"OVIDIUS" UNIVERSITY CONSTANTA
FACULTY OF "NATURAL SCIENCES AND AGRICULTURAL SCIENCES"

PhD Thesis

summary

BIOCHEMICAL STUDY OF *Escherichia coli* BACTERIA ISOLATED FROM THE SEA WATER AND SOME IMPLICATIONS IN HUMAN PATHOLOGY

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physical-chemical factors.

SUMMARY

OBJECTIVES AND PURPOSE OF THE PAPER

The Black Sea used to be one of the most productive seas, with pelagic and benthic organisms recording a remarkable abundance (Antipa, 1941). Compared to that period, the ecosystem structure was strongly affected, registering a significant reduction in biodiversity (Gomoiu, 1981). Isolation from the world ocean, associated with significant fluvial intake (Danube, Dnieper, Dniester), determined the exposure of the Black Sea to different anthropic pressures. The problems caused by eutrophication and pollution began in the second half of the ’70. Large quantities of inorganic and organic compounds penetrated into the sea annually, both by the rivers and by discharges of municipal and industrial wastewater have caused dramatic changes at all levels of the ecosystem. The increase of nutrients and hazardous substances in the water caused major changes in coastal ecosystems, with significant impact on biological diversity and marine activities (fishing, recreational activities) (Bologa et al., 1995; Petranu et al., 1999; Gomoiu, 2004).

Fecal contamination of surface waters is a serious environment and public health issue. In complex systems, fecal pollution can come from several sources, including wastewater discharge, agricultural and urban pluvial spills. Identifying and eliminating the source of contamination is not simple, because evaluating fecal pollution generally relies on a limited number of surface water samples to measure the density of fecal indicators (Gordon et al. 2002; Byappanahalli et al., 2003).

Escherichia coli is a fecal coliform bacteria that exists in the animal and human intestine. The presence of the *Escherichia coli* bacteria in water is a strong indicator of recent contamination with animal dejection or wastewaters. During rains, melting snow or other precipitation, these bacteria can be carried in bays,

rivers, lakes or groundwater, and when these waters are used as drinking water and not treated or inadequately treated, the bacteria can get into the drinking water (Llopis et al., 2004).

Numerous studies have indicated that *Escherichia coli* can resist in benthos, being subsequently detected in surface waters. Residual pollution persists even at low temperatures, where fecal coliform levels in wastewater initially rapidly decrease, but then stabilize at 1% to 10% of the total initial population. Moreover, *Escherichia coli* isolated from septic tanks proved to be less genetically variable, forming a distinct clone, unlike *Escherichia coli* streams isolated from inhabitants of households serviced by these systems.

Although most *Escherichia coli* streams are nonpathogenic and live in healthy human and animal intestines, there still are some *Escherichia coli* streams that may express virulence factors acquired from pathogenic species, factors responsible for the occurrence of severe clinical forms of infection.

The *Escherichia coli* bacteria is also a sanitary indicator, as its presence in the water provides some indication on contamination with other highly pathogen enteric microorganisms (*Salmonella sp., Shigella sp.*, or even different enteroviruses) having the intestinal habitat as source (Bărzoi, 1985).

The purpose of this study is to investigate the antibiotic resistance profile and distinctive aspects of virulence and pathogenicity, both phenotypically and genotypically as well as the influence of the physical-chemical factors, in a lot of 100 *Escherichia coli* strains isolated from sea water on the Black Sea coast.

For this purpose the following objectives were proposed:

- Isolating and identifying the *Escherichia coli* strains from sea water on the Black Sea coast;
- The phenotypic study of resistance to antimicrobial substances, antibiotics, and divalent transition metals markers in the isolated strains;
- the study of the adhesion capacity in *Escherichia coli* strains an abiotic (plastic polymer) and biotic (HeLa cells) substrate;
- characterization of the soluble virulence factors in *Escherichia coli* strains*;*
- establishing a correlation between phenotypic and genotypic aspects of antibiotic resistance and virulence;
- the study of the influence of physical-chemical parameters: cultivation medium, incubation temperature, presence / absence of O2, pH variations, composition of the cultivation medium on the phenotypic aspects of antibiotic resistance and phenotypic expression of virulence factors associated with the cell wall (adezins) and with soluble (enzyme / toxin).

PART II. PERSONAL CONTRIBUTIONS
PHENOTYPIC AND GENOTYPIC STUDY OF RESISTANCE AND VIRULENCE FACTORS IN A SET OF *Escherichia coli* STRAINS ISOLATED FROM THE SEA WATER AND OF POSSIBLE IMPLICATIONS IN HUMAN PATHOLOGY

INTRODUCTION

The purpose of the present paper was to achieve a comprehensive study of the phenotypic and genotypic level of resistance to antibiotics and of the degree of virulence, as well as the influence of physical-chemical factors on the expression of these characters with determining role in pathogenicity of these strains, on a significant lot of *Escherichia coli* strains isolated from sea water in the Black Sea coast area.

The study objectives referred to:

- isolating and identifying the de *E. coli* strains from sea water in the Black Sea coast area
- the phenotypic study of the resistance to antimicrobial antibiotic substances and to divalent transition metals markers in the isolated strains;
- the study of the adhesion capacity in *E. coli* strains an abiotic (plastic polymer) and biotic (HeLa cells) substrate;
- characterization of the soluble virulence factors in *E. coli* strains*;*
- establishing a correlation between phenotypic and genotypic aspects of antibiotic resistance and virulence;
- the study of the influence of physical-chemical parameters: cultivation medium, incubation temperature, presence / absence of O2, pH variations, composition of the cultivation medium on the phenotypic aspects of antibiotic resistance and phenotypic expression of virulence factors associated with the cell wall (adezins) and with soluble (enzyme / toxin).

CHAPTER 3. MATERIALS AND METHODS

3.1. COLLECTING AND TRANSPORT OF ANALYZED SAMPLES

For the microbiological analysis, namely for isolating the *E. coli* strains, the collection and transport of the seawater samples was performed in compliance with aseptic conditions according to current standards.

3.1.1. COLLECTION OF ANALYZED SAMPLES

The collection was performed at 10 stations along the Black sea coast (fig. 23): Năvodari, Mamaia – two stations, Constanţa, Eforie Nord, Eforie Sud, Costineşti, Olimp-Neptun, Mangalia, Vama-veche; with a bimonthly frequency, in the summer season (from May to September).

3.1.2. TRANSPORT OF ANALYZED SAMPLES

The collected samples were transported to the laboratory as quickly as possible (in maximum 6 hours), in cases refrigerated with refrigerating agents, maintaining a temperature of 5±3°C. (EN ISO 19458, 2006).

3.2. ISOLATION AND IDENTIFICATION OF *Escherichia coli* STRAINS

3.2.1. ISOLATING THE *Escherichia coli* STRAINS

For isolating the *E. coli* strains, we used the membrane filter technique; the analyzed volume was of 100 ml from the actual sample and successive decimal dilutions up to 10^{-2}. For the dilutions, we used the *peptone salin diluent* (tryptone) medium, with the following composition (SR EN ISO 9308-1, 2000). After filtration, the membrane was applied on a 50 mm diameter Petri dish, with *lactose-TTC* agar medium with sodium heptadecyl sulfate with the following composition (SR EN ISO 9308-1, 2000):

3.2.2. IDENTIFYING THE *Escherichia coli* STRAINS

For identifying the *E. coli* bacteria, we used the following tests:

- the oxidase test;
- the indole production test;
- other biochemical identification tests (polytrope tests, API kits).

3. 3. THE PHENOTYPIC STUDY OF SENSITIVITY TO ANTIBIOTICS AND TO THE SALTS OF DIVALENT TRANSITION METALS

3.3.1. EFFICIENCY *IN VITRO* EVALUATION OF A SUBSTANCE WITH ANTIMICROBIAL ACTION

The antibiotic resistance phenotypes of the *E. coli* strains isolated from sea water were highlighted by testing their sensibility to antibiotics, using the Kirby-Bauer diffusion antibiogram;

The antibiotics used (Oxoid disks) for antibiotic susceptibility testing were selected from different groups of antibiotics, according to NCCLS (2004) indications for *Enterobacteriaceae*: ampicillin (AMP 10µg); cefoxitin (FOX 30µg); ceftazidime (CAZ 30µg); ceftriaxone (CRO 30µg); imipenem (IMP 10µg); ciprofloxacin (CIP 5µg), nalidixic acid (NA 30µg); gentamicin (G/CN10µg), amikacin (AK/AN 30µg), tobramycin (NN10µg); trimethoprim / sulfamethoxazole (SXT 1.25µg/23.75µg), chloramphenicol (C 30µg), nitrofurantoin (F/M 300µg).

For testing the sensitivity to different metals, we used complex combination of divalent transitional metals: Zn, Mn, Cu, Co, Ni ($MnC_{12}H_{31}N_{10}O_5$, $NiC_{12}H_{28}N_{10}O_4$, $CuC_{12}H_{32}N_{10}O_6$, $ZnC_{12}H_{32}N_{10}O_6$).

3.3.2. DIRECT DETECTION OF β-LACTAMASES

Productions of β-lactamases using direct tests (nitrocefin hydrolysis). Nitrocefin is available in purified powder form from Becton Dickinson (Oxford, UK) disks containing antibiotic (OXOID).

3.4. HIGHLIGHTING THE PHENOTYPES OF VIRULENCE FACTORS

3.4.1. ADHERENCE TO INERT CELLULAR SUBSTRATE

The spectrum of virulence factors associated with the cellular wall in *E. coli* strains isolated from sea water, was highlighted through qualitative and quantitative methods concerning:

- The capacity study of adherence to inert / abiotic substrate;
- The capacity study of adherence to cellular/biotic substrate;

3.4.1.1. The capacity study of adherence to an inert substrate

The ability of *E. coli* strains to produce extracellular polysaccharide and form biofilms on an inert substrate was investigated in the present study, through the *Slime* factor production test, using the microtitration method (Christensen et al., 1982).

3.4.1.2. The capacity study of adherence to cellular substrate

The study of the adhesion and invasion capacity of bacteria to a cellular substrate was achieved using the Cravioto method (adapted after Lazăr, 2003), using the HeLa cellular line from the cervix neoplasm (ECACC # 93021013).

3.4.2. HIGHLIGHTING THE SOLUBLE VIRULENCE FACTORS

The analysis of soluble virulence factors in de *E. coli* strains isolated from sea water was achieved through enzymatic tests highlighting the presence / absence of: esculinase, amylase, pore-forming enzymes (hemolysin - spot and CAMP-like hemolysis; lipase; lecithinase), lizin-decarboxylase, proteases (caseinase, gelatinase), mucinase, DNase.

3.5. DETECTING RESISTANCE AND VIRULENCE GENES

3.5.1. ISOLATION, PURIFICATION AND HIGHLIGHTING PLASMIDIAL DNA

For analysis of resistance and virulence genes in *E. coli* strains isolated from sea water, the following steps were taken:

- isolation,
- purification
- highlighting the plasmidial DNA

For the isolation, purification and highlighting plasmidial DNA we used the Birnboim, Doly & Ish-Horowitz technique (Vassu et al., 2001).

3.5.2. HIGHLIGHTING THE RESISTANCE GENES TO DIFFERENT CLASSES OF ANTIBIOTICS IN *Escherichia coli* STRAINS ISOLATED FROM SEA WATER

To highlight the genes that encode enzymes inactivating β-lactam antibiotics and sulphonamides, we applied simplex and multiplex – PCR techniques.

3.6. THE STUDY OF THE INFLUENCE OF PHYSICAL-CHEMICAL FACTORS ON THE EXPRESSION OF SOLUBLE VIRULENCE FACTORS AND OF SENSITIVITY TO THE SALTS OF SOME TO DIVALENT TRANSITION METALS OF THE *Escherichia coli* STRAINS ISOLATED FROM SEA WATER

We have used the previously described methods to determine the bacterial sensitivity to complex combinations of transitional divalent metals, Zn, Mn, Cu, Co, Ni and of virulence factors, with the indication that the experiments were repeated under various conditions, in which a physical or chemical parameter was varied.

3.6.1. STUDY OF THE INFLUENCE OF PHYSICAL FACTORS

We analyzed the action of temperature, of O_2 partial pressure and of pH value on the expression of the virulence factors and resistance to divalent transitional metals.

3.6.1.1. Study of the temperature influence

The previously presented experiments were repeated at different incubation temperatures: 4°C, 22°C, 37°C, 44°C and 56°C.

3.6.1.2. Study of the influence of O_2 presence/absence

For the study of the influence of O_2 presence/absence, the experimental variants were incubated at: 4°C, 22°C, 37°C, 44°C and 56°C, for 24 hours, in aeropauses conditions (O_2 presence), and in anaerobiosis conditions (O_2 absence). The anaerobiosis conditions were achieved by adding paraffin oil.

3.6.1.3. The study the influence of pH value on the culture medium

For the study the influence of pH value on the culture medium, the bacterial strains were grown in an medium with pH adjusted to the desired value, with hydrochloric acid solutions, 10% solution for pH 5.0 value and 0, 1 N sodium hydroxide solution for pH 9.6 value.

3.6.2. STUDY OF THE INFLUENCE OF CHEMICAL FACTORS

The composition of a culture medium is very diverse and depends on the nutritional requirements of the microorganism to be cultivated.

3.6.2.1. The study of the influence of glucose concentration on the culture medium

For the study of the influence of glucose concentration on the culture medium, the strains were seeded in 1 ml nutrient broth with concentration of 1.5% and 3% glucose.

