NUTRITIONAL PARTICULARITIES OF PATHOGENIC CONDITIONED MICROORGANISMS FROM THE MICROBIOTA OF THE THERAPEUTIC WATERS

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Abstract. For their growth and multiplication, microorganisms need both major (C, O, H, N, S, P, K, Mg, Ca) and minor (Zn, Mn, Na, Cl, Cu, Ni) biogenic elements; they must find in the environment the source of C (energy) and a source of N, essential elements for the synthesis of specific molecules: proteins, nucleic acids. A microbiological analysis of 148 samples from 2017-2018 aimed at seeing whether different concentrations of nitrogen compounds in therapeutic waters, correlated with pH variation, can limit or stimulate the development of pathogenic conditioned microorganisms. The presence of total coliform bacteria in therapeutic waters was correlated with faecal coliforms but not with *Pseudomonas aeruginosa* or clostridiums. Intestinal enterococci were identified along with other microbiological indicators of faecal pollution (*Escherichia coli*, pseudomonadae, anaerobic bacteria), with the exception of total coliforms. Moreover, intestinal enterococci were identified in therapeutic waters regardless of their pH (R = 0.0264) but their numerical densities varied in direct proportion to their mineralization (R = 0.248) and also with the ammonium ion concentration (R = 0.3662).

Keywords: microbiota, nutrition, water, biogen, therapeutic.

Rezumat. Particularităţi nutriţionale ale microorganismelor condiţionat patogene din microbiota apelor terapeutice. Microorganismele au nevoie pentru creştere şi multiplicare de elemente biogene majore (C, O, H, N, S, P, K, Mg, Ca) şi minore (Zn, Mn, Na, Cl, Cu, Ni); ele trebuie să găsească în mediu sursa de C (energie) şi o sursă de N, elemente esenţiale pentru sinteza moleculelor specifice: proteine, acizi nucleici. Dacă concentraţii diferite ale compuşilor cu azot din apele terapeutice, corelate cu variațiile de pH, pot limita sau stimula dezvoltarea microorganismelor condiționat patogene, s-a urmărit prin analiza microbiologică a 148 de probe, în intervalul 2017-2018. Prezenţa bacteriilor coliforme totale din apele terapeutice a fost corelată cu cea a coliformilor fecali dar nu şi cu *Pseudomonas aeruginosa* sau cu clostridiile. Enterococii intestinali au fost identificaţi împreună cu alţi indicatori microbiologici ai poluării fecale (*Escherixhia coli,* pseudomonade, bacterii anaerobe), cu excepţia coliformilor totali. De asemenea enterococi intestinali au fost prezenţi în ape terapeutice indiferent de pH-ul lor (R=0.0264) dar densitatea lor numerică a variat direct proporțional cu mineralizarea lor (R=0.248) dar şi cu concentrația ionilor de NH_4^+(R=0.3662).

Cuvinte cheie: microbiota, nutriție, apa, biogen, terapeutic.

INTRODUCTION

For their growth and multiplication, as well as for other manifestations of their biological activity, microorganisms need the same biogenic elements found in the structure of any biological system, differentiated in major bioelements (C, O, H, N, S, P, K, Mg, Ca and Fe) and minor bioelements (Zn, Mn, Na, Cl, Mo, Se, Co, Cu, W, Ni) (CABRAL & MARQUES, 2006). In all natural ecosystems there are fluctuations in nutrient and energy concentrations for which micro-organisms permanently modulate their energy-producing reactions (through genetic regulation of enzyme synthesis involved - induction/repression) in order to be in agreement with energy-consuming reactions and ensure cell survival (ESPIGARES et al., 1996).

Mineralized water can provide a significant contribution to the nutrients required by microorganisms (Ca^{2+} and Mg^{2+}) (JIN LI et al., 2010). Natural mineral waters have been described as complex living environments containing a various microbiota consisting of aerobic heterotrophic native microorganisms (*Pseudomonas* spp., *Aeromonas hydrophila*), total and faeces coliforms (*Escherichia coli)* but also opportunistic pathogens. The typology of pathogen microorganism species identified in the aquatic environment has increased over the last decade, with the emergence of new species of bacteria from faeces sources, capable of developing in the water distribution system.

