NEW DATA ON MICROORGANISMS ISOLATED FROM CERAMIC MATERIALS OF THE ROMULA ARCHAEOLOGICAL SITE, ROMANIA

BĂTRÎNESCU-MOTEAU Costin, NEAGU Simona, LUCACI Anca-Ioana, RUGINESCU Robert, COJOC Roxana, PODOSU (VLAD) Aurelia, PURCĂREA Cristina, NEGRU Mircea, ENACHE Mădălin

Abstract. In the context of preserving cultural heritance against biodegradation, this study focused on the identification and characterization of new microorganisms isolated from several types of archaeological objects represented by Roman pottery used for transport, food and construction materials. The chemical composition of the ceramic samples revealed similar building materials composed of six common elements, with variable Ba, Sn and traces elements. The number of colony-forming units on nutrient broth and MH media showed that the content is relatively similar for the three types of materials. The hydrolytic extracellular activity of some selected strains showed that five types of extracellular hydrolases have been identified in the case of construction materials. Four isolates selected from the construction material samples stood out as producing factors with antagonistic activity and four strains as their target, as putative controlling mechanism of material-associates microcosm.

Keywords: archaeological objects, archaeological microorganisms, microbial strains, microbial enzyme.

Rezumat. Noi date privind microorganismele izolate din materiale ceramice ale sitului arheologic Romula, România. În contextul conservării patrimoniului cultural împotriva biodegradării, acest studiu s-a concentrat pe identificarea și caracterizarea de noi microorganisme izolate din mai multe tipuri de obiective arheologice reprezentate de ceramica romană folosită pentru transport, hrană și materiale de construcţie. Compoziţia chimică a probelor ceramice a evidenţiat materiale de construcţie similar compuse din şase elemente comune, cu Ba, Sn şi oligoelemente variabile. Numărul de unităţi formatoare de colonii pe geloză şi mediu MH a demonstrat o similitudine din punct de vedere al conţinutului cu cele trei tipuri de materiale. Activitatea hidrolitică extracelulară a unor tulpini izolate de la nivelul materialelor de construcţie a evidenţiat prezenţa a cinci tipuri de hidrolaze extracelulare. Patru izolate selectate de pe materialele de construcţie au prezentat capacitatea de a produce factori cu activitate antimicrobiană iar patru tulpini s-au comportat ca ţinte ale acestora, ca potenţial mecanism de control al microcosmosului asociat materialelor.

Cuvinte cheie: obiecte arheologice, microorganisme arheologice, antagonism microbian, enzimă microbiană.

INTRODUCTION

Microorganisms play a key role in metabolizing several compounds in various habitats from arid deserts to polar areas, and from fresh water to salted ones (MERINO et al., 2019). This broad distribution reflects their capacity to use different substrates for accumulating energy, the community complexity being modelled mainly by the corresponding colonization support (OMELON, 2016). In this context, archaeological objects are also suitable for colonization by microorganisms. Cultural heritages are often subject to either biodeterioration or biodegradation considering their long time exposure to several environmental and anthropogenic factors (CIFFERI, 1999; ELSEROGY et al., 2016). The colonization of heritage objects is often initiated by photosynthetic microorganisms which use sunlight to transform the CO_2 into biomass, a future substrate for heterotrophic bacteria and fungi involved in major biogeochemical cycles of carbon, nitrogen, and sulphur (ZHANG et al., 2019). Therefore, the complexity of the microbial community of cultural heritage materials, although initiated by a biofilm of photosynthetic bacteria, is expected to be high (PINAR et al., 2009).

During the Roman Empire (2^{nd} - 3^{rd} centuries AD) the Romula site was the largest urban, economic and cultural centre in the Roman province of Dacia Inferior (Malvensis) located North of the Lower Danube sector, on the northern border of the Roman Empire (NEGRU et al., 2016). Archaeological studies during the last half century revealed one of the main ceramic centres in this Roman province (POPILIAN, 1976). In the northern industrial sector of this city, eight ceramic workshops were discovered comprising 25 kilns for burning ceramic building materials, ceramic pots, lamps, and other clay objects (POPILIAN, 1976; NEGRU et al., 2020).