3.6.2.2. The study of the influence of NaCl concentration on the culture medium

For the study of the influence of NaCl concentration on the culture medium, the bacterial strains were seeded in 1 ml nutrient broth with the following concentrations of salt: 0%, 0.5%, 2%, 3%, 4%, 5%, 6%, 7% and 10%.

CHAPTER 4
RESULTS AND DISCUSSIONS

4.1. PHENOTYPES OF RESISTANCE TO ANTIBIOTICS OF THE *Escherichia coli* STRAINS ISOLATED FROM SEA WATER

The *E. coli* strains isolated from sea water, showed high sensitivity to most antibiotics they were tested with, namely: imipenem (IMP) 99%, cefoxitin (FOX) 99%, nitrofurantoin (F/N) 99%, tobramycin (NN) 98%, ceftriaxone (CRO) 98%, ceftazidime (CAZ) 97%, gentamicin (G) 96%, amikacin (AK) 96%, ciprofloxacin (CIP) 93%, chloramphenicol (C) 92%, nalidixic acid (NA) 92%, biseptol (SXT) 89%; being resistant only to ampicillin (AMP) 86% (resistance otherwise known as constituent for members of the *Enterobacteriaceae* family) (fig. 40, 41).

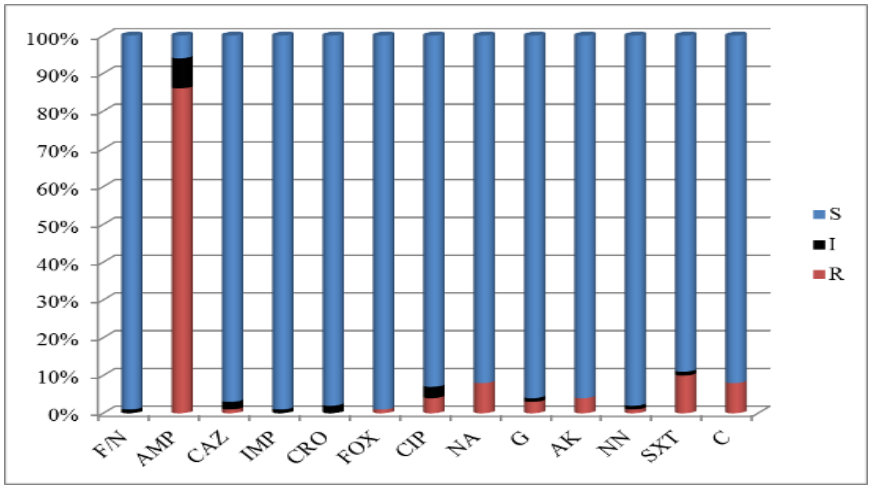


Figure 40. Graphical representation (%) of sensitivity / resistance to antibiotics in de *E. coli* strains isolated from sea water (S = sensible, I = intermediary, R = resistant).

Nevertheless, in resistant *E. coli* strains isolated from sea water, we have observed that the tested strains presented 1 to 8 antibiotic resistance markers, the most frequent associations being (fig. 42):

- AMP + SXT (10%);
- AMP+C (9%);
- AMP+CIP (8%);
- AMP+ NA (7%).

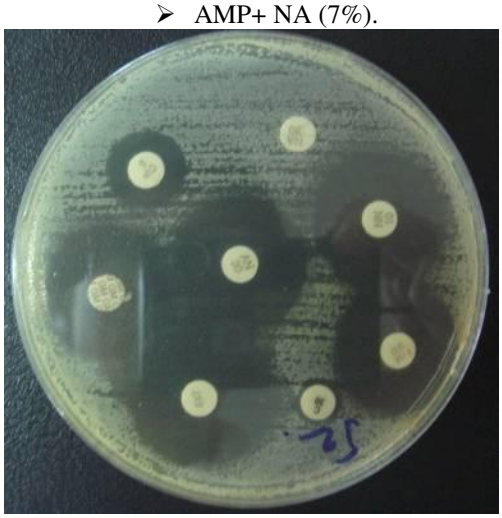


Figure 41. Antibiogram of an *E. coli* (no. 52) strain isolated from sea water.

The study of β – lactamases production by rapid nitrocefin hydrolyze test showed that 44% of the *E. coli* strains isolated from sea water expressed constitutive β – lactamase (fig. 43).

These aspects argue for the need to study antibiotic resistance phenotypes in strains isolated from the medium, aiming to identify the factors responsible for the spread of antibiotic resistance mechanisms in the external medium, with potential negative impact on human health.

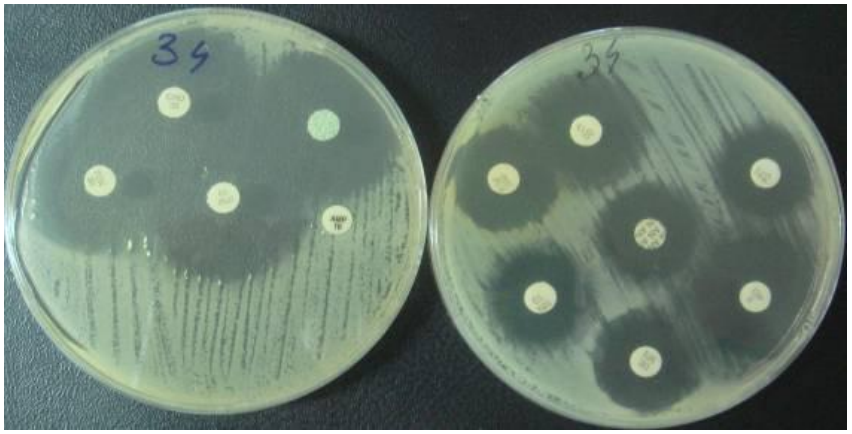


Figure 42. Antibiotic resistance phenotypes in *E. coli* strains isolated from sea water (1 resistance marker- up and 6 resistance markers - down).

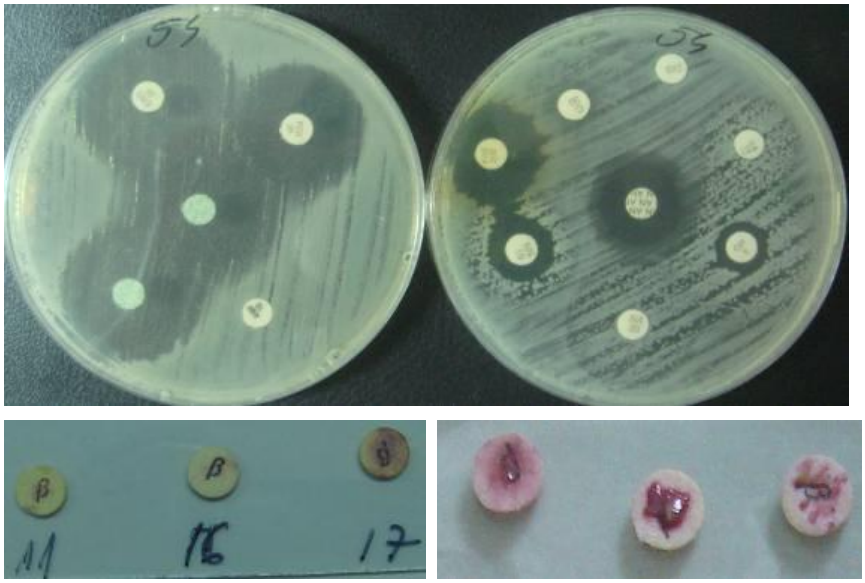


Figure 43. Highlighting β - lactamases by rapid nitrocefin hydrolyze test, in *E. coli* strains isolated from sea water (left-negative strains; right-positive strains)

4.2. SENSIBILITATEA TULPINILOR DE *Escherichia coli* IZOLATE DIN APA DE MARE LA SĂRURI ALE METALELOR BIVALENTE TRANZIŢIONALE

The present study on the resistance of *E. coli* strains isolated from sea water to divalent transitional metal salts (Zn, Mn, Cu, Co, Ni), demonstrated that most tested strains are resistant to these metals' salts (fig. 44), Ni 99%, Mn 98%, Cu 98%, Co 95%, and Zn 72% (fig. 45).

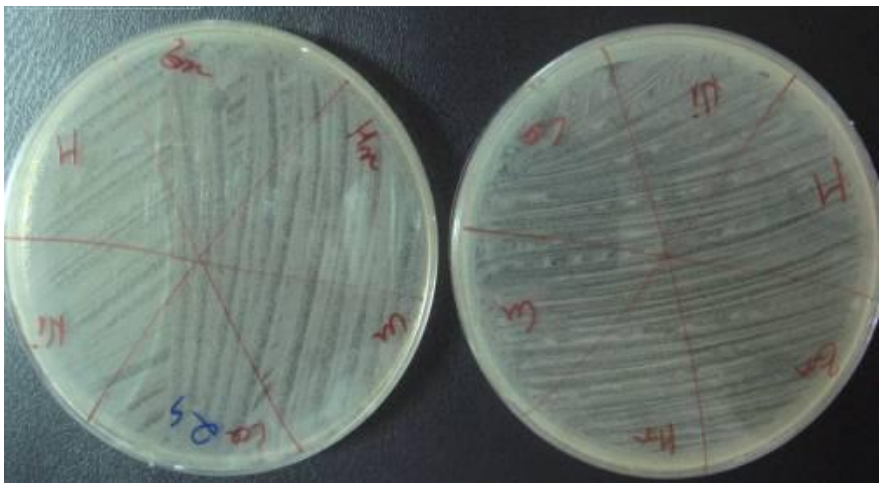


Figure 44. Testing the sensitivity of *E. coli* strains isolated from sea water to divalent transitional metal salts through the adapted diffusimetric method.

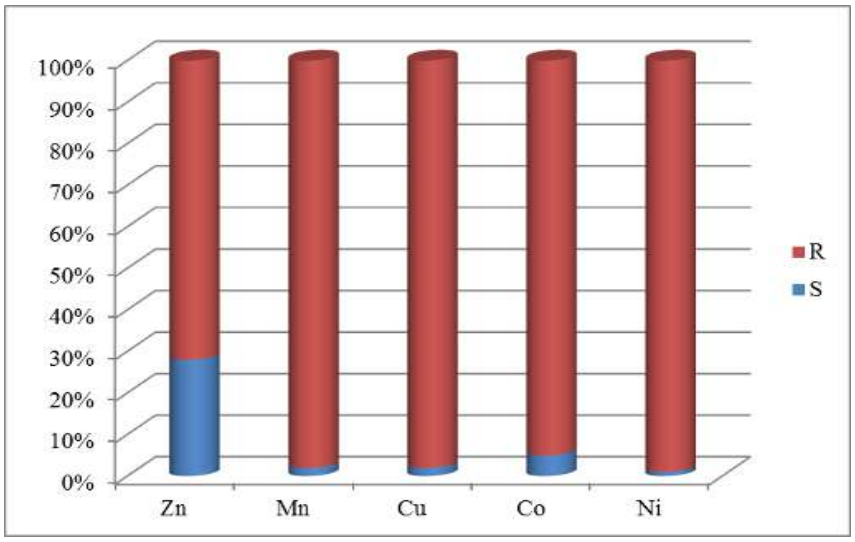


Figure 45. Graphical representation (%) of the levels of sensitivity / resistance in *E. coli* strains isolated from sea water to divalent transitional metal salts (S = sensible, R = resistant).

4.3. THE SPECTRUM OF VIRULENCE FACTORS ASSOCIATED WITH CELL WALL AND SOLUBLE, IN *Escherichia coli* STRAINS ISOLATED FROM SEA WATER

4.3.1. THE ADHESION CAPACITY TO AN ABIOTIC SUBSTRATE OF THE *Escherichia coli* STRAINS ISOLATED FROM SEA WATER

The study of the adherence capacity to an inert/abiotic substrate through the Slime factor production test, showed that *E. coli* strains isolated from sea water colonized the abiotic/inert substrate (represented by plastic) in 60% proportion (fig. 46).

The adherence capacity to an inert/abiotic substrate by plastic of the *E. coli* strains isolated from sea water, could be observed during the qualitative testing method of the adherence to the cellular substrate, after cultivating the bacterial strains in liquid medium, in 3,5 cm diameter Petri dishes (fig. 47).

4.3.2. THE ADHESION CAPACITY TO A BIOTIC SUBSTRATE OF THE *Escherichia coli* STRAINS ISOLATED FROM SEA WATER

This study has shown that a high percentage of the analyzed strains (63%) had the adherence capacity to the cellular substrate – HeLa cells, presenting adherence indicators of 30% with *patterns*: localized, aggregative şi diffuse (fig. 48)

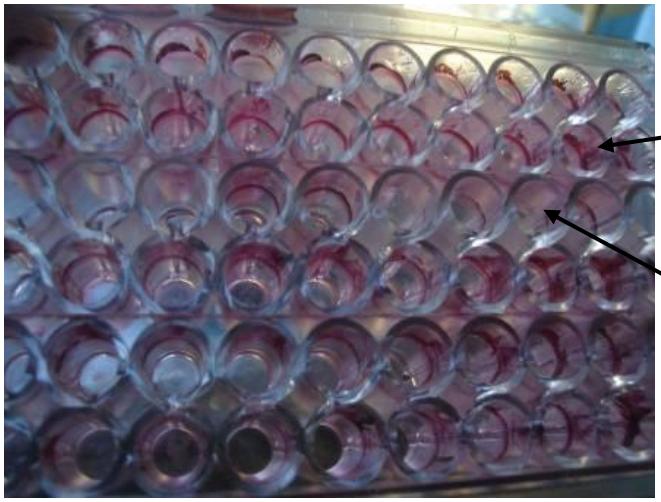


Figure 46. Representation of the experimental model used to test the capacity of the *E. coli* strains isolated from sea water to colonize the inert substrate (represented by plastic).