In order to analyse the risk of the presence of pathogens in natural mineral water, the starting point was to understand the ecology of these microorganisms, but also to study the interactions between native bacteria in water and potential pathogens in different habitats (distilled/bi-distilled water, drinking water, underground water) that offer variable nutritional opportunities. In contrast to eutrophic waters, oligotrophic natural mineral waters have shown a much lower diversity of species, with a small number of species probably reflecting the limited availability of organic C in these oligotrophic habitats (KERR et al., 1999).

The presence of *Escherichia coli* in non-carbonated mineral waters has no antagonistic effect on native microbiota; *E. coli* cells lysed during storage release their content in the environment, these substances being used by local microorganisms, which explains their increase in inoculated samples as opposed to non-inoculated samples (LOY et al., 2005). Ammonia is one of the key molecules in the biogeochemical cycle of nitrogen; its presence in surface water can be caused by direct contamination with agricultural fertilizers and/or microbial degradation of proteins, nucleic acids and urea. Ammonia is rapidly oxidized in the environment, usually present in natural waters in concentrations lower than 0.1 mg/l. Concentrations higher than this indicate a recent high-level contamination with waste where the ammonia level is very high, around 101 or 102 mg / l (LAZĂR et al., 2016).

ESPIGARES et al. (1996) conducted a comparative study on chemical and microbiological indicators of pollution (total and faecal coliforms, faecal streptococci and sulphite-reducing clostridiums) in the Guadalquivir River (Spain) and its tributaries. Total coliforms have been correlated with faecal coliforms, but not with faecal streptococci or clostridiums. The presence of faecal streptococci and clostridium was correlated with that of other indicators, except for total coliforms. All these microbiological indicators were negatively correlated with the dissolved oxygen and correlated positively with dissolved organic C, ammonia (PAYMENT et al., 2003). CABRAL & MARQUES (2006) noted that ammonia was significantly correlated with all tested microbiological parameters (total and faecal coliforms, faecal streptococci and enterococci) in the study of the Febros River (NW Portugal).

MATERIAL AND METHODS

The degree of microbiological pollution from 148 samples of mineral therapeutic waters (MTW) was quantified over the period February 2017- December 2018, identifying the bacterial indicators of pollution that affect their way of association in matrices with a different profile regarding their chemical composition. Concentrations of nitrogen compounds (recognized bacterial nutrients), pH, MTW mineralization were also evaluated.

The method used to quantify the microorganisms was that of membrane filtration (from cellulosic esters with a pore diameter of 0.45μm). The number of coliform bacteria and *Escherichia coli* was obtained by counting specific colonies developed on culture media according to SR EN ISO 9308-1:2015; for the identification of intestinal enterococci the working methodology according to SR EN ISO 7899-2:2002 was used and the number of *Pseudomonas aeruginosa* bacteria was obtained according to SR EN ISO 16266:2008. SR EN 26461-2:2002 was used as standard method for detection and enumeration of the sulphite-reducing anaerobe bacteria. The results of the microbiological analysis were expressed as colony forming units (CFU) per unit volume. The pH of the mineral therapeutic waters was also monitored in the study, for which the electrometric method according to SR EN ISO 10523:2012 was used; the weight of ammonium ions from the analysed waters was identified by the manual spectrometric method according to SR ISO 7150-1: 2001 and concentrations of nitrate ions were calculated by the spectrometric method with sulfosalicylic acid according to SR ISO 7890-3:2000. For the determination of the nitrite ions, the spectrometric method and SR EN 26777:2002 were used.

The obtained results were processed in Excel Office Microsoft and statistically analysed by calculating linear regression and the Pearson correlation coefficient, as the values of $R^2 \leq 1$ represented a direct correlation between the variables, $-1 \leq R^2 < 0$ - inverse correlation and $R^2 = 0$ - the absence of a correlation between the variables.

RESULTS

From the bacteriological analysis of 148 samples of mineral therapeutic waters, within 24 months (February 2017- December 2018), 32 samples (21.62%) were evaluated as microbiologically contaminated because pollution indicating microorganisms were identified single or in association, with different densities, sometimes exceeding the maximum admissible reference concentrations from Government Decision 1020/2005 for natural mineral water and Government Decision 459/2002 for bathing water (Table 1).

Table 1. Microbiological indicators of pollution identified in therapeutic mineral waters with variable biogenic profile.

