tested antibiotics - 19 (38%) strains had multiresistant phenotype, and 8 (16%) strains produced ESBLs (broad-spectrum β-lactamases). Evaluation of the relationship between biofilm-formation and other virulence factors such as antibiotic resistance, presence of haemolysins, and morphotype of the strains studied, showed that biofilm formation was not directly related to other virulence determinants.

Bacteria with high biofilm-forming capability cause serious trouble in medical practice as a source of both contaminations of indwelling medicinal devices, and nosocomial infections. Biofilm microorganisms develop antibiotic resistance more rapidly than plankton. For this reason, novel anti-biofilm strategies address the search for substances that may suppress the biofilm growth of pathogens without killing the microorganisms themselves.

In **Publication III. 2.4**. are presented the results of a search for plant substances corresponding to these requirements. Three strains of urinary clinical isolates of *E. coli*, two uropathogenic (UPEC) and one from asymptomatic bacteriuria (ABU), with pre-established biofilm proficiency were compared. Antibiotic resistance of the strains was determined by the disk-diffusion assay. Each of the UPEC strains was resistant to two antibiotics while the ABU strain was multiresistant. The antibacterial and antibiofilm effects of 14 extracts in different organic solvents from four medicinal plants (*Rhodiola rosea*, *Arnica montana*, *Petasites albus*, *Petasites hybridus*) were tested. The dried extracts were dissolved as stocks in ethanol. Disk diffusion assay with different amounts of the extracts showed no antibacterial activity against the selected strains. All of the extracts modulated biofilm growth, and four had significant biofilm suppression effects on the UPEC strains while they stimulated the attached mode of growth of the ABU strain. Meanwhile, the extracts had no significant influence on the growth curves of the UPEC but could delay the growth of the ABU strain. The registered opposite effects on pathogenic and non-pathogenic strains by Rr1, Rr2, Am1, and Am2 confirm that they have good potential for antibiofilm applications in medical practice.

The study presented in **Publication III. 2.5**. addresses, whether products released by antagonistic bacteria – Lactobacillus isolates from vaginal and dairy-product samples, could be useful for controlling *E. coli* biofilms. The effects of diluted cell-free supernatants (CFS) from late-exponential *Lactobacillus* cultures on the growth and biofilm production of *E. coli* were tested. Most of the CFS applied as 10^{-2} had no impact on bacterial growth, biofilm development however was influenced even by 10^{-4} of CFS. Biofilm modulation varied between different CFS and *E. coli* combinations from inhibition to activation; however, three of the tested CFS showed consistency in biofilm suppression. This was not due to antibacterial activity since Live/Dead fluorescence labeling showed insignificant differences in the number of dead cells in control and treated samples. Some *E. coli* strain-specific mechanisms of response to the three CFS included a reduction in hydrophobicity and motility. Released exopolysaccharides isolated from the three CFS stimulated sessile growth, but proteinase K reduced their inhibitory activities implying participation of protein or peptide biofilm suppression factor(s).

Escherichia coli is the most frequently isolated, but not the only etiological agent, causing urinary tract infections. Often such conditions are caused by other members of the Enterobacterakes order or Gram-positive species, such as *Staphylococcus saprophyticus*, *Enterococcus spp*., fungi, etc. The two species *Enterococcus faecalis* and *E. faecium* are the third most commonly isolated pathogens in catheter-associated urinary tract infections (CAUTI). The biofilm-forming capabilities of 72 strains of *Enterococcus faecalis*, isolated from the urogenital tract of outpatients are explored in **Publication III. 3.11.** The obtained results demonstrate that urinary tract infections (UTI), caused by enterococci are more frequent among children up to 10 years of age, while genital tract infections (GTI) are most

often observed in women of reproductive age. Antimicrobial resistance was low, with higher levels for UTI compared to GTI strains. The results demonstrate 100% susceptibility to penicillins, which are the most effective agents for the treatment of infections caused by *E. faecalis*. The resistance to fluoroquinolones was less than 19 %, with clearly defined cross-resistance. Biofilm formation was established for 26% of the tested strains after 24 h of cultivation on tryptic soy broth, with OD630 values for the biofilms in the range 0.050-0.200. This categorizes the strains as low-grade biofilm-forming strains.