With a view to preventing against potential biodegradation of the ancient pottery exposed to various biological agents, the current study focused on evaluating the quantitative microbial density associated with a batch of ceramic vessels from the Roman archaeological site of Romula including a terra sigillata bowl, a plate of fine pasta, and amphorae fragments, and screened their potential to produce different metabolites, including enzymes and antimicrobial antagonistic factors in order to identify new microbial systems for preservation and restoration of cultural heritages using natural bioactive compounds. Moreover, the chemical composition and physicochemical properties of the pottery materials were also determined and discussed in relation with the determined microbial data.

MATERIALS AND METHODS

The investigated samples were represented by ceramic fragments of three categories of archaeological objects represented by pottery for transport, food and construction materials. The samples were obtained from the archaeological site Romula, located in the proximity of Caracal in Olt County, Romania (Fig. 1). The ceramic fragments originated from the archaeological construction site fund, and were not classified as heritage objects according to the National law, while not being certified as classified objects, and thus available for investigation by destructive and non-destructive methods for obtaining scientific information (O.G. 43/2000 approved by Law no. 378/2001).

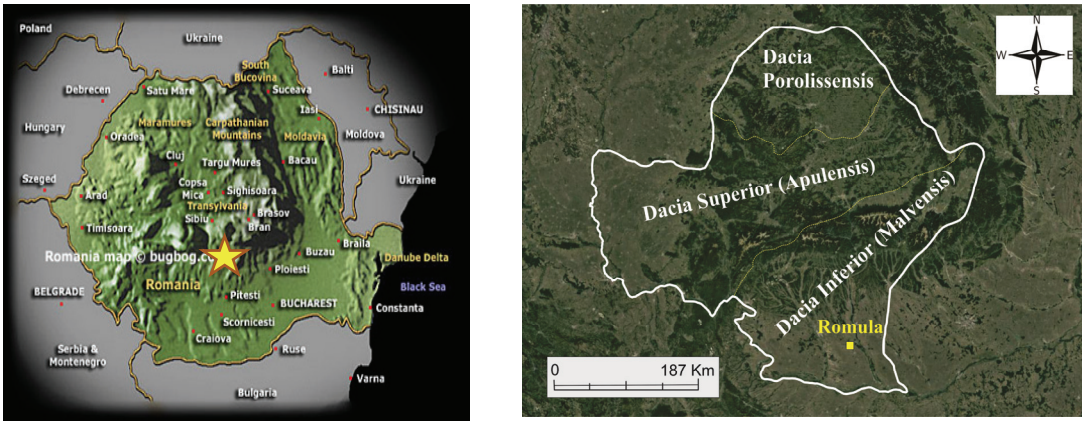


Figure 1. The geographically localization of Romula archaeological site - Google Earth, Copyright Image 2022 Maxar Technologies.

The chemical composition of the ceramic samples was performed by X-ray fluorescence using an XRF Rigaku ZSX100e Supermini system (Rigaku, Japan), following the manufacturer protocol. After grounding with a pestle, the chemical composition of powdered material was recorded by the system and reported as mass percentage (%).

In order to *estimate the colony-forming units,* the fragments were weighed and placed in an Erlenmeyer flask containing 50 mL of physiological serum and incubated for 7 days at 25°C under stirring conditions (150 rpm). The presence of microorganisms in the washing serum was assessed 2 and 7 days after incubation, and quantitated by the serial decimal dilution method in MH and nutrient solid media after incubation at 37°C and 28°C for 48 hours and seven days respectively (MH medium). The microbial density was estimated as the number of bacterial colony-forming units per mL of washing serum.

The microbial strains were isolated using the MH culture medium with the following composition (g/L): NaCl 100, yeast extract 10, $MgCl_2 \times 6H_2O$ 7, $MgSO_4 \times 7H_2O$ 9.6, $CaCl_2 \times 2H_2O$ 0.36, KCl 2, $NaHCO_3$ 0.06, NaBr 0.026. The pH value of the medium was adjusted to 7.2 before autoclaving at 121° C (LUCACI et al., 2021), and nutrient broth medium and further cultivated at 28°C for 48 hours.

The pH values have been determined by using the Hanna HI4522 pH meter.

The protein concentration of washing serum was determined by using the Lowry protocol with bovine serum albumin as standard (LOWRY et al., 1951).

Extracellular hydrolytic activities (protease, amylase, esterase, gelatinase, and cellulase) were assessed as previously reported (BENMEBAREK et al., 2020; RUGINESCU et al., 2022) by cultivation at 28°C for 48 hours on solid media supplemented with the corresponding substrates (casein, gelatine, carboxymetylcellulose, Tween 80 and starch, all chemicals from Merck, Germany) and the extracellular activities were identified based on halo formation due to substrate consuming around of microbial colonies formed.