Sample of microbiologically impure MTW	Coliform Bacteria (CFU/100ml)	*Escherichia coli* (CFU/100ml)	Intestinal enterococci (CFU/100ml)	*Pseudomonas aeruginosa* (CFU/100ml)	Anaerobic sulphite reducing bacteria (CFU/100ml)
Govora, Vâlcea county The Spring 2 The Spring 3 The Spring 9	 $2{,}7\cdot10$ $1{,}4\cdot10^2$ $1{,}9\cdot10^2$	 0 0 0	 0 0 $1{,}4\cdot10^2$	 0 0 0	 0 0 0
Amara Lake, Ialomiţa county The southern gulf Lebada Hotel Point	 $2{,}8\cdot10^2$ $1{,}1\cdot10^2$	 $4{,}8\cdot10^1$ $0{,}8\cdot10^1$	 $1{,}9\cdot10^2$ $0{,}6\cdot10^1$	 0 0	 $0{,}2\cdot10^1$ 0
Techirghiol, Constanţa county-Techirghiol Lake (BT)	$2{,}8\cdot10^2$	$1{,}2\cdot10^2$	$9{,}5\cdot10^1$	$0{,}1\cdot10^1$	$6{,}6\cdot10^1$
Brădetu, Argeş County- 1 IBF Source	$0{,}1\cdot10^3$	0	0	0	0
Sărata, Bacău county-FH1 Spring	$0{,}1\cdot10^2$	0	$3{,}3\cdot10^1$	$0{,}1\cdot10^3$	$0{,}1\cdot10^2$
Pucioasa, Dâmboviţa county Well nr.1	**$2{,}3\cdot10^2$**	$1{,}1\cdot10^1$	$0{,}2\cdot10^1$	0	$0{,}1\cdot10^1$
Topliţa, Harghita county Source 19804IFLGS Source F1 ICSPC	 $0{,}2\cdot10^1$ $0{,}4\cdot10^1$	 0 0	 0 0	 0 0	 0 $0{,}1\cdot10^1$
Geoagiu,.Hunedoara county Source F3	$6{,}8\cdot10^1$	0	0	$0{,}1\cdot10^1$	0

Călimăneşti, Vâlcea county – 6 springs mixture 1009 FORADEX well	$3,2\cdot10^1$ $0,7\cdot10^1$	0 0	$0,1\cdot10^2$ 0	0 0	0 0
Pietrari, Vâlcea county- Pietrari Spring	$0,1\cdot10^1$	0	$0,8\cdot10^1$	0	0
Sovata, Mureş county - Black lake (BT)	$0,3\cdot10^1$	$0,1\cdot10^1$	0	0	0
Tuşnad, Harghita county – 2 Ileana Spring (BT)	$4,2\cdot10^1$	0	$0,2\cdot10^1$	0	0
Băile Herculane-Caraş Severin county F4573 bis Source Apollo II Spring	$0,1\cdot10^1$ $0,6\cdot10^1$	0 0	0 0	$0,6\cdot10^1$ $1,3\cdot10^1$	$0,1\cdot10^1$ 0
Crişeni, Sălaj county - F1 Source	0	0	0,1	0	0
Eforie Nord, Constanţa county – Water from Black See	$7,8\cdot10^1$	$0,1\cdot10^1$	$2,7\cdot10^2$	$5,4\cdot10^1$	$2,9\cdot10^2$
Ocna Sibiului, Sibiu county – Avram Iancu Lake	$2,3\cdot10^2$	0	$0,4\cdot10^2$	0	$3,6\cdot10^1$
Slănic Prahova, Prahova county – Baia Baciului lake (BT)	$0,5\cdot10^1$	0	$9,6\cdot10^1$	0	$4,3\cdot10^1$
Slănic Moldova, Bacău county - Spring nr.3	$0,1\cdot10^1$	0	0	0	0
Tăşnad, Satu-Mare county – 4715 Source	$0,1\cdot10^1$	0	0	0	0
Covasna, Covasna county – FVII IAFAAA Source	$0,3\cdot10^1$	0	0	0	0
Băile Felix, Bihor county F4003 Source FP2 Source	$1,8\cdot10^1$ $0,8\cdot10^1$	0 0	0 0	0 $1,7\cdot10^1$	0 0
Crasna, Sălaj county– F1H Source	$0,3\cdot10^1$	0	0	0	0
Nicolina Iaşi, Iaşi county– F 3506 Source	$0,7\cdot10^1$	0	0	0	0
Bizuşa, Sălaj county- Bizuşa Băi Nord well	$2,5\cdot10^2$	$0,4\cdot10^1$	0	$1,3\cdot10^2$	0
Târgu-Ocna, Bacău county- The lake with no bottom	$1,3\cdot10^2$	$0,1\cdot10^1$	0	0	$0,6\cdot10^1$

BC = Coliform Bacteria; E. coli = *Escherichia coli*; E = Enterococci; P. a = *Pseudomonas aeruginosa*; BASR = Anaerobic Sulfite-Reducing Bacteria

In 37.5% of cases, the unique microbiological pollutant was coliform bacteria or enterococci, the maximum incidence being represented by coliform bacteria identified in 12 samples (Fig. 1).