In **Publication III. 3.12**. are presented the results from studies of the taxonomic composition, antifungal resistance, and some virulence factors of *Candida* strains isolated from samples of outpatients. The majority of isolates were identified as *C. albicans* (84%), followed by *Candida glabrata* (7%), *Candida krusei* (4%), *Candida parapsilosis* (3%), and *Candida tropicalis* (2%). The most affected age group includes women between 21 and 40 years old. Antifungal resistance was low and mainly associated with *C. glabrata*, with total susceptibility to the tested antifungal drugs over 95%. Analysis of the hydrolytic enzyme activities and the biofilm formation abilities showed that only 8% of the strains produced gelatinase and phospholipase, 6% produced caseinase, and 5% esterase. Seven of the tested Candida strains (7.2%) formed stable biofilm after 24 h cultivation in Sabouraud dextrose broth supplemented with 6% glucose. This study revealed no significant correlation between the antifungal susceptibility and the studied virulence factors of *Candida spp*. isolates from the genital tract of outpatients. All biofilm forming strains were sensitive to antifungal drugs and could not produce any hydrolytic enzymes. In general, the tested strains demonstrate predominantly commensal characteristics. Further studies are needed to confirm the hypothesis that the fungal infection is more a result of the change in the balance of the normal microbiota.

Publication III. 3.4. is focused on the alarming drug resistance increase and on the search for alternative therapeutic agents. A series of cyclic enaminoketones or dimedone were selectively amidoalkylated at the α-carbon atom of the enaminone. The new 2-substituted derivatives of 2,3-dihydrobenzimidazole are interesting both from a synthetic point of view and as potential bioactive compounds. Eight of the synthesized benzimidazole derivatives displayed antimicrobial activity. Some of the compounds had a clear bactericidal effect when tested against *Staphylococcus aureus*, while the effect against *Enterobacter aerogenes* was bacteriostatic.

III. Contributions in the field of environmental microbiology:

- **Evaluation of the microbiological status of water bodies; assessment of the effect of the environmental parameters and anthropogenic impact of net-cage aquaculture on the bacterial communities;**
- **Establishment of the structure and functional activity of the microbial communities in wetlands in the Maritza River Basin, Southern Bulgaria, through next-generation sequencing and physiological profiling;**
- **Construction of biosorbents for heavy metals by immobilization of waste bacterial biomass; establishing the optimal conditions for biosorption and possibilities for application in the processes of bioremediation of polluted waters.**

Contributions to the field of microbial ecology can be outlined in several directions. One of them is the determination of the microbiological status of two large and economically significant heavily modified water bodies - Dospat (**Publication III. 2.7.**) and Kardzhali reservoirs (**Publication III. 3.8**. and **Publication III. 3.6.).**

The two reservoirs are intensively used for net-cage fish farming, which can lead to water quality degradation and changes in the composition of the microbiota around the cages. In Bulgaria, there are no studies on the impact of these aquaculture facilities on the bacteriological status of the hydroecosystem of the water bodies in which they are located. In addition, in the last two decades, there is a significant increase in urbanization and coastal development, additionally increasing the risk of pollution from non-point sources. This calls for increased attention to sanitary parameters of water quality, including assessment of microbiological status (total number of heterotrophic microorganisms, and sanitary state indicators).

The microbiological study of water quality in the Kardzhali reservoir (**Publication III. 3.8.**) includes the determination of total coliforms (TC) and coli-titer at two stations in the aquatory of the water body and one station at the Arda River in August 2011. The total coliforms (TC) in the reservoir vary from 1900±674 cfu/100 ml in station I, to 1293±194 cfu/100 ml in station II, while the TC value of River Arda reaches 1698±134 cfu/100 ml. In reservoir Kardzhali, the smallest volume of water in which *Escherichia coli* cells were found, varies between 5 and 15 ml, while for the River Arda the value of coli-titter is equal to 1. With the highest percentage, regarding the presence of microbiological species in the reservoir waters, is the genus *Klebsiella* (70%), followed by *Citrobacter* (15%), *Enterobacter* (10%), and *Serratia* (5%), respectively represented by the species *Klebsiella oxytoca, Citrobacter freundii, Enterobacter cloacae* and *Serattia marcescens*. In the Arda River two genera were found - *Serratia* (50%) and *Salmonella* (50%).