The antagonistic activity between the isolated strains was evaluated using each strain both as a target and as a producer. In this technique, the target bacterial strain was cultivated in liquid medium for 24 h until reached the stationary phase. 1 mL of broth culture containing about 10^8 CFU/mL was streaked on solid agar medium, and 50 µL of cell suspension (10^8 CFU/mL) from each tested producer strain was added to wells made on the inoculated plates. The inoculated plates were incubated for 48 h, and the presence of an inhibition halo indicated an antagonistic activity.

RESULTS

Fourteen ceramic samples obtained from the archaeological site Romula were investigated in this study (Fig. 2).

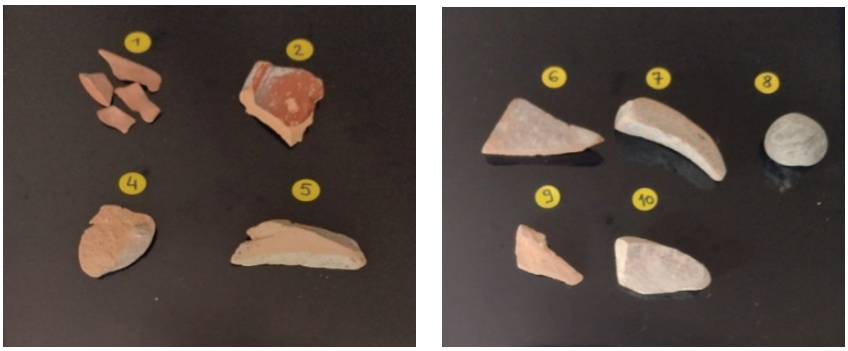


Figure 2. Different archaeological objects. The significance of number corresponding with number from Table 1.

Generally, they consisted of small dimension fragments with weight varying from one gram (sample 1 – food pottery fragment) to nine grams (sample 7 - transportation pottery) (Table 1). The samples 11-16 (not shown) corresponded to finely grounded ceramic (ceramic dust).

Table 1. Description of the investigated archaeological samples.

Sample number	Sample codification	Weight (grams)	Sample type
1	1. ROM TS 36	1	Food pottery
2	2. TS ROM 108	5	Food pottery
3	3. ROMULA IS 4728 AP S14 AC2	8	Construction material
4	4. ROM AMF 10827	4	Transportation pottery
5	5. ROM AMF 9121	4	Transportation pottery
6	6. ROM AMF 10666	6	Transportation pottery
7	7. ROM AMF 10542	9	Transportation pottery
8	8. ROM AMF 14862	8	Transportation pottery
9	9. ROM AMF 8442	1	Transportation pottery
10	10. ROM AMF 8571	6	Transportation pottery
11	11. ROM 2017 IS4730 (C1)	5	Construction material
12	12. ROM 2017 IS4729 (C2)	8	Construction material
13	13. ROM 1503 C (C3)	4	Construction material
14	14. ROM 1050/C 10C (C4)	3	Construction material

The chemical composition of ceramic dust samples 11, 12, 13 and 14 determined by XRF analysis (Table 2) indicated variable concentrations of the commonly found compounds calcium (Ca), silica (Si), iron (Fe), aluminium (Al), potassium (K), and titanium (Ti). Among these, Si and Ca were the most abundant, ranging from 8.18 to 63.35 in case of Ca and from 18.69 to 56.84 mass percentages in case of Si (Table 2).

Table 2. Chemical composition of some building materials from Romula archaeological site.

Chemical elements (mass %)	Sample 11	Sample 12	Sample 13	Sample 14
CaO	52.56	63.35	14.51	8.18
SiO_2	25.65	18.69	50.36	56.84
Fe_2O_3	9.30	6.34	11.94	12.21
Al_2O_3	5.16	4.15	10.75	13.23
K_2O	2.54	2.07	4.74	5.33
TiO_2	2.32	1.70	1.66	2.13
BaO	0	1.31	0	0
SnO_2	0	0	1.76	0
Trace of:	MgO; P_2O_5; SO_3; Cl; MnO; SeO_2; Br; SrO; ZrO_2; Ag_2O; Dy_2O_3.	P_2O_5; SO_3; Cl; Br; SrO; Ga_2O_3; Nb_2O_5; Eu_2O_3; Tb_4O_7; Fr.	MgO; P_2O_5; SO_3; Cl; MnO; SrO; ZrO_2; Ag_2O; Yb_2O_3; Cr_2O_3; ZnO; Rb_2O; Tc; U_3O_8.	MgO; P_2O_5; SO_3; Cl; MnO; SrO; Ag_2O; CuO; Rb_2O; ReO_2.