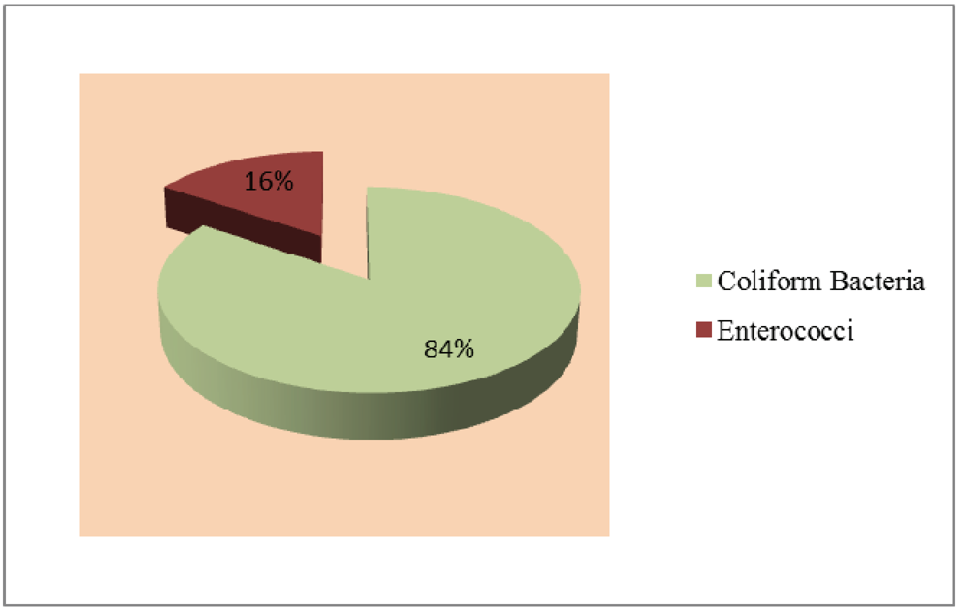


Figure 1. The percentage presence of the unique micropollutant from mineral therapeutic waters.

In the case of microbial associations constituting the contaminating microbiota of the analysed mineral waters with a different biogenic profile (Table 2), coliform bacteria and intestinal enterococci were the most frequently identified group (16.67%); in a similar percentage of 11.12%, the following groups were isolated as sources of contamination: coliform bacteria + intestinal enterococci + sulphite-reducing anaerobic bacteria and coliform bacteria + sulphite-reducing anaerobic bacteria (***. Universitatea din Bucureşti, 2014; ***. România, SR ISO/2000; 2001; Government Decision 459/2002; Government Decision 1020/2005; SR ISO/2008; 2012; 2015).

Table 2. The biogenic profile (nitrogen compounds, pH, mineralization) of therapeutic waters analysed.