The highest levels of contamination with sewage water, based on the study of coli-titter (1ml) and coli-index (200 cfu *E. coli*/1L) in the three studied stations, are established for the waters of the Arda River. The absence of pathogenic species, combined with a decrease in the number of TC, coli-titter, and coli-index in station II is evident for the self-purifying capability of the studied reservoir. The rise in the number of TC at station I in proximity to Glavatarci village, as well as the presence of fecal coliforms (FC), indicates a secondary site of contamination with sewage waters in the region.

Research in this area was continued by monitoring the seasonal dynamics of major indicator groups (*Escherichia coli*, fecal streptococci (FS), and *Clostridium perfringens*) in the waters of the Kardzhali reservoir in the period April - March 2011/12, by the collection of water samples from six sites in the reservoir aquatory (**Publication III. 3.6**.). Except for August and October, the count of *E. coli* and FS did not exceed 30 cfu/100 ml and 20 cfu/100 ml respectively. In August was established an increase in the values for the two microbial indicators and this trend continued during November. Statistically significant differences were established for the *E. coli* and FS count between the stations located in the control zone of the reservoir and those at the net-cage farms and Glavatartsi village, subjected to strong anthropogenic influence. The presence of *Clostridium perfringens* in the studied samples was established only during August, mainly at the stations located near the net-cage farms and

near the dam. A total of 220 strains of fecal coliforms were isolated and identified, to establish the basic species composition. All of them fall into 5 genera of the *Enterobacteriaceae* family. The monitored microbiological parameters show pronounced seasonal dynamics in the Kardzhali Dam and the Arda River, with peak values of all indices in August. The established species composition of the coliform group suggests that microbiological pollution is mainly due to increased environmental pressure caused by human activities in the area. On the other side, the low percentage of the FC and *E. coli* in the area of stations V and VI is indicative of the high self-purification capability of the Kardzhali reservoir.

Studies were carried out in the Kardzhali dam to establish the relationship between ecological parameters, microbiological indicators of water quality, and phytoplankton, with a respect to the development of intensive cell aquaculture (**Publication III. 2.14**.). Data sets of eighteen parameters from 5 monitoring sites during 2016–2018 were used for analysis. We have applied multivariate methods, aiming to identify the key parameters affecting the communities, including the impact of the net-cage farms. The ANOSIM (analysis of similarities), showed significant differences in the values of physicochemical factors between the control site and the area for aquaculture, with higher nitrate, total nitrogen, and COD (chemical oxygen demand) content near the net-cages. The results were confirmed by the high R-value ($R=0.87$; $p<0.01$). The conducted PCA (principal component analysis) showed that the physicochemical parameters grouped in principal components (PCs), explaining 90.5% of the total data variation. PC1 was formed by nitrogen forms and COD, PC2 represents the physical source of the variability (pH and dissolved oxygen) and PC3 was loaded with total phosphorus (0.537) and ammonium nitrogen (0.764) concertation. The parameters with the highest impact on the abundance of heterotrophic bacteria (TVC) include temperature, TN, and COD, while the phytoplankton community was negatively correlated with Secchi depth and COD. The redundancy analysis confirmed that the location of the sampling station significantly affects the studied variables and that net-cage aquaculture is a major anthropogenic factor in the Kardzhali reservoir.