Moreover, traces of elements of several compounds (magnesium, sulphur, phosphorus, chlorine, manganese, selenium, bromine, strontium, zirconium, silver, dysprosium, gallium, europium, terbium, niobium, uranium, chromium) were also identified. Overall, the chemical composition of the analysed samples was relatively similar except for Barium (Ba) and Tin (Sn) that were present only in samples 12, and 13, respectively, and several trace elements showing a larger variation among the ceramic samples (Table 2).

The estimated number of the total colony-forming units (CFU) after two and seven days' growth on nutrient agar and MH culture media with different compositions and salinity appeared to be affected by the structure of the pottery samples (Table 3). In the case of nutrient agar, the CFU numbers were very similar among the tested samples, excepting some of the construction materials samples. The data shown in Table 3 revealed that the number varied from 3.1×10^5 (sample 1) to 9.8×10^6 (sample 10) after two days of growth and from 2.8×10^5 (sample 1) 9.6×10^6 (sample 10) after seven days of incubation. The obtained results revealed that after seven days of incubation, the number of CFU gradually decreases. Due to their rather diverse chemical composition, the presence of some bacteria in the analysed sample suggested that they may be exploited as a source of microelements for growth in first 48 hours of incubation. In the case of samples 11, 12, 13, and 14 the bacterial growth has been generally observed after seven days of incubation but in a very small number. Most probably the results are mainly argued by the construction material structure of the investigated samples. Compared to nutrient agar, on MH medium, the CFU number was significantly lower, ranging from 1×10 (sample 9) to 2×10^4 (sample 1) after two days of incubation, and from 5 (sample 10) to 3×10 (sample 3) after seven days of growth. Comparing the results obtained after two and seven days of growth, a decrease in the number could be observed, as in the case of nutrient agar.

Table 3. Estimation of number of colony-forming units related to investigated samples (CFU/mL). n.d.= no data recorded.

Sample	Nutrient agar 2 days	Nutrient agar 7 days	MH medium 2 days	MH medium 7 days
1.ROM TS 36	3.1×10^5	2.08×10^5	2×10^4	3.8×10^2
2.TS ROM 108	6.8×10^6	6.2×10^6	1.5×10^2	5.3×10
3. ROMULA IS 4728 AP S14 AC2	3.9×10^6	2.8×10^6	5.8×10	3×10
4. ROM AMF 10827	8.3×10^6	7.6×10^6	3.6×10^2	3.8×10^2
5. ROM AMF 9121	2.7×10^6	2.3×10^6	2.2×10^3	2.8×10^2
6. ROM AMF 10666	2.7×10^6	2.2×10^6	8×10^2	1.3×10^2
7. ROM AMF 10542	4.2×10^6	4×10^6	4.6×10^2	7.4×10
8. ROM AMF 14862	3.8×10^6	3.3×10^6	1.6×10^2	3.1×10^3
9. ROM AMF 8442	4.2×10^6	4.1×10^6	1×10	0
10. ROM AMF 8571	9.8×10^6	9.6×10^6	$7.5x\times10$	5
11. ROM 2017 IS4730 (C1)	2×10^5	2.6×10^5	0	0
12. ROM 2017 IS4729 (C2)	nd	3.8×10^4	0	0
13. ROM 1503 C (C3)	7	1.1×10^5	0	0
14. ROM 1050/C 10C (C4)	nd	3×10^4	0	0