Sample of MTW microbiologically impure	NH_4^+ ($mg \cdot dm^{-3}$)	NO_2^- ($mg \cdot dm^{-3}$)	NO_3^- ($mg \cdot dm^{-3}$)	Mineralization ($mg \cdot dm^{-3}$)	pH
Govora, Vâlcea county					
The Spring 2	1,3	≤ 0,06	5,7	2228,2	7,52
The Spring 3	0,5	≤ 0,06	3,1	1619,0	7,18
The Spring 9	2,9	≤ 0,06	8,3	1774,4	7,53
Amara Lake, Ialomiţa county					
The southern gulf	≤ 0,03	≤ 0,06	13,6	14830,1	8,28
Lebada Hotel Point	≤ 0,03	≤ 0,06	11.2	15015.6	8.05
Techirghiol, Constanţa county- Techirghiol Lake (BT)	≤0,03	≤0,06	14.5	82183.6	8,25
Brădetu, Argeş County- 1 IBF Source	0,8	0,10	3,2	1404,5	7,64
Sărata, Bacău county- FH1 Spring	1,6	0,16	4,9	203231,7	7,26
Pucioasa, Dâmboviţa county Well nr.1	0,6	0,10	2.8	3633.5	7,08
Topliţa, Harghita county					
Source 19804IFLGS	≤ 0,03	≤ 0,06	5,8	1484,3	6,52
Source F1 ICSPC	≤ 0,03	≤ 0,06	3,4	1480,3	6,48
Geoagiu, Hunedoara county Source F3	0,6	0,10	4.35	1102.45	7,31
Călimăneşti, Vâlcea county – 6 springs mixture					
1009 FORADEX well	0,2	≤ 0,06	5,7	14149,7	6,93
	0,5	≤ 0,06	5,7	14035,1	7,02
Pietrari, Vâlcea county- Pietrari Spring	17,2	0,15	6,7	146256,1	6,03
Sovata, Mureş county - Black lake (BT)	3,7	0,20	8,6	54783,7	6,57
Tuşnad, Harghita county – 2 Ileana Spring (BT)	2,5	0,10	3,8	4881,0	6,27
Băile Herculane- Caraş Severin county					
F4573 bis Source					
Apollo II Spring	0,4	0,08	7,5	7592,0	7,12
	1,3	0,10	8,5	2754,9	7,37
Crişeni, Sălaj county - F1 Source	1,2	0,07	4,2	4964,2	7,26
Eforie Nord, Constanţa county – Water from Black See	0,6	0,07	3,7	12377,0	7,28
Ocna Sibiului, Sibiu county – Avram Iancu Lake	145,2	0,09	6,2	169614,4	6,04
Slănic Prahova, Prahova county – Baia Baciului lake (BT)	15,3	0.07	12,2	17761,2	6,25
Slănic Moldova, Bacău county - Spring nr.3	4,5	0,10	3,8	15875,7	7,53
Tăşnad, Satu-Mare county – 4715 Source	9,3	0,08	3,8	9030,4	7,51
Covasna, Covasna county – FVII IAFAAA Source	0,5	≤0,06	1,1	8100,0	6,72
Băile Felix, Bihor county					
F4003 Source	2,4	≤0,06	3,0	917,0	7,05
FP2 Source	1,6	≤0,06	0,5	827,9	7,52
Crasna, Sălaj county– F1H Source	1,8	0,10	7,9	5268,3	8,01
Nicolina Iaşi, Iaşi county– F 3506 Source	10,8	0,15	≤0,06	25171,1	7,51
Bizuşa, Sălaj county- Bizuşa Băi Nord well	≤0,03	≤0,06	5,0	1970,4	6,52
Târgu-Ocna, Bacău county- The lake with no bottom	2.8	≤0,06	5.1	18533.3	7.12

The following microbial clusters have been identified as pollution agents for mineral therapeutic water with the lowest frequency (2.78%): BC + *E. coli*, BC + *E. coli* + E, *E. coli* + E, BC + *E coli*+ E + P.a + BASR, BC + *E. coli* + Pa, E + P.a + BASR (Fig. 2).

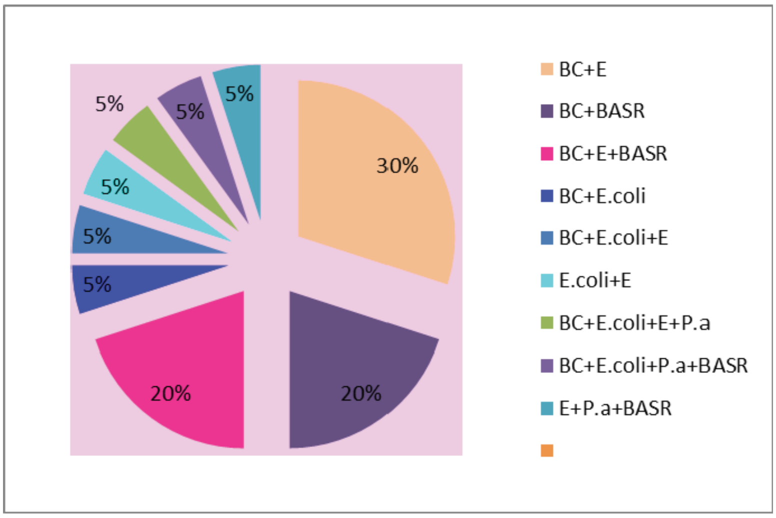