The analysis of the waters of the Dospat reservoir aims to establish the microbiological status of the water body and to identify possible secondary sources of contamination in the water body (**Publication III. 2.7**.). Six stations located in the reservoir area and one station on the Dospatska River were selected for assessment of the main microbiological indicators. The seasonal dynamics of the total viable count (TVC 20°C), total coliforms (TC), E. coli, fecal streptococci (FS), and *C. perfringens* were studied for the period April 2011 – March 2012. The values for the TVC 20^{o}C were within the range of 1.103cfu/100 ml to 39.103 cfu/100 ml, without statistically significant differences between stations. The average values for TC varied from 10 cfu/100 ml to 100 cfu/100 ml and in August it rose to 1000 cfu/100 ml with higher numbers near the net cage farms. The presence of *E. coli*, FS and *C. perfringens* during the spring months was not established. A slight increase of the sanitary-state indicator was observed in August with values of 26 cfu/100 ml, 20 cfu/100 ml, and 10cfu/100 ml for *E. coli*, FS and *C. perfringens* respectively, with similar trends in November. Coliforms were characterized by low species diversity with the domination of *Serratia marcescens*, *Pantoea aglomerans*, *Hafnia alvei*, and *Enterobacter*

cloacae at stations tree, four and five. The analysis of the VC 20°C and TC showed a pronounced seasonal dynamic in Dospat Dam. The waters of River Dospatska were characterized with higher levels of microbial load in comparison with the open aquatory of the water body for the whole period of the study. The higher level of occurrence of the monitored microbial parameters, recorded at station III evidence that net cage farm acts as a secondary source of organic contamination in the area.

In addition to the established microbiological status of the two large reservoirs, the antibiotic resistance of bacterial strains isolated from the sediments under the net-cage farm in the Kardzhali dam was assessed (**Publication III. 2.6**.). Antimicrobial resistance is a direct result of selective pressures caused by the overuse of antibiotics, including the misuse of antibiotics in veterinary medicine and aquaculture. Surveillance of bacterial susceptibility to 16 antimicrobial agents was performed for 160 Gram-negative strains (*Pseudomonas mandelii* - 100 strains; *Hafnia alvei* - 30 strains; and *Raoultella ornithinolytica* - 30 strains). No significant differences in the resistance to the tested antibiotics were observed between the strains isolated from under the cag farm and from the control area (analysis of variance, $P > 0.05$). Widespread resistance to penicillins and certain cephalosporin antibiotics was observed in both stations. None of the studied strains showed resistance to the aminoglycoside antibiotics gentamicin and amikacin, or ciprofloxacin. Minimal Inhibitory Concentrations (MIC) were determined for five of the tested antimicrobial agents by the microdilution antibiotic sensitivity assay. The data indicate that amikacin, tetracycline, and ciprofloxacin effectively suppress the growth of the tested microorganisms. The isolates from genus *Pseudomonas* showed the highest MIC and were characterized by the highest percentage of antibiotic resistance.

Microorganisms inhabiting freshwater environments are an integral part of aquatic ecosystems. Very few data are available regarding the profiles of the microbial communities in the reservoirs in Bulgaria, despite their key role in the biogeochemical processes. In **Publication III. 2.8.** the first comprehensive metagenome analysis of the planktonic bacterial community of two large and economically important dams in Bulgaria - Batak Dam and Tsankov Kamak Dam is presented. Analysis of the metagenomic amplicon datasets, including quality filtering, clustering of Operational Taxonomic Units, and taxonomy assignment revealed that 78.45%of the microbial communities between the two reservoirs were overlapping. The diversity (H) and Pielou's evenness (J) indices declined along the longitudinal axis of both reservoirs. The estimated values for the Shannon diversity index are typically observed in oligotrophic lakes. The microbial communities of both reservoirs were dominated by Proteobacteria, followed by Actinobacteria and Bacteroidetes all comprised over 95% of the relative abundance, regardless of the reservoir's large hydrogeological differences. The bacterioplankton was characterized by high phylogenetic heterogeneity in the taxonomic structure, being distributed among 211 genera. The genera *Limnohabitans* and *Rhodoferax* held the absolute predominance, implying their significance in the aquatic food webs. The obtained data can contribute to a better systematic understanding of the microbial diversity of freshwater environments.