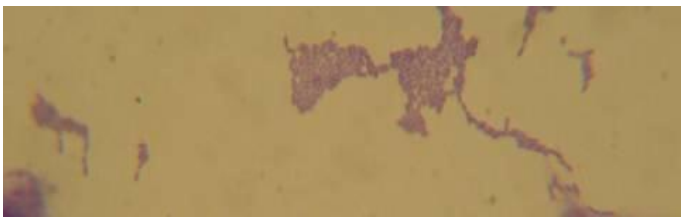


Figure 47. Optical microscopy image with *E. coli* strains isolated from sea water adhering to inert substrate (plastic plate), forming aggregates where bacteria are orderly arranged (Giemsa, x100 coloration).

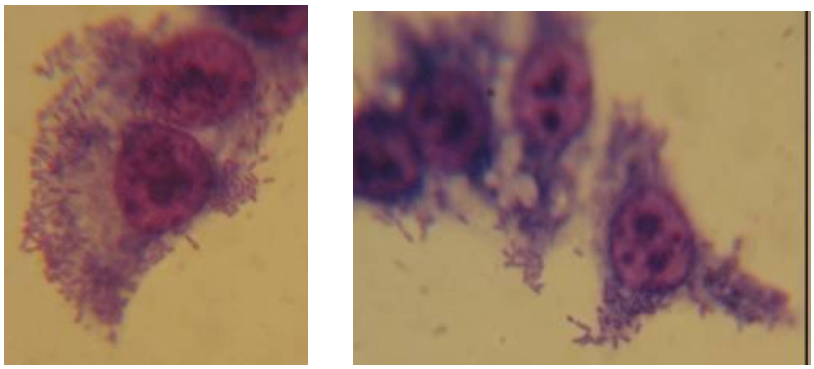


Figure 48. Optical microscopy image of the HeLa cells infected with *E. coli* strains isolated from sea water, adhering to cells in the culture (Giemsa, x100 coloration).

4.3.3. QUANTITATIVE ADHERENCE AND INVASION RATE OF THE *Escherichia coli* STRAINS ISOLATED FROM SEA WATER

The quantitative test of adherence and invasion capacity in the *E. coli* strains isolated from sea water surprisingly showed an important number of strains (70%) also presented the capacity to invade HeLa cells, demonstrating this strain's potential to invade and destroy the host cells (fig. 49).

The *E. coli* strain's capacity of invasion is demonstrated by the creation of a pathovar reuniting there strains, namely the enteroinvasive pathovar. The EIEC strains penetrate the colon's epithelial cells, where they multiply, determining lesions of the digestive mucosa. Our results could be explained by the fact that HeLa cells represent a line of epithelial origin.

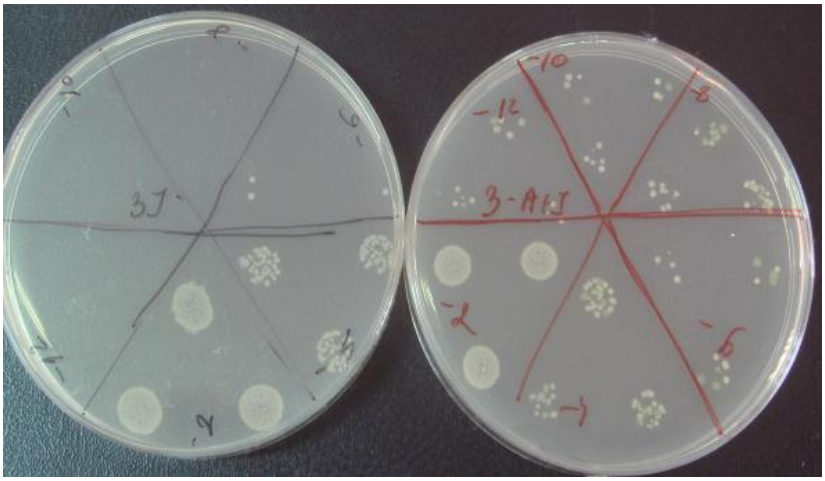


Figure 49. Quantitative study of the invasion capacity – left, respectively of the adherence and invasion capacity – right, in *E. coli* strains isolated from sea water.

4.3.4. THE EXPRESSION OF THE SOLUBLE VIRULENCE FACTORS IN *Escherichia coli* STRAINS ISOLATED FROM SEA WATER

The results of the study producing the 10 soluble virulence factors by seeding the microbial strains I mediums with incorporated specific substrate have shown that the tested strains were positive for the production of mucinase 100% (fig. 50), lizin-decarboxylase 93% (fig. 51), esculinase 67% (fig. 52), hemolysins being produced in much lower percentages (CAMP factor with *Staphylococcus aureus* β- hemolytic ATCC 25923 – 7%, spot ones 6%) (fig. 53). The tested strains are negative for all the other soluble tested factors, as shown in figure 54.

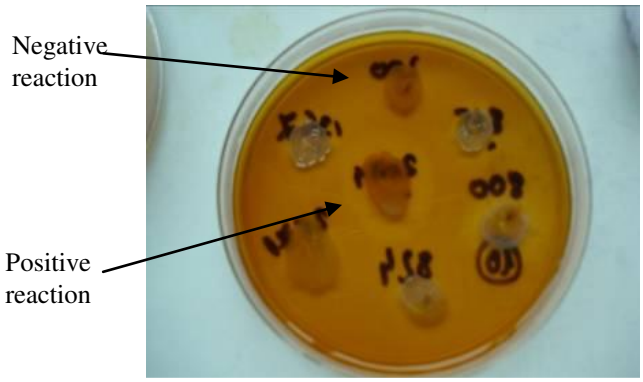


Figure 50. Highlighting the production of mucinase in *E. coli* strains isolated from sea water.

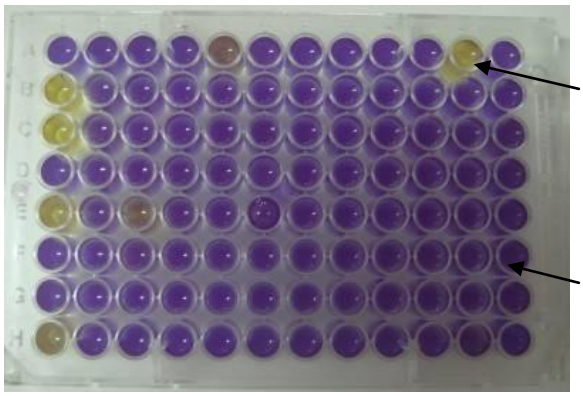


Figure 51. Production of lizin-decarboxylase *E. coli* strains isolated from sea water.

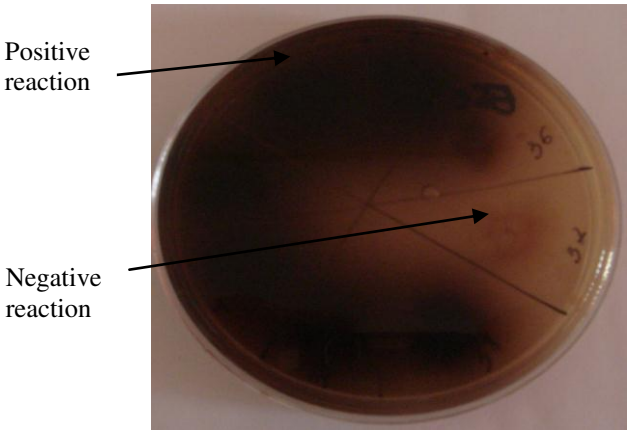


Figure 52. Highlighting the esculinase with esculetol production (black precipitate) in *E. coli* strains isolated from sea water.

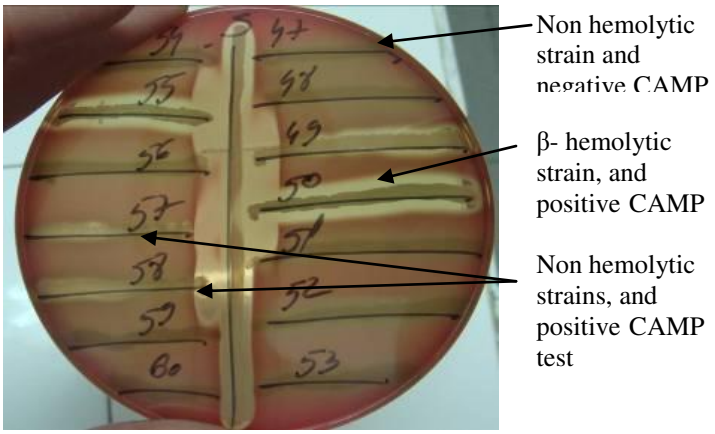


Figure 53. Production of spot hemolysins and CAMP test with *Staphylococcus* β- hemolytic ATCC 25923 in *E. coli* strains isolated from sea water.

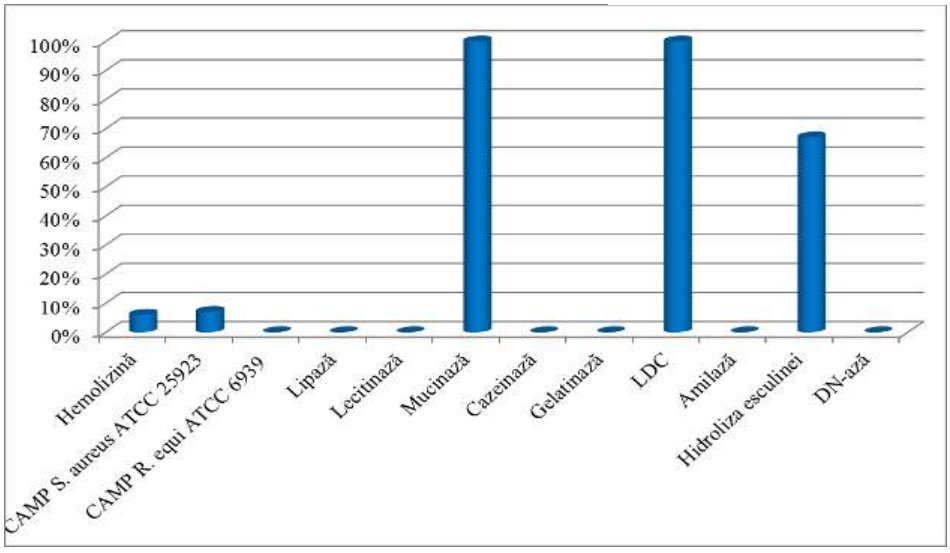


Figure 54. Graphical representation (%) the level of positivity of different soluble virulence factors in *E. coli* strains isolated from sea water.

4.4. GENETIC DETERMINISM OF RESISTANCE AND VIRULENCE IN *Escherichia coli* STRAINS ISOLATED FROM SEA WATER

4.4.1. THE STUDY OF THE PRESENCE OF PLASMIDIAL DNA

The study of the presence of plasmids in *E. coli* strains isolated from sea water has sown that 25% of them presented with at least one plasmid with variable molecular weight (fig. 56).

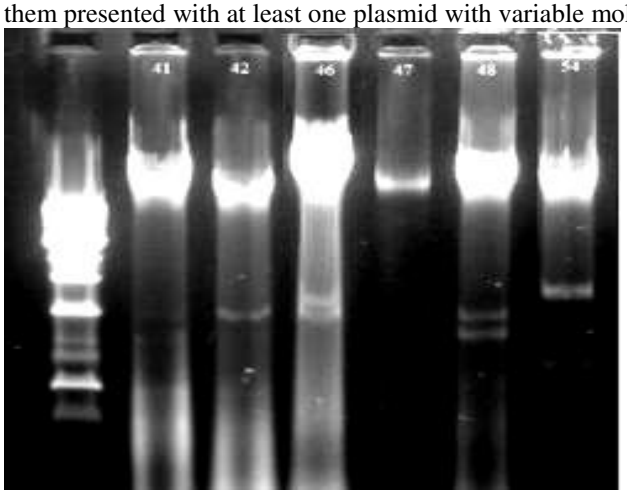


Figure 56. DNA profile migrated in 0,7% agarose gel, in *E. coli* strains isolated from sea water.

4.4.2. STUDY OF THE RESISTANCE GENES

In the present study, all *E. coli* strains resistant to sulfonamides were investigated for the presence of genes *sul*1, *sul*2 and *sul*3 by PCR. The *sul*1 gene has been detected in 55% of the sulfonamide resistant isolates, the *sul*2 gene in 22% of isolates, while the *sul*3 gene has not been found in marine isolates (fig. 57). The investigation of the presence of β-lactamase resistant genes: bla_{TEM1}, $bla_{SHV1,}$ bla_{OXA1} an bla_{CTX-M} by PCR techniques has shown that all strains were negative for the presence of these genes.

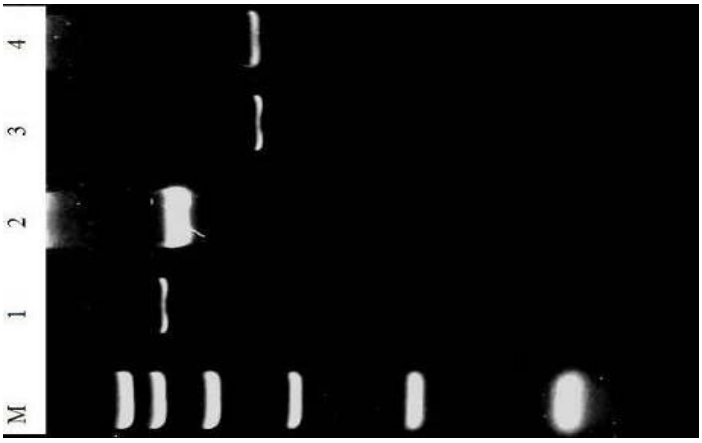


Figure 57. Highlighting by agarose gel migration of sulphonamides resistance gene amplicons, obtained by PCR multiplex, in *E. coli* strains isolated from sea water (strips 1, 2 - samples amplified with *sul*2 primers; strips 3, 4 - samples amplified with *sul*1 primers).