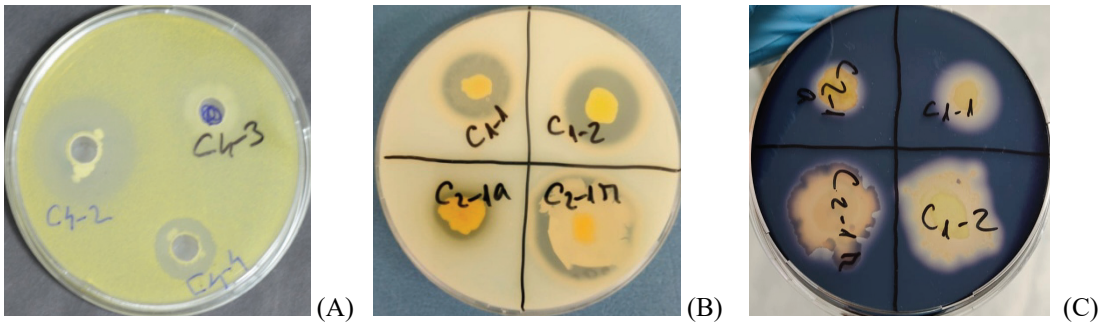
The pH values of the physiological serum used for isolation of microorganisms (CFU) did not show significant differences between samples after 2 or 7 days of incubation. Considering condition of strains isolation, the neutral values supported data obtained for the CFU number. Moreover, the protein content after 7 days of incubation varied from 13.71 µg/mL (sample 6) to 122.17 µg/mL (sample 3), supporting also the obtained number of microbial colonies on both nutrient agar and MH medium (Table 4). A relatively high content of protein, more than 100 µg/mL, has been obtained for the construction material (Table 4).

Table 4. pH values and protein content of microbial community obtained from ceramic samples.

Sample	pH values 2 days after growth	pH values 7 days after growth	Protein content (µg/mL)
Physiological serum	7.58		
1. ROM TS 36	7.8	8.1	28.93
2. TS ROM 108	8	8.15	16.35
3. ROMULA IS 4728 AP S14 AC2	8.7	8.27	122.17
4. ROM AMF 10827	8.55	8.5	118.8
5. ROM AMF 9121	8.4	8.45	83.73
6. ROM AMF 10666	8.25	8.3	13.71
7. ROM AMF 10542	8.4	8.5	105.51
8. ROM AMF 14862	8.2	8.24	118.46
9. ROM AMF 8442	8.05	8.3	68.42
10. ROM AMF 8571	8.33	8.54	97.6
11. ROM 2017 IS4730 (C1)	8.25	8.36	121.15
12. ROM 2017 IS4729 (C2)	8.34	8.26	109.16
13. ROM 1503 C (C3)	8.17	8.17	99.80
14. ROM 1050/C 10C (C4)	8.21	8.27	110.12

Fourteen colonies were isolated from the investigated ceramic samples, purified, and tested for the production of antagonistic interaction factors and extracellular hydrolytic activities.

Among the tested extracellular hydrolytic activities, proteases were detected in the case of 12 strains, amylases and esterases for 8 strains, cellulase for 7 strains, and gelatinases for 5 strains (Fig. 3B-C; Table 5). Most of these activities are involved in degrading compounds which can be used as food sources for microorganisms. In some cases, combined activities were observed.



(A) (B) (C)

Figure 3. The aspect the production of antagonistic factor from some investigated strains (A); proteinase (B) and amylase (C) production from some strains.

Table 5. Production of several extracellular hydrolytic enzymes by strains isolated from ceramic samples. The number represents the halo diameter in centimeters. The letter "s" means that a halo has been observed but with a diameter that is difficult to measure.

Enzymes	Proteinase	Amylase	Esterase	Gelatinase	Cellulase
Strains					
1-1	6	5	9		6
1-2	4	7	2		1
1-2R	10		11	1	
2-1A	2		7		4
2-2	s			1	4
2-3	s		2		
3-1	s	6	10		
4-1				1	2
4-2	7			s	
4-3	8	7	9	1	
4-4	6	s			
4-5	7	4			5
4-6	7	4	9		4
4-7		4			

The evaluation of the potential antagonistic activity of the isolated strains (Figs. 4, 3A; Table 6) revealed that 5 strains tested positive for production of antagonistic factors, among which strain 4-4 and 4-6 acting against strain 1-2 as target. All strains producing antagonistic factor acted towards strain 1-2 as a target. Moreover, the producing strain 4-2 acted against strains 1-1, 4-3, 4-5 and 4-6 as a target. The producing strain 2-1A acted against 4-5 as a target and strain 4-5 acting as producer for strain 4-7 as a target. This preliminary data indicated strain 4-2 as a promising candidate for further test for potential antimicrobial activity against pathogenic strains as an alternative to current antibiotics.