Another area of research in the field of microbial ecology is the characterization of microbial communities in wetlands in the Maritsa River basin, southern Bulgaria,

(**Publication III. 2.11**; and **Publication III. 2.18**.). Wetlands are important ecological territories or aquatories, and their microbial community structure, including biofilms, play a significant role in primary productivity, nutrient cycling, and water pollution. Constructed wetlands are low-cost ecological facilities based on the idea of biological treatment methods for remedying anthropogenic pollution. Wetlands are located in areas of low elevation and play an important role in purification near urban areas. Studies of interspecific communities and their ability to develop metabolic networks and biofilms may be useful in bioremediation processes of contaminated habitats.

The status of microbial communities and their ability to form biofilms in two Natura 2000 protected wetlands - "Zlato Pole" and Tsalapitsa rice fields in southern Bulgaria was analyzed (Publication **III. 2.11**.). The numbers of heterotrophic bacteria (TVC22 and TVC 37), actinomycetes, fungi, and sanitary state indicators were determined for dry soil samples and sediments collected from Zlato pole wetland and Tsalapitsa rice paddies. The number of heterotrophic microorganisms (TVC22 and TVC 37) and indicators of sanitary status (FS, FC, and *Escherichia coli*) in the two rice paddies near the city of Plovdiv is higher in comparison to the control zone Zlato pole – the maximum was recorded in the rice paddy near Tsalapitsa village (C_1 and C_2 was $12.6x10^6$ $cfu.g^{-1}$ and $26x10^6$ $cfu.g^{-1}$, respectively). In the studied samples, the bacterial complex takes a dominant position and it exceeds the number of both fungi and actinomycetes at least 1.5 times. The cluster analysis showed a high similarity between the soils surrounding the paddy fields and separated sediment from Zlato pole (ZP2) because of the lowest organic load. Biofilm formation analysis showed a good correlation between microbial community structure and biofilm formation capacity.

The wetlands located along the Bulgarian part of the Maritsa River basin are part of the Ramsar Convention and represent rare specific ecosystems. Our study investigated the spatial variation and physiological diversity of soil microbial communities in natural wetlands and constructed rice paddies in the Maritza River basin, protected under the Birds Directive 2009/147/EC as natural habitats (**Publication III. 2.18**.). The metabolic activity of microorganisms in the studied wetlands differed significantly, proving that the profile of the substrate used is not determined by a single environmental factor, but by a wide range of parameters such as soil water content, pH, organic matter, and nitrogen sources. The PCA and cluster analysis revealed that the long-term agricultural exploitation is related to changes in soil properties and bacterial communities, grouping the rice paddies in a separate cluster. The continuous rice cropping in the Tsalapitsa wetland leads to soil acidification and is related to a higher overall metabolic activity but a lower catabolic richness and substrate diversity which makes the microbial communities sensitive to stress and external factors. The higher substrate diversity, along with the sandy river-like sediments, the low concentration of organic nitrogen, organic matter, and phosphates, are evident for the good ecological potential of the Zlato Pole wetland. This is also confirmed by the higher metabolic activity regarding the more difficult utilization of polyols and amino acids due to the lack of easily digestible carbohydrates.

The bacterial microbiome in the natural wetland of Zlato Pole and protected, periodically flooded rice fields in the Maritza River basin was analyzed (**Publication III. 2.15.**) using a metagenomic approach, based on high-throughput sequencing (NGS). Alpha-

diversity analysis showed a significant variation between the three study sites for Chao1 and ACE (abundance-based coverage estimator) richness estimators. A positive correlation was established with pH, with the highest values detected for the rice paddies and the lowest, for the Zlato Pole sediments. The obtained sequences were assigned into 37 known bacterial phyla with over 97% bacterial sequences classified within only nine of them. The bacterial communities in rice paddies sediments were more evenly distributed, whereas the Zlato Pole sediment was the most biased. The consortiums in the rice paddies were dominated by *Proteobacteria*, followed by *Actinobacteria* and *Acidobacteria*. The bacterial assemblages in those sites could not be distinguished by analysis of similarity. The Zlato Pole sediment community held an isolated position, where *Acidobacteria* was replaced by *Firmicutes* and *Bacteroidetes* and the microbiome showed an extremely high abundance of Gammaproteobacteria and *Bacilli*. The dominance of Gammaproteobacteria and the presence of *Deinococcus-Thermus* phylum, along with lower nutrient concentration and the absence of correlation with the environmental parameters, is evidence of constant anthropogenic pressure around the area.