4.4.3. GENETIC VIRULENCE DETERMINISM IN *Escherichia coli* STRAINS ISOLATED FROM SEA WATER

In the present study, the *E. coli* strains isolated from sea water have been investigated through the PCR technique for the presence of genes codifying proteins involved in adherence: *aggR*, transcriptional activator of genes for type I expression fimbrias in enteroaggregative *E. coli* (EaggEC), of the genes for afimbrial adezins (*aaf*I / II), and of the genes involved in the synthesis of some toxins – the genes for thermostable enterotoxin (*EAST /* 1). All strains were negative for the presence of these genes.

In the case of the present study of the phenotypic expression of soluble virulence factors, out of the 10 soluble virulence factors, 2 factors (lipase and DNase) could not be highlighted, in none of the cultivation conditions. Given that some genes may exist without phenotypic expression, the present study has investigated all strains for the presence of *EcpldA* and *EchelD* genes by PCR. The *EcpldA* gene has been detected in 69%, and the *EchelD* gene has been detected in 73% of the isolates (fig. 58).

The densitometric analysis of the jpeg images, using ImageJ 1.43 (NIH, USA, http://rsb.info.nih.gov/ij software has shown that the intensity of the 370 pb amplicons strip corresponding to the helicase (DNase), in 65, 69, 79, 81 and 84 strains is 2,12; 1,47; 3,04; 1,43 and 1,49 times lower. This is due either to the existence of more clone populations, some presenting the gene and some not, or to the decrease of the number of copies per cell. Moreover, the 24, 67 and 77 strains lose the helicase gene and are not observed on the specific strip at 370 pb (fig. 59).

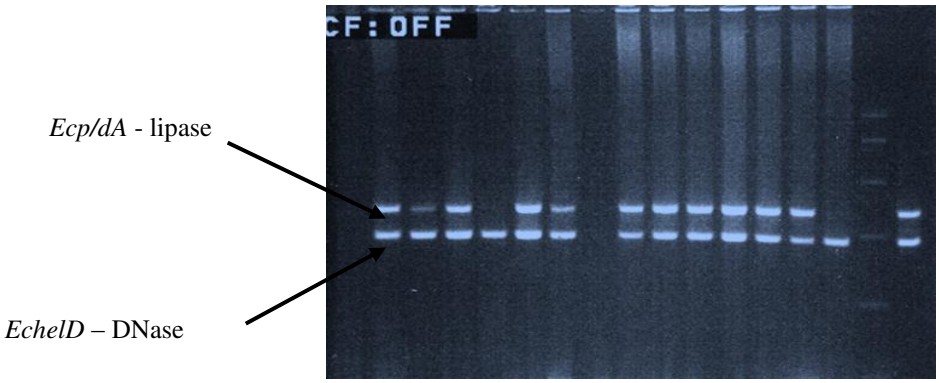


Figure 59. Migration of PCR amplicons in2% agarose gel, in *E. coli* strains isolated from sea water.

4.5. RESULTS OF THE STUDY IN THE INFLUENCE OF PHYSICAL-CHEMICAL FACTORS ON THE EXPRESSION OF SOLUBLE VIRULENCE FACTORS AND OF SENSITIVITY TO THE SALTS OF SOME TO DIVALENT TRANSITION METALS OF THE *Escherichia coli* STRAINS ISOLATED FROM SEA WATER

4.5.1. THE INFLUENCE OF PHYSICAL-CHEMICAL FACTORS ON THE EXPRESSION OF BACTERIAL SENSITIVITY TO SOME TRANSITIONAL DIVALENT METAL

4.5.1.1. The influence of the consistency of the culture medium

The consistency of the culture medium for bacteria (liquid *vs* solid) did not influence the antimicrobial activity of divalent transitional metal salts in *E. coli* strains isolated from sea water, antimicrobial activity being slightly increased when bacteria were grown in solid medium, with the exception of the Mn salt (fig. 60).

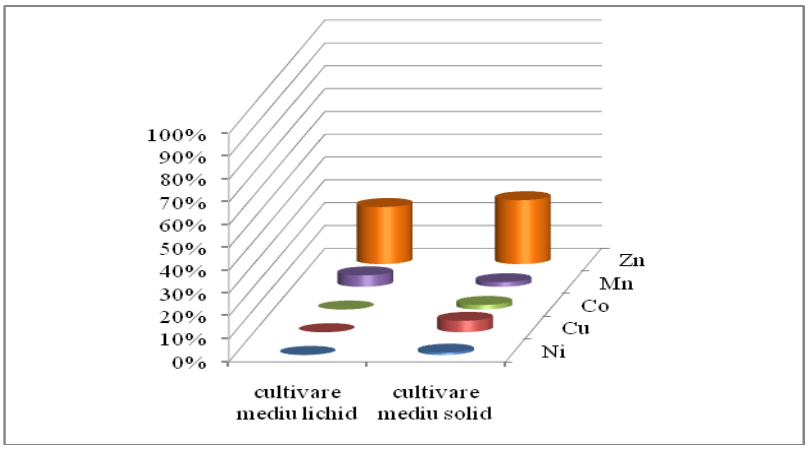


Figure 60. The influence of the consistency of the culture medium on the antimicrobial activity (%) of divalent transitional metal salts in *E. coli* strains isolated from sea water.

.4.5.1.2. The influence of temperature in aerobiosis conditions

The levels of sensitivity were best expressed at a temperature of 44°C, followed by 22°C. The only metal that registered a total resistance phenomenon, indifferent of the bacteria cultivation temperature, was Ni (fig. 61).

At the temperature of 44°C and 22°C Zn was the only one to manifest a bactericide effect.

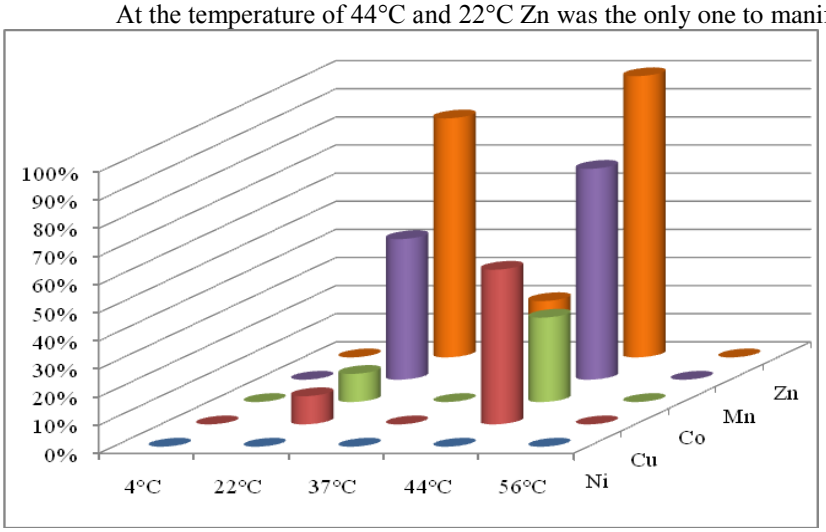


Figure 61. Temperature's influence on the antimicrobial activity of divalent transitional metal salts (%), in aerobiosis conditions, in *E. coli* strains isolated from sea water.

4.5.1.3. The influence of temperature in anaerobiosis conditions

We can observe that the levels of sensitivity were also best expressed at a temperature of 44°C, followed by 22°C. The only metal that registered a total resistance phenomenon, indifferent of the bacteria cultivation temperature, was Ni (fig. 62).

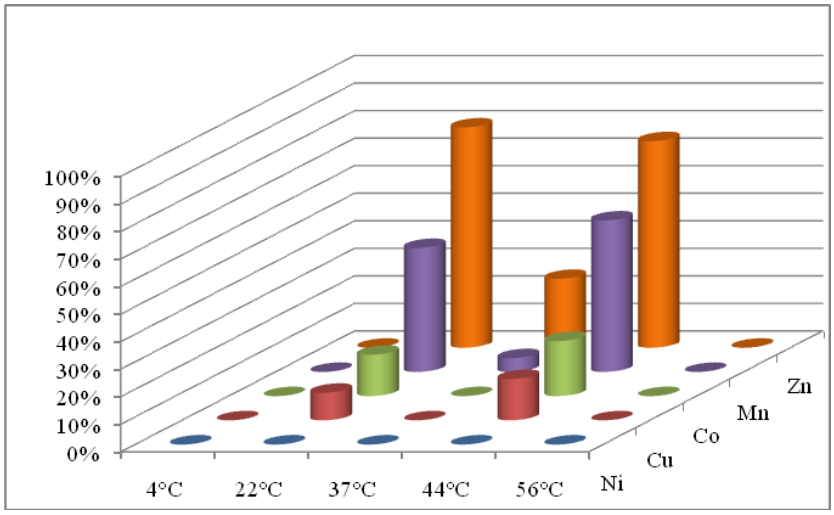


Figure 62. Temperature's influence on the antimicrobial activity of divalent transitional metal salts (%), in anaerobiosis conditions, in *E. coli* strains isolated from sea water.

4.5.1.4. The influence of the presence / absence of oxygen

The growth of bacteria in aerobiosis and anaerobiosis conditions has shown that the presence/absence of O_2 did not influence the phenomenon of bacterial resistance to studied salts. It should be noted that at 37°C, in both aerobe and anaerobe conditions; the antimicrobial activity of Ni, Co and Cu was completely eliminated, and the susceptibility rates for Mn and Zn were drastically reduced (fig. 63).

4.5.1.5. The influence of the culture medium's pH variation

We could notice that the levels of sensitivity were best expressed at alkaline pH; the most constant results were registered for the salts of Cu, Co, and Zn (fig. 64). Mn sensitivity slightly increased at acid pH values in comparison with neutral pH, while at alkaline pH, the sensitivity for Mn and Zn significantly increased (fig. 64). Ni had antimicrobial activity only at alkaline pH (fig. 64).

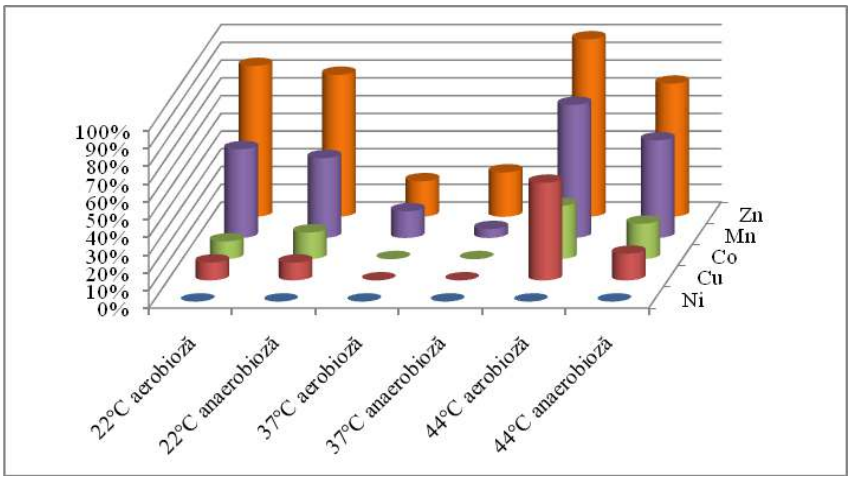


Figure 63. The influence of the O_2 presence/absence on the antimicrobial activity of divalent transitional metal salts (%), in anaerobiosis conditions, in *E. coli* strains isolated from sea water.

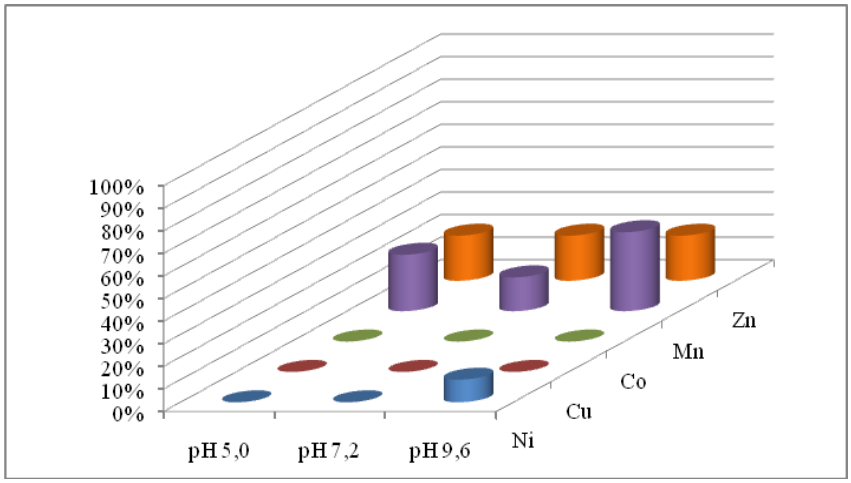


Figure 64. The influence of the culture medium's pH variation on the antimicrobial activity of divalent transitional metal salts (%), in anaerobiosis conditions, in *E. coli* strains isolated from sea water.

4.5.1.6. The influence of the glucose concentration from the culture medium

High glucose concentration (3% *versus* 1,5%) significantly increased bacteria sensitivity to Mn and Zn salts and with only 5 % for Ni and Cu salts. The only metal not manifesting any antimicrobial action both in low (1,5%) and in high glucose concentrations (3%) was Co (fig. 65).