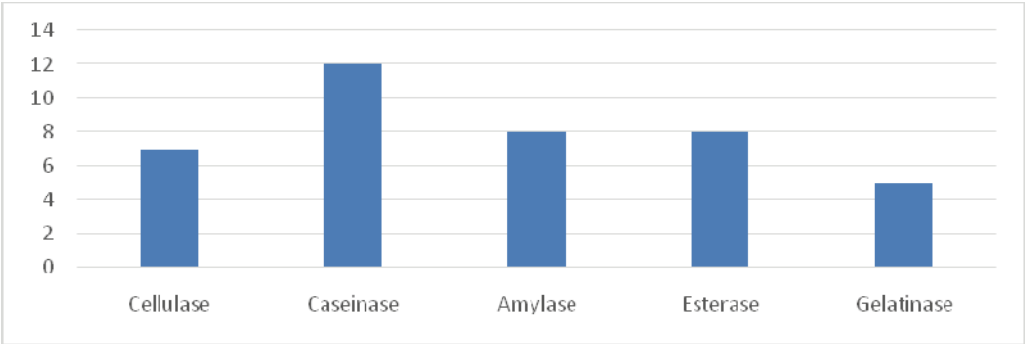


Figure 4. The number of strains identified as producers of extracellular hydrolytic activities. The number on the ordinate axis represents the number of strains.

Table 6. The production of factors with antagonistic activity. The number from the table indicated the halo diameter expressed in centimetres. The 0 representing the absence of activity.

Producers Target	1-1	1-2	1-2R	2-1A	2-2	2-3	3-1	4-1	4-2	4-3	4-4	4-5	4-6	4-7
1-1	-	0	0	0	0	0	0	0	20	0	0	0	0	0
1-2	0	-	0	12	0	0	0	0	32	0	20	0	1	0
1-2R	0	0	-	0	0	0	0	0	0	0	0	0	0	0
2-1A	0	0	0	-	0	0	0	0	0	0	0	0	0	0
2-2	0	0	0	0	-	0	0	0	0	0	0	0	0	0
2-3	0	0	0	0	0	-	0	0	0	0	0	0	0	0
3-1	0	0	0	0	0	0	-	0	0	0	0	0	0	0
4-1	0	0	0	0	0	0	0	-	0	0	0	0	0	0
4-2	0	0	0	0	0	0	0	13	-	0	0	0	0	0
4-3	0	0	0	0	0	0	0	10	15	-	0	0	0	0
4-4	0	0	0	0	0	0	0	0	0	0	-	0	0	0
4-5	0	0	0	18	0	0	0	18	1	0	0	-	0	0
4-6	0	0	0	0	0	0	0	0	16	0	0	0	-	0
4-7	0	0	0	0	0	0	0	0	0	0	0	25	0	-

CONCLUSIONS

In the current study, three types of materials were analysed from ancient ceramic used for transport amphorae, tableware, and construction materials from the Romula site, Romania, in order to estimate their associated microbial content and isolate and characterize ceramic-associated strains with putative protecting role against biodegradation of these materials. The chemical composition of the ceramic revealed majorly similar materials, and variable trace elements that could play a role in inhibiting the growth of substrate-associated microbes. The isolated microbial strains from the Roman pottery tested positive for five types of extracellular hydrolases, which should contribute to a selective microbial environment for associated communities. Among these, five microbial isolates stand out as producing factors with antagonistic activity and another six as their targets. Both these functional characteristics give a glimpse on unravelling the dynamics of the microbial community associated with the ancient pottery material for further unravelling the natural protective mechanisms against degradation.

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Bătrînescu-Moteau Costin, Neagu Simona, Lucaci Anca-Ioana, Ruginescu Robert,
Cojoc Roxana, Podosu (Vlad) Aurelia, Purcărea Cristina, Enache Mădălin
Institute of Biology of Bucharest of the Romanian Academy, 296 Splaiul Independenței, Bucharest, 060031, Romania.
E-mails: costinbatrinescu@gmail.co; simona.neagu@ibiol.ro; ioana.lucaci@ibiol.ro; robert.ruginescu@gmail.com; roxana.cojoc@ibio.ro; vladaurelia24@yahoo.com; cristina.purcarea@ibio.ro; madalin.enache@ibiol.ro

Negru Mircea
Faculty of Legal and Administrative Sciences, Spiru Haret University, 13 Ion Ghica Street, Bucharest, 030045, Romania.
Faculty of Social Sciences, University of Craiova, 13 Alexandru Ioan Cuza Street, Craiova, 200764, Romania.
E-mail: mircea_negru_arch@yahoo.com

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