The present study provides the first detailed analysis of the bacterial diversity in two different wetlands in the Maritsa River Basin. The results revealed a significant difference in the bacterial community structure between the permanently inundated sediments of the Zlato Pole wetland and the seasonally flooded sediments of the protected area "Orizishta Tsalapitsa". Multivariate analyses grouped soil microbiomes in the Maritza River basin based on soil type, wetland type, and land reclamation activities. The results confirm the significant importance of environmental factors on microbial community structure. Microbial communities drive biogeochemical cycles in wetlands; they support soil function and are easily affected by anthropogenic pressure.

A current trend in modern ecology is the removal of various pollutants whose presence in the biosphere is continuously increasing due to intensive industrial and urban growth. Contamination by metals, in particular, deserves special attention due to their toxicity and potential to bioaccumulate via the food chain. Biosorption is a cost-effective and environment-friendly technology, an alternative to conventional wastewater treatment methods. Biosorption is a metabolically independent process, in which dead microbial biomass is capable of removal and concentrating metal ions from aqueous solutions. Dead microbial biomass shows many advantages over living cells: low cost, absence of nutrient medium and maintenance of pure microbial cultures, high sorption and desorption rate, work over a wide pH range, use of simple equipment, rapid and easy regeneration of biomass used. Free microbial biosorbents are of small size and low density, insufficient mechanical stability, and low elasticity, which causes problems with metal ion desorption, the separation of the sorbent from the medium, and its regeneration. By immobilizing microbial biomass on suitable carriers the disadvantages of free biosorbents are eliminated and more opportunities for practical use of biosorption become available. The study in **Publication III. 2.12.** examines different immobilization techniques and carriers, certain basic features and possibilities of using immobilized microbial biosorbents for the removal and concentration of metals from aqueous solutions.

Biomass from bacteria - Gram-positive (*Bacillus sp., Corynebacterium sp*.), Gram-negative (*Escherichia sp., Pseudomonas sp*.) and cyanobacteria (*Anabaena sp., Sinechoestys sp.*), molds (*Aspergillus sp., Rhizopus sp*.), basidiomycetes (*Trichosporon sp., Trametes sp*.), yeast (*Saccharomyces sp., Candida sp*.), waste microbial biomass from the production of antibiotics, enzymes, amino acids, and other biotechnological manufacturing processes can be used as biosorbents. Waste biomass from *Bacillus cereus* immobilized in the sodium alginate and co-immobilized with activated carbon or with bentonite into alginate gel was studied for Pb(II), Cd(II), and Hg(II) removal from aqueous solutions. The composite biosorbent consisting of waste *B. cereus* biomass co-immobilized with activated carbon into alginate beads was selected as the most prospective for heavy metals removal. Immobilization increased both the removal capacity and the mechanical strength of the biosorbent. Major process parameters were optimized and maximum removal efficiency of 92.13% was reached for Pb(II) ions at pH 5.0, biosorbent dosage 2 g/L, temperature 25°C, agitation speed 120 rpm for 120 min (**Publications III. 2.13.)**.

The possibility of applying *Bacillus thuringiensis* waste biomass as a biosorbent for Pb(II), Cd(II), and Hg(II) from model aqueous solutions was also investigated (**Publication III. 2.17**.). Heat inactivated and alkali-treated biomass showed ability for removal of metal ions from single solutions in the following order Pb(II) > Cd(II) > Hg(II). It was proved that the major groups involved in biosorption are hydroxyl/amino, alkyl, carbonyl and phosphoryl groups. The influence of different factors as pH, initial sorbate concentration, biosorbent concentration, contact time was evaluated. The highest removal capacity for Pb (II) was reached at optimal process parameters pH 5.0, biomass dosage 1 g.dm-3 and contact time 90 min. The studied biosorbent is prospective because it successfully removes heavy metals not only from single but even from ternary solutions, which means that is effective at model conditions closer to real wastewaters.