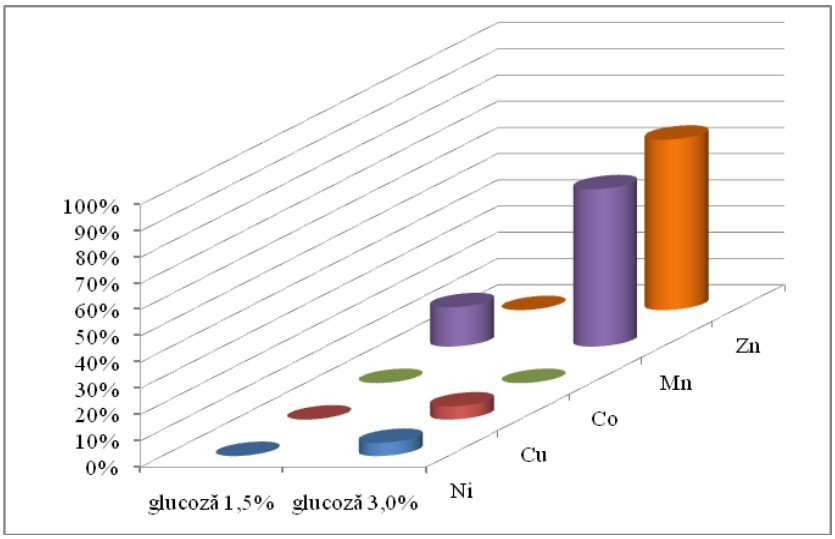


Figure 65 The influence of the variation of the glucose concentration in the culture medium on the antimicrobial activity of divalent transitional metal salts (%), in anaerobiosis conditions, in *E. coli* strains isolated from sea water.

4.5.1.7 The influence of the NaCl concentration from the culture medium

Microbial sensitivity to divalent transitional metal salts was strongly influenced by the salinity variations in the culture medium. Low NaCl concentrations (0% and 0,5%), as well as the % NaCl concentration induced the same sensitivity profile (for example, sensitivity to Mn and Zn). In 3%, 4%, 6%, 7% NaCl concentrations, we noticed the presence of sensitivity models in 3 or even 4 markers, like the salts of Zn, Mn and Cu or Zn, Mn and Ni or Zn, Mn, Ni and Cu. At the highest concentration of 10% NaCl, we noticed the manifestation of lower sensitivity levels for all tested divalent transitional metal salts (fig. 66).

Zn had bactericide action in 3% and 4% NaCl concentrations in the culture medium (fig. 66).

The only NaCl concentration where Mn had bactericide action was 3% (fig. 66).

Zn and Mn manifested antimicrobial action in all tested NaCl concentrations (fig. 66).

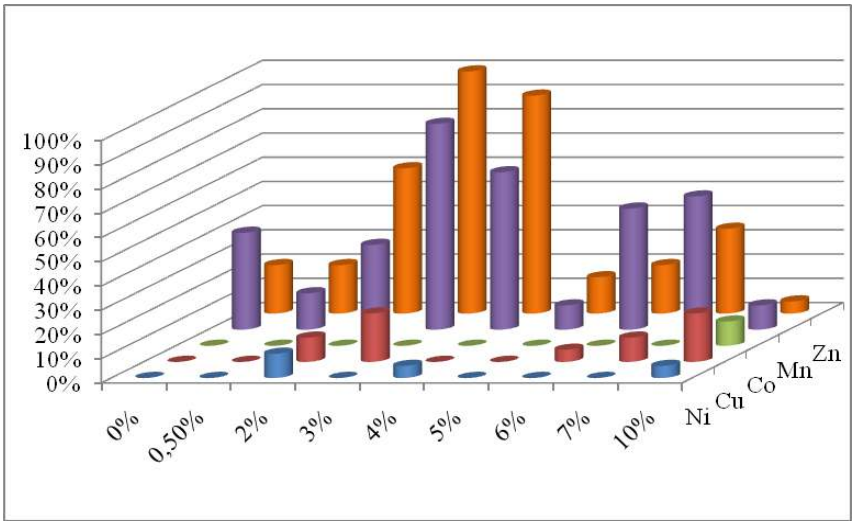


Figure 66. The influence of the NaCl concentration variation in the culture medium on the antimicrobial activity of divalent transitional metal salts (%), in anaerobiosis conditions, in *E. coli* strains isolated from sea water.

4.5.2. THE INFLUENCE OF PHYSICAL AND CHEMICAL FACTORS ON THE BACTERIAL ADHERENCE CAPACITY TO A CELLULAR SUBSTRATE OF THE *Escherichia coli* STRAINS ISOLATED FROM SEA WATER

4.5.2.1. The influence of the consistency of the culture medium

Bacteria growth in liquid media caused their adherence on the cellular substrate, represented by HeLa cells, indicating the fact that bacteria development in liquid medium favors the initiation of an infectious process (fig. 67).

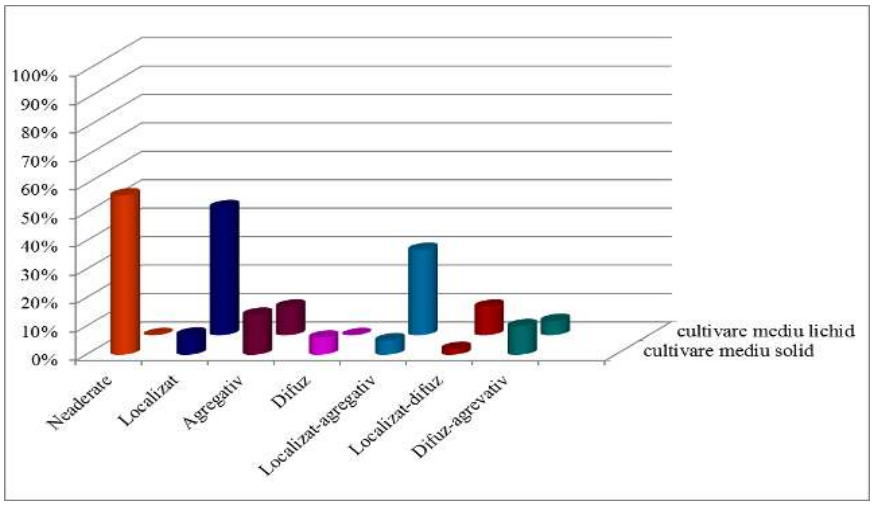


Figure 67. The influence of the consistency of the culture medium on the bacterial adherence capacity to the HeLa cellular substrate in *E. coli* strains isolated from sea water.

4.5.2.2. The influence of temperature in aerobiosis conditions

As you can observe in figure 68, the maximum bacterial adherence capacity to the HeLa cellular substrate was expressed at a temperature of 37°C. Regardless of the incubation temperature, the most frequently expressed adherence *pattern* was the localized one.

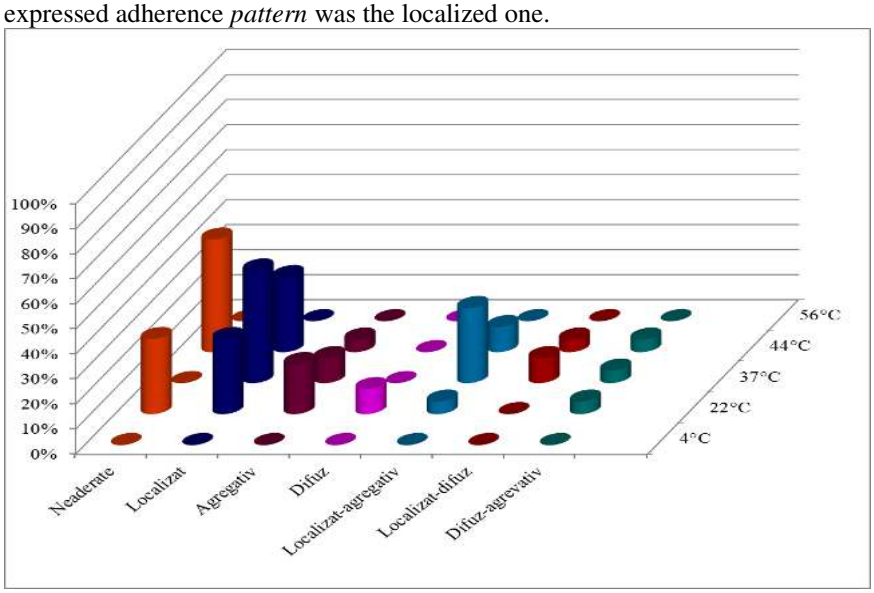


Figure 68. The influence of temperature on the bacterial adherence capacity to the HeLa cellular substrate (%), in aerobiosis conditions, in *E. coli* strains isolated from sea water.

4.5.2.3. The results of the study on the influence of temperature in anaerobiosis conditions on the bacterial adherence capacity to a cellular substrate

As you can observe in figure 69, bacterial adherence capacity to the HeLa cellular substrate, in anaerobiosis conditions was also best expressed at a temperature of 37°C. Regardless of the incubation temperature, the most frequently expressed adherence *pattern* was the localized one.

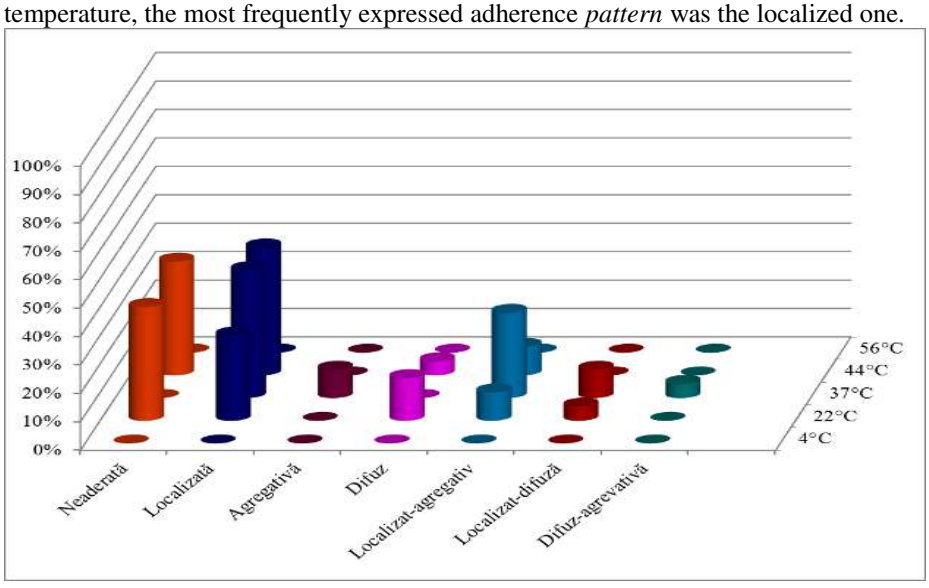


Figure 69. The influence of temperature on the bacterial adherence capacity to the HeLa cellular substrate(%), in *E. coli* strains isolated from sea water, in anaerobiosis conditions.

4.5.2.4. The influence of O_2 presence/absence

Growing bacteria in aerobiosis and anaerobiosis conditions indicated that the presence/absence of O_2 does not influence the bacterial adherence capacity to the HeLa cellular substrate, when the strains are cultivated 37°C (fig.70).

It should be noted that at 22°C, the absence of O_2 induced a decrease of the bacterial adherence capacity to the HeLa cellular substrate, and the aggregative *pattern* was not expressed (fig.70).

Growing bacteria at a temperature of 44°C in aerobiosis and anaerobiosis conditions induced a slight decrease of the bacterial adherence capacity to the HeLa cellular substrate, revealing the adherence *patterns*: localized, diffuse and localized-aggregative (fig. 70).

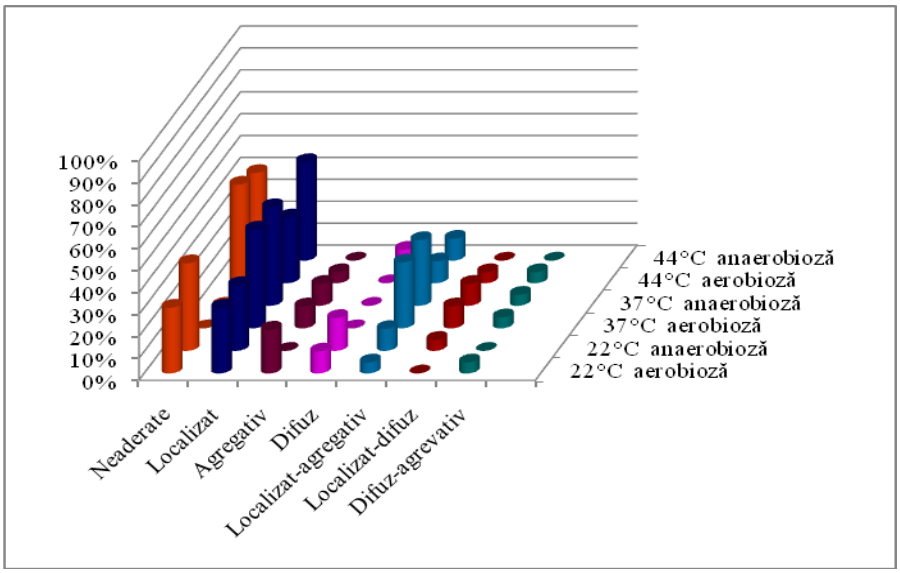


Figure 70. The influence of O_2 presence/absence on the bacterial adherence capacity to the HeLa cellular substrate (%), in *E. coli* strains isolated from sea water.

4.5.2.5. The influence of the culture medium's pH variation

As shown in figure 71, the only bacteria cultivation condition reducing by only 15% the bacterial adherence capacity to the HeLa cellular substrate is the pH acid level in the culture medium. Both in acid pH and in alkaline pH, all adherence *patterns* were expressed.

Maintaining the bacterial adherence capacity to the HeLa cellular substrate in the conditions of an alkaline pH reveals the capacity of *E. coli* strains isolated from sea water to initiate an infectious process, both in conditions of physiological pH and alkaline pH.

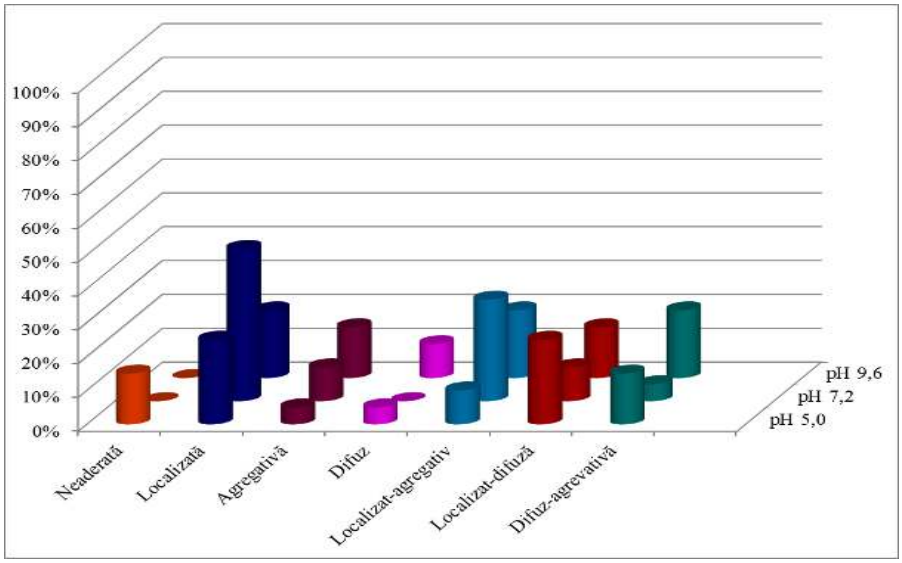


Figure 71. The influence of the culture medium's pH variation on the bacterial adherence capacity to the HeLa cellular substrate (%), in *E. coli* strains isolated from sea water.

4.5.2.6. The influence of the glucose concentration from the culture medium

This study has revealed the fact that the glucose concentration from the culture medium does not have significant influence on the bacterial adherence capacity to the HeLa cellular substrate. The *patterns* that were not expressed in neither one of the two glucose concentrations were the aggregative and the diffuse-aggregative ones (fig. 72).

Low glucose concentration from the culture medium (1,5%) favors bacterial adherence the HeLa cellular substrate (fig. 72).

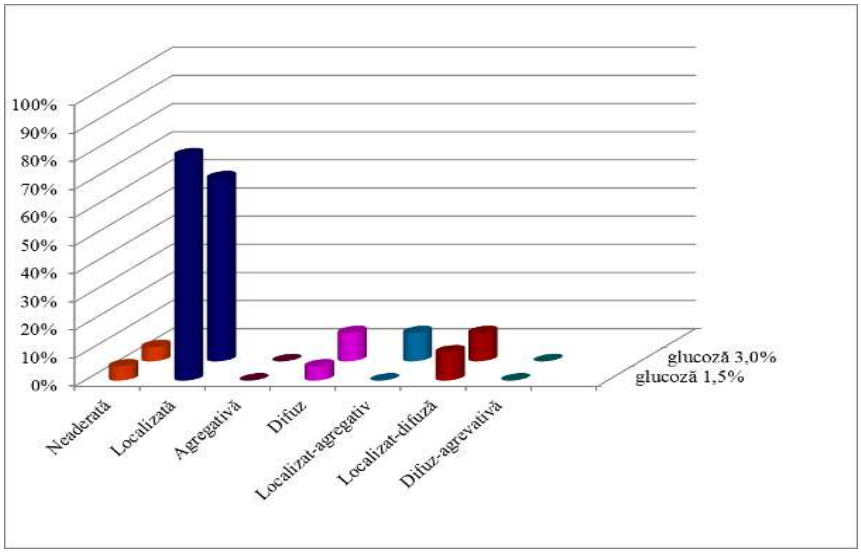


Figure 72. The influence of the glucose concentration from the culture medium on the bacterial adherence capacity to the HeLa cellular substrate (%), in *E. coli* strains isolated from sea water.

4.5.2.7. The influence of the NaCl concentration from the culture medium

As this study has shown, the only NaCl concentration not influencing the bacterial adherence capacity to the HeLa cellular substrate is of 0,5%, unlike the 4% and 5% concentrations, that have easily influenced the bacterial adherence capacity to the cellular substrate, and the 2%, 6%, 7% and 10% have significantly influenced this phenomenon. The most frequently expressed *pattern* was the localized one, regardless of the NaCl concentration in the culture medium. The NaCl concentrations allowing the expression of the most adherence *patterns* were the 0.5% and 5% ones, with 4 adherence *patterns*, the 0%, 3%, 4%, 6% NaCl concentrations, with 3 adherence *patterns*, the 2% and 10% concentrations, with 2 adherence *patterns*, and the only NaCl concentration inducing only one adherence *pattern* was of 7%. The only salt concentration expressing the diffuse *pattern* was of 3%. Also, the only salt concentration expressing the diffuse-aggregative *pattern* was 0.5% (fig. 73).

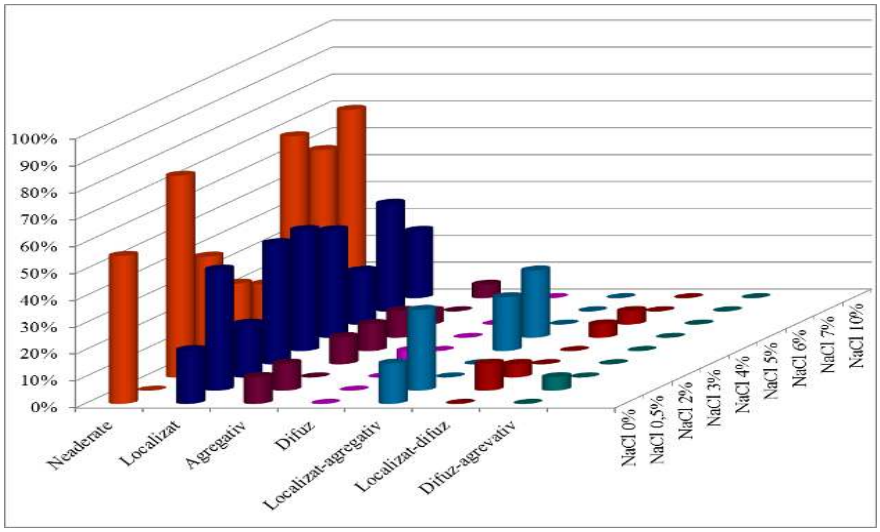


Figure 73. The influence of the NaCl concentration from the culture medium on the bacterial adherence capacity to the HeLa cellular substrate (%), in *E. coli* strains isolated from sea water.

4.5.3. THE INFLUENCE OF PHYSICAL AND CHEMICAL FACTORS ON THE EXPRESSION OF SOLUBLE VIRULENCE FACTORS IN *Escherichia coli* STRAINS ISOLATED FROM SEA WATER

4.5.3.1. The influence of the consistency of the culture medium

As shown in figures 74 şi 75, of the 11 soluble virulence factors tested in *E. coli* strains isolated from sea water, 8 of them expressed high levels; the highest level of expression was noticed for amylase, when for obtaining primary cultures we used a liquid medium.

These results demonstrate that both the capacity of invasion and the toxigenesis in marine *E. coli* strains are much better expressed when the bacteria are developed in a liquid medium.

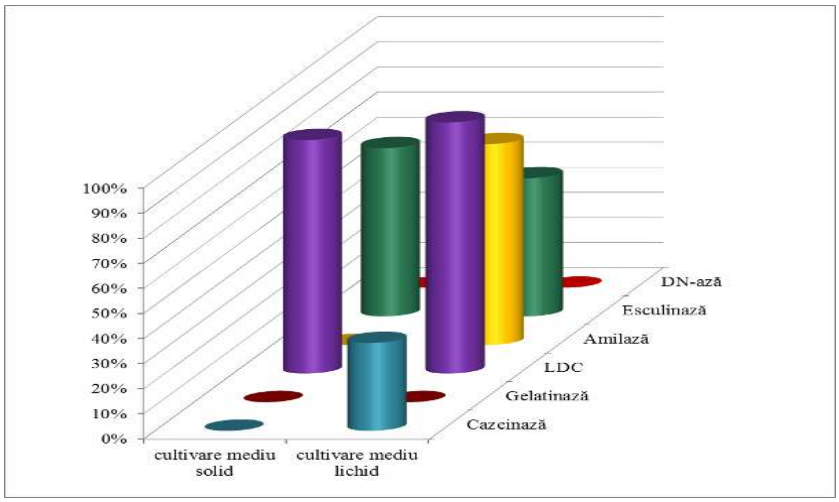


Figure 74. The influence of the consistency of the culture medium on the level of the phenotypic expression (%) of the enzymes involved in the process of invasion and survival in *E. coli* strains isolated from sea water, in aerobiosis conditions.

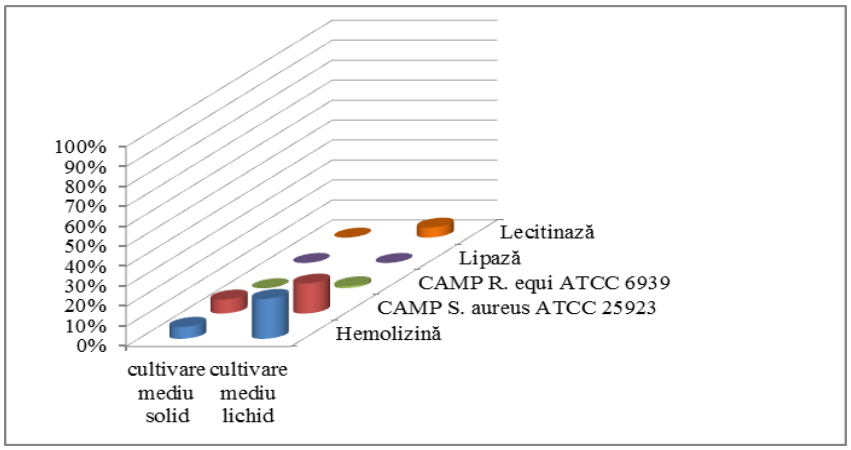


Figure 75. The influence of the consistency of the culture medium on the level of the phenotypic expression (%) of the pore forming toxins in *E. coli* strains isolated from sea water, in anaerobiosis conditions.

4.5.3.2. The influence of temperature in aerobiosis conditions

In terms of temperature of incubation, the broadest spectrum of virulence expression factors was observed at 37°C, in strains incubated in anaerobe conditions (fig. 76, 77). These results could explain the pathogenicity of *E. coli* strains involved in intra-abdominal infections.

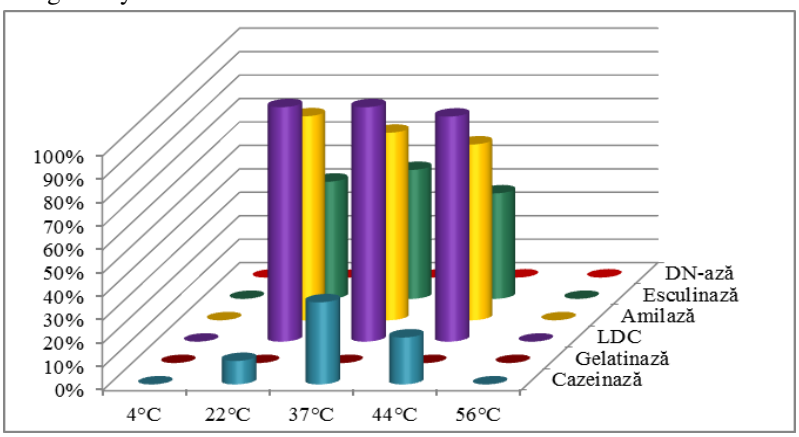


Figure 76. The influence of temperature in aerobiosis conditions on the level of the phenotypic expression (%) of the enzymes involved in the process of invasion and survival in *E. coli* strains isolated from sea water.

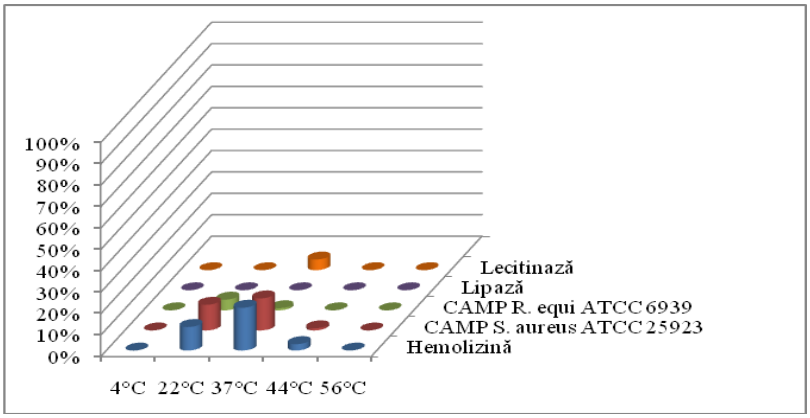


Figure 77. The influence of temperature in aerobiosis on the level of the phenotypic expression (%) of the pore forming toxins in *E. coli* strains isolated from sea water.