Contributions of ecological but also applied importance could also include the results reported in **Publication III. 2.9**. The research presents the effect of novel edible coatings based on low molecular weight chitosan on some properties of fresh-cut melon fruits – weight loss, total soluble solids, total acidity, mechanical strength, and bacteria growth. Three different compositions were used as coatings – pure chitosan, chitosan, and Ca-lactate and alginate/chitosan multilayers. It was shown that the additional alginate layer substantially improves the protective properties of pure chitosan coating, resulting in the preservation of cell structure. In the presence of chitosan coating alone, there was an initial reduction in total counts of about two times within the initial day of the experiment. This difference remained constant during the whole storage time. Chitosan + Ca and chitosan + alginate coatings also demonstrated antimicrobial activities, but they ensure less protection than the chitosan alone.

IV. Contributions towards the field of education.

1. Author of a textbook: S. Kostadinova. „Microbial metabolism“, Plovdiv University Press “Paisii Hilendarski”, 2021, 287 pages, ISBN 978-619-202-641-7.

The textbook on “Microbial Metabolism” is intended for use by students from different biological specialties of Plovdiv University “Paisii Hilendarski”.

Catabolic and anabolic processes in microorganisms are presented, with emphasis on prokaryotes, which are distinguished by great metabolic diversity, enabling them to assimilate a variety of substrates and to develop in all possible habitats on Earth. Catabolic processes and the ability of bacteria to utilize different substrates and generate energy due to their specific enzymatic activity are discussed in detail. The biosynthetic processes of the main building components of the microbial cell (carbohydrates, lipids, proteins, nucleic acids) from the precursor molecules are commented. The unique metabolism in specific groups of microorganisms, such as methanogenic archaebacteria, sulfate-reducing bacteria, halobacteria, and phototrophic prokaryotes, which occupy specific ecological niches and are of great importance in physiological processes in the biosphere, is discussed.

Knowledge of the metabolism of microorganisms is the basis for elucidating their role in the environment, including their involvement in biogeochemical cycles, the establishment of antagonistic and symbiotic relationships, and the possibility of their application in biotechnological processes.

2. Co-author of a textbook for practical classes in Microbiology:

S. Kostadinova, V. Gochev, M. Markhova, T. Girova, D. Georgiev, I. Iliev. Textbook for practical classes in Microbiology. 2017. Plovdiv University Press "Paisii Hilendarski", 265 pages**,** ISBN 978-619-202-240-2.

The microbiology manual is developed in accordance with the curricula for the training of students in bachelor and master specialties at the University of Plovdiv "Paisii Hilendarski".

The exercises in the manual are grouped thematically, allowing teachers to compile the necessary set for the respective course. The manual includes 10 main sections - "Microscopic techniques", "Basic laboratory culture methods", "Morphology of microorganisms", "Biochemical activity of microorganisms", "Effect of environmental factors on microorganisms", the manual also includes exercises in the field of "Sanitary microbiology", "Role of microorganisms in the cycling of substances", "Microbial genetics" and "Medical microbiology". The exercises aim to provide students with knowledge of modern experimental techniques in the field of microbiology, as well as the skills to interpret and report the results obtained.

3. Co-author of a textbook: „Biological membranes"

I. Denev, St. Spasieva, D. Stefanova, E. Daskalova, M. Gevezova, M. Markhova, S. Kostadinova. **Biological membranes**. **2016**, Electronic edition, **Plovdiv University Press "Paisii Hilendarski"**, 181 pages, ISBN 978-619-202-111-5.

This textbook presents the current knowledge of the structure and function of biological membranes based on scientific discoveries in recent decades. The textbook is structured in three sections. The first section discusses the biochemical composition and structure of biological membranes as well as the structural features of membrane components. The second section is devoted to the main functions of membranes - transport of low- and high-molecular compounds, receptors and signaling. The third section presents the specific membranes in organisms - the structure and functions of bacterial membranes; the unique

composition and structure of archaebacterial membranes, as well as some intracellular membrane structures in prokaryotes are considered. Internal membranes in plant and animal cells are described.

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