4.5.3.3. The influence of temperature in anaerobiosis conditions

Similarly, in anaerobiosis conditions, it was again the temperature of 37°C that allowed the expression of the most soluble virulence factors, for both the enzymes involved in the invasion and survival process and for the pore forming ones (fig. 78 and 79).

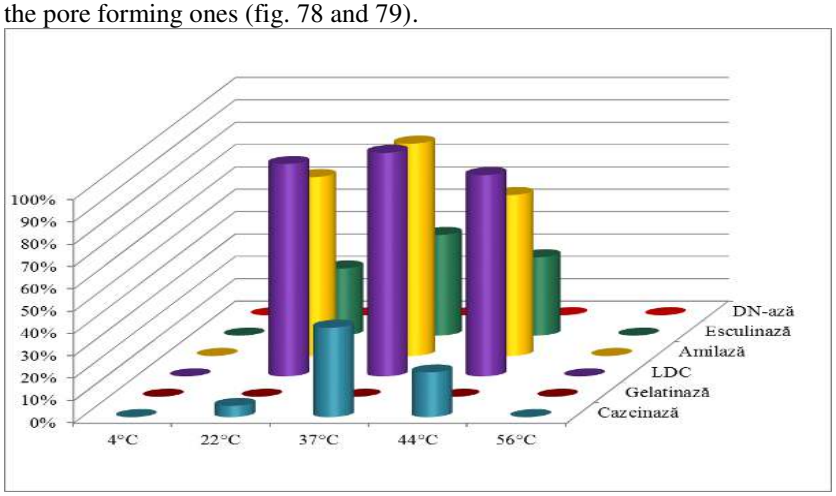


Figure 78. The influence of temperature on the level of the phenotypic expression (%) of the enzymes involved in the process of invasion and survival, in anaerobiosis conditions, in *E. coli* strains isolated from sea water.

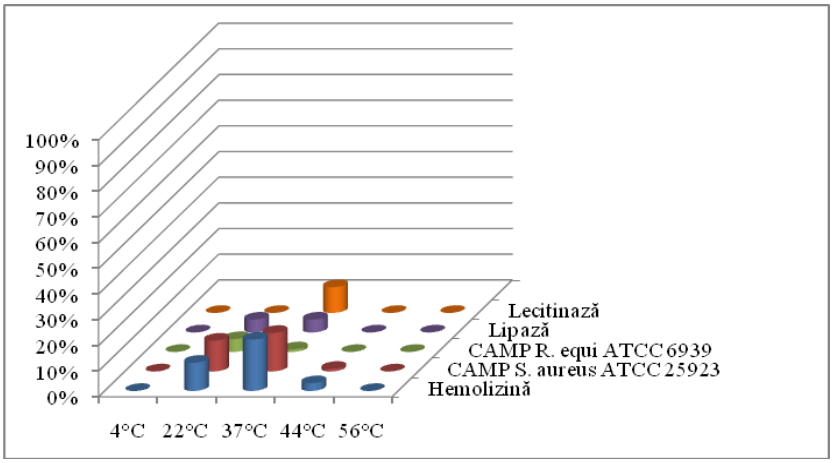


Figure 79. The influence of temperature on the level of the phenotypic expression (%) of the pore forming toxins, in anaerobiosis conditions, in *E. coli* strains isolated from sea water.

4.5.3.4. The influence of O_2 presence/absence

The expression of the enzymes involved in the invasion and survival process is slightly inhibited in anaerobiosis conditions, regardless of the incubation temperature (fig. 80, 81).

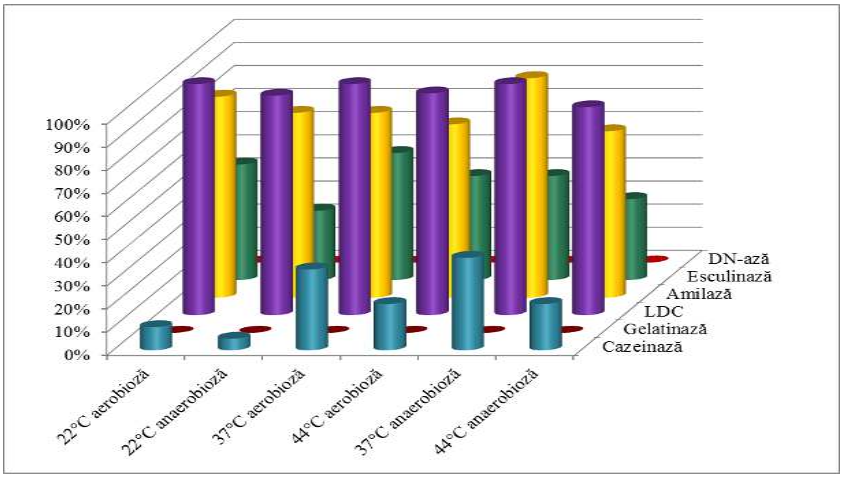


Figure 80. The influence of O_2 presence/absence on the level of the phenotypic expression (%) of the enzymes involved in the process of invasion and survival in *E. coli* strains isolated from sea water.

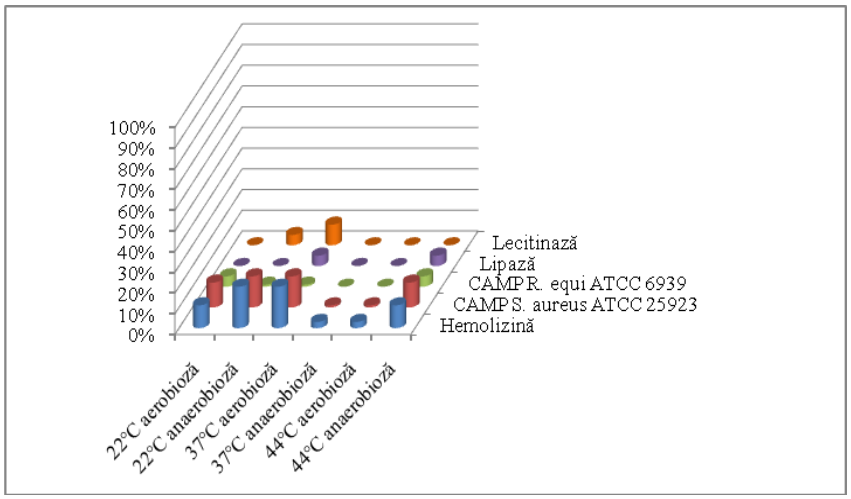


Figure 81. The influence of O_2 presence/absence on the level of the phenotypic expression (%) of the pore forming toxins, in anaerobiosis conditions, in *E. coli* strains isolated from sea water.

4.5.3.5. The influence of the culture medium's pH variation

The lecithinase was slightly expressed only in physiological pH (7,2), this also being the pH value at which the most soluble virulence factors were expressed. The caseinase and amylase expression is favored by acid pH. The lysine decarboxylase was the only enzyme on which the pH variation of the culture medium made no modification, expressing in 100% in all pH variations (fig. 82, 83). The pH variation did not significantly influence the expression of the virulence factors, who presented a similar profile, with a remarkable growth of the proteolytic potential, emphasized by the high level of caseinase in pH 5 (fig. 82, 83).

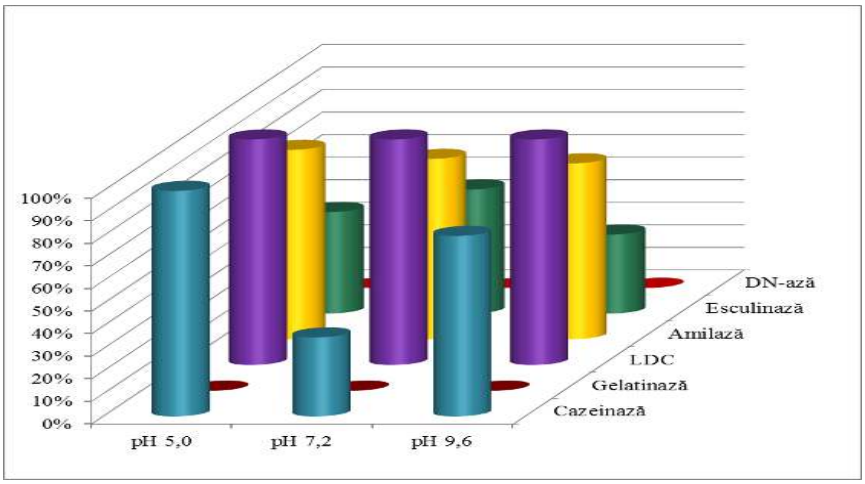


Figure 82. The influence of the culture medium's pH variation on the level of the phenotypic expression (%) of the enzymes involved in the process of invasion and survival in *E. coli* strains isolated from sea water.

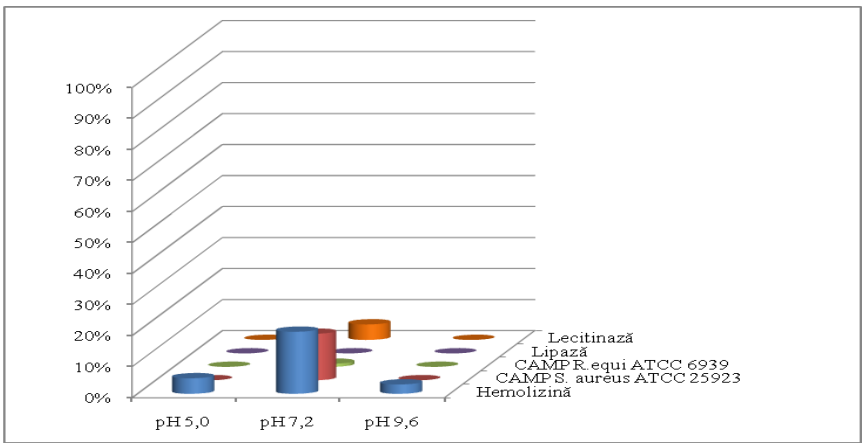


Figure 83. The influence of the pH variation on absence on the level of the phenotypic expression (%) of the pore forming toxins, in *E. coli* strains isolated from sea water.

4.5.3.6. The influence of the glucose concentration from the culture medium

This study has found that the soluble virulence factors were better expressed in lower glucose concentrations (1,5%) (fig. 84 and 85).

The higher glucose concentration (3%) from the culture medium slightly inhibited the expression of lysine decarboxylase, amylase (fig. 84), hemolysin and of the CAMP factor with *S. aureus* ATCC 25923 (fig. 85). This glucose concentration from the culture medium induced major influences in the expression of esculinase (fig. 86) and caseinase (fig. 84).

The 1,5% glucose concentration stimulated the esculinase production (fig. 86), demonstrating the fact that at a low glucose concentration, bacteria synthesize enzymes involved in metabolizing other C sources, like the case of esculin, a complex heteroside, revealing the potential of these strains to initiate an infectious process.

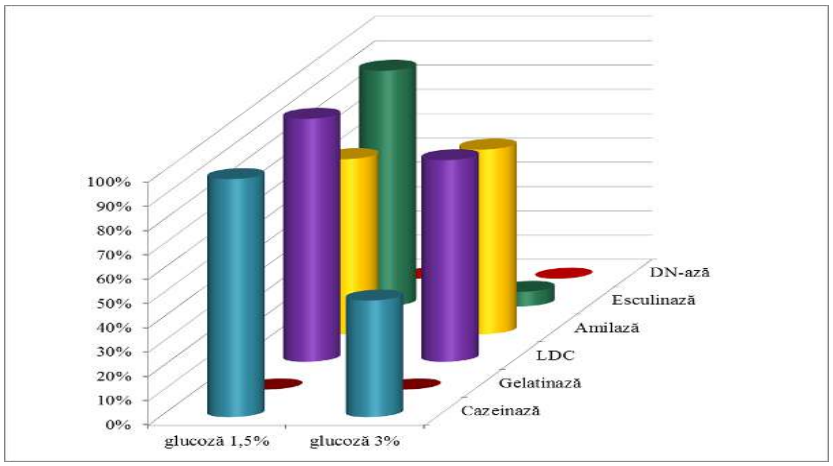


Figure 84. The influence of the glucose concentration from the culture medium on the level of the phenotypic expression (%) of the enzymes involved in the process of invasion and survival in *E. coli* strains isolated from sea water.

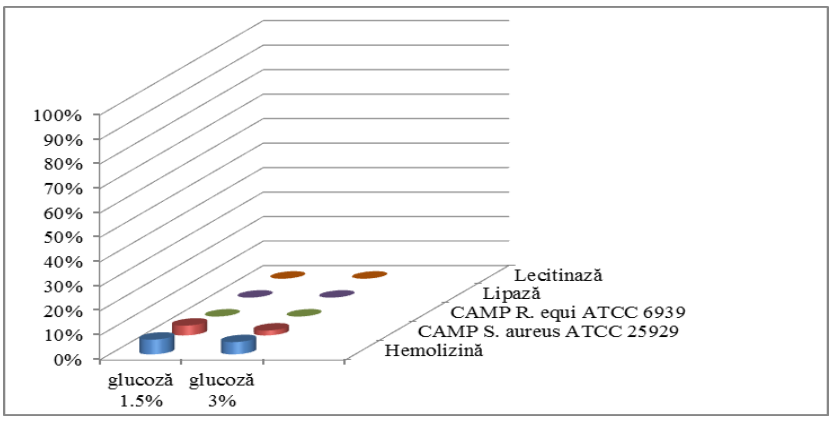


Figure 85. The influence of the glucose concentration from the culture medium on the level of the phenotypic expression (%) of the pore forming toxins, in *E. coli* strains isolated from sea water.

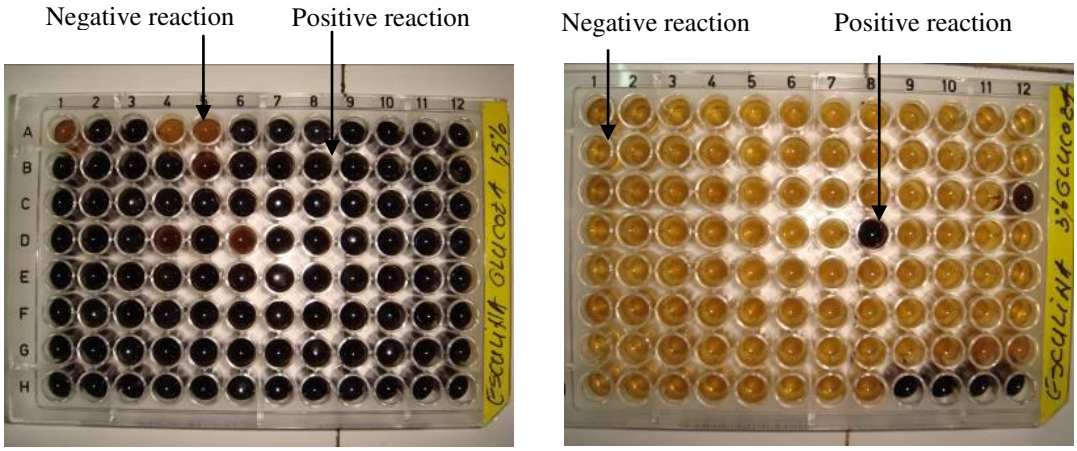


Figure 86. The influence of the glucose concentration from the culture medium on the expression of esculinase, in *E. coli* strains isolated from sea water (a and b).

a. the expression of esculinase in the presence of 1,5% glucose concentration in the culture medium

b. the expression of esculinase in the presence of 3% glucose concentration in the culture medium

4.5.3.7. The influence of the NaCl concentration

The variations of the NaCl concentration in the culture medium generally inhibited the expression of the soluble virulence factors; the greatest percent of soluble virulence factors was expressed in 6% and 2% NaCl concentrations, followed by 0,5% (fig. 87, 88).

The NaCl concentration in the culture medium significantly influenced the expression of esculinase (fig. 89) and caseinase (fig. 87).

In high NaCl concentrations in the culture medium, we observed a growth in the virulence potential, with a maximum value reached at 6% NaCl, demonstrating the fact that high osmolarity accentuates the potential of these strains to initiate an infectious process.

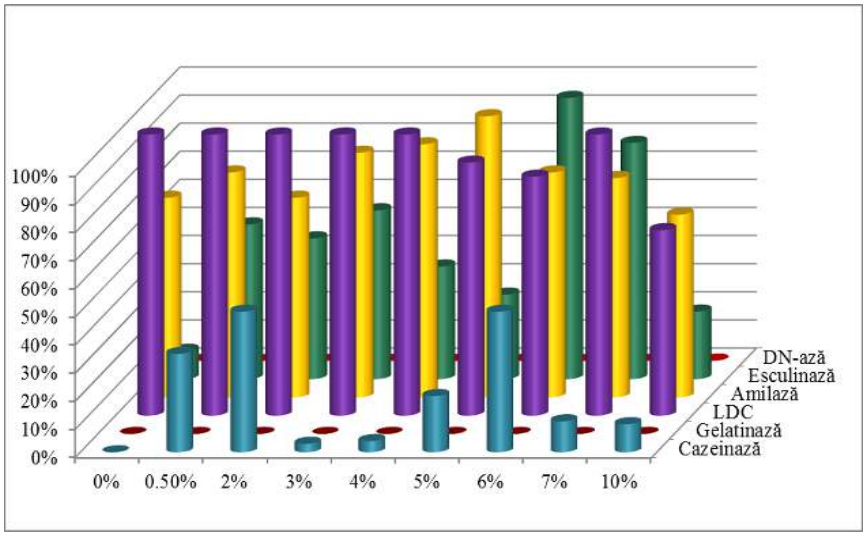


Figure 87. The influence of the NaCl concentration from the culture medium on the level of the phenotypic expression (%) of the enzymes involved in the process of invasion and survival in *E. coli* strains isolated from sea water.

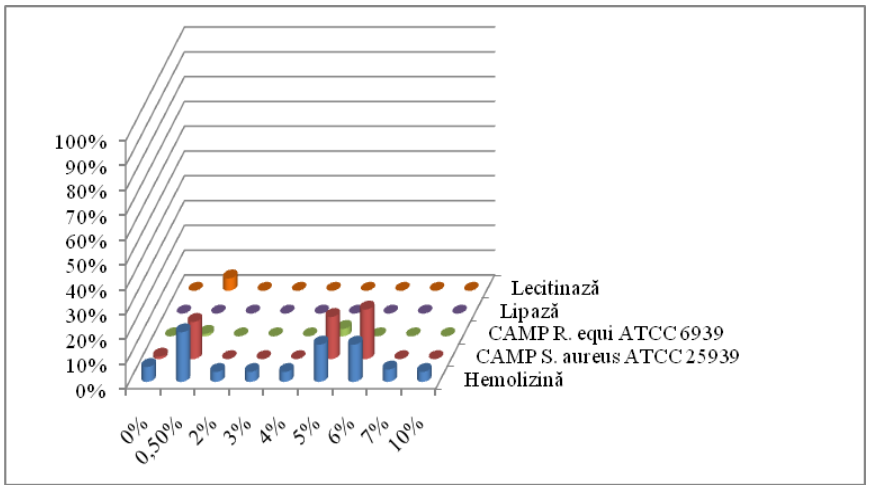
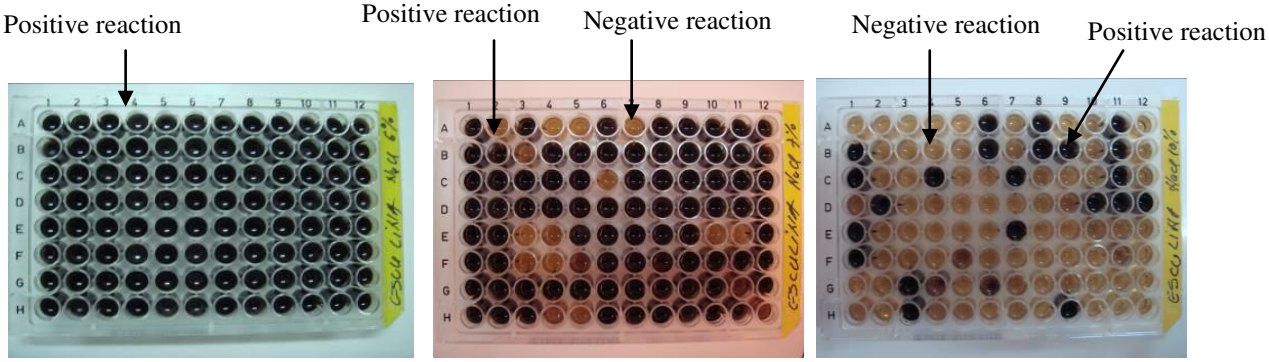


Figure 88. The influence of the NaCl concentration from the culture medium on the level of the phenotypic expression (%) of the pore forming toxins, in *E. coli* strains isolated from sea water.

Of the 10 analyzed soluble virulence factors, only amylase, esculinase, caseinase, lizin-decarboxylase and hemolysins were expressed in all used NaCl concentrations, presenting different expression intensities.

The soluble virulence factors with the most constant expression in different medium conditions were: amylase, esculinase, caseinase, lizin-decarboxylase and hemolysins.

A slight expression was noticed for O lecithinase and CAMP factor, both for *S. aureus* ATCC 25923, and fr *R. equi* ATCC 6939, regardless of the incubation conditions.



a. esculin hydrolyze in the presence of 6% NaCl concentration, in the culture medium

b. esculin hydrolyze in the presence of 7% NaCl concentration, in the culture medium

c. esculin hydrolyze in the presence of 10% NaCl concentration, in the culture medium

Figure 89. The influence of the NaCl concentration from the culture medium on esculin hydrolyze (a, b and c)

CONCLUSIONS

From the analysis of the data from this complex biochemical study of the *Escherichia coli* strains isolated from sea water, but with potential implications in human pathology, we can formulate the following conclusions:

1. The *E. coli* strains isolated from sea water presented with resistance and multi-resistance profiles, correlated with the presence of some plasmids of different molecular weights.
2. The concurrent resistance mechanisms to different classes of antibiotics could be mediated by the presence of nonspecific flow pumps necessary for adapting and survival in hypersalinity conditions.
3. The only highlighted antibiotic resistance genes were *sul*1 and *sul*2, coding for resistance to biseptol.
4. The sensitivity profiles of the de *Escherichia coli* strains isolated from sea water to divalent transitional metals (Ni, Co, Cu, Mn and Zn) varied depending on the experimental conditions (cultivation temperature, O_2 presence/absence, pH and glucose and NaCl concentrations in the culture medium), the highest antimicrobial activity being manifested by the Zn and Mn salts.
5. The highest levels of sensitivity were registered at the temperature of 44°C, alkaline pH, high glucose concentrations and average salinity, and the lowest ones at the temperature of 37°C, neutral pH, low glucose concentrations and low salinity.
6. The Zn salt has bactericide effect at temperatures of 44°C and 22°C, in aerobiosis conditions, with 3% and 4% NaCl concentrations in the culture medium, and the Mn salt at 3% NaCl concentrations in the culture medium.
7. The Ni salt manifested antimicrobial action in alkaline pH conditions (9,6) and with 2%, 4% and 10% NaCl concentrations, and the Co salt at 22°C and 44°C in both aerobiosis and anaerobiosis, with 10% NaCl concentration in the culture medium.
8. The high levels of positivity in the adherence rates to abiotic and biotic surfaces noticed in *Escherichia coli* strains isolated from sea water plead for their potential to colonize human mucous surfaces or implanted prosthetic devices, thus being capable to initiate and develop an infectious process, sustained by the secretion of soluble enzymes involved in virulence.
9. The adherence capacity to a cellular substrate represented by HeLa cells was significantly influenced by the variation of the NaCl concentration in the culture medium.
10. The adherence *pattern* to the cellular substrate expressed most frequently was the localized one.
11. The maximum adherence capacity to a cellular substrate of 100% was highlighted by growing bacteria in liquid culture medium with alkaline pH of 9.6 and physiological conditions (pH 7,2 at 37°C, aerobiosis).
12. The expression of varied adherence *patterns* was highlighted in bacteria cultivated in a culture environment with 5,0 acid pH and9,6 alkaline pH.
13. In terms of soluble virulence factors, the most constant expression in different experimental conditions was obtained for lizin-decarboxylase, amylase and hemolysin, and the largest variations for the production of esculetol (Fe chelator) and caseinase.
14. The highest expression of virulence factors was noted at a temperature of 37°C, pH 7.2, 0.5% NaCl concentration and 1.5% glucose.
15. The increase in aerobe/anaerobe conditions did not influence the sensitivity to various divalent transitional metal salts or the expression of virulence factors.
16. The enzymes involved in the invasion and survival process were phenotypically expressed with higher frequencies than the pore forming enzymes, in all experimental conditions.
17. Cultivation on liquid mediums favored both the adherence capacity to a cellular substrate and the expression of enzymes, for both the enzymes involved in the invasion and survival process, and the pore forming toxins.
18. The PCR reaction did not highlight protein coders involved in adherence and toxigenicity in classical enteric pathotypes.
19. The presence of lipase and DNase could not be phenotypically distinguished in none of the used experimental conditions, instead, in the analyzed strains, we found genes for phospholipase and helicase, both involved in pathogenicity (by producing massive tissue destruction, gas gangrenes, lung and skin infections due to host cell membrane destabilization), theoretically capable of being transferred between strains of the same type and sometimes even between different species and types through the phenomena of conjugation, transformation and transduction.
20. The present study demonstrates that the marine environment represents an ecologic system appropriate for the existence and maintenance of a complex reservoir of antibiotic resistance and virulence, with increased risk of colonization of organisms in general and with possible implications for the health of the human host in particular; the physical and chemical condition favoring the initiation of an infectious process of exogenous origin being represented by the liquid medium, with hypersalinity (NaCl 6%) temperature of 37°C and acid pH (5,0).

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LUCRĂRI ŞTIINŢIFICE PUBLICATE

A. Lucrări ştiinţifice publicate în reviste recunoscute C.N.C.S.I.S.

1. **Pănuş E.,** Roşoiu N., Chifiriuc C., Israil A. M., Banu O., Lysinedecarboxylase expression assay in *Escherichia coli* strains isolated in Romanian Seaside from marine and drinking water, ARS Medica Tomitana, XIII, 2(49), 15-18, **(2007).**
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4. **Pănuş E.,** Chifiriuc M.C., Banu O., Mitache M., Bleotu C., Roşoiu N., Lazăr V., Comparative study of resistance and virulence markers in *Escherichia coli* strains isolated from hospital surfaces, clinical specimens and drinking/marine waters, Biointerface Research in Applied Chemistry., Open access journal, 1, (1), 24-30, **(2011). Revistă indexată ISSN 2069-5837 – 8 citări în reviste cotate ISI cu factor de impact.**
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B. Lucrări prezentate la manifestări ştiinţifice internaţionale şi publicate sub formă de rezumat

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C. Participări la congrese ştiinţifice naţionale şi internaţionale

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11. 19th European Congress of Clinical microbiology and Infections Diseases (ECCMID), **(Helsinki Finland, 2009).**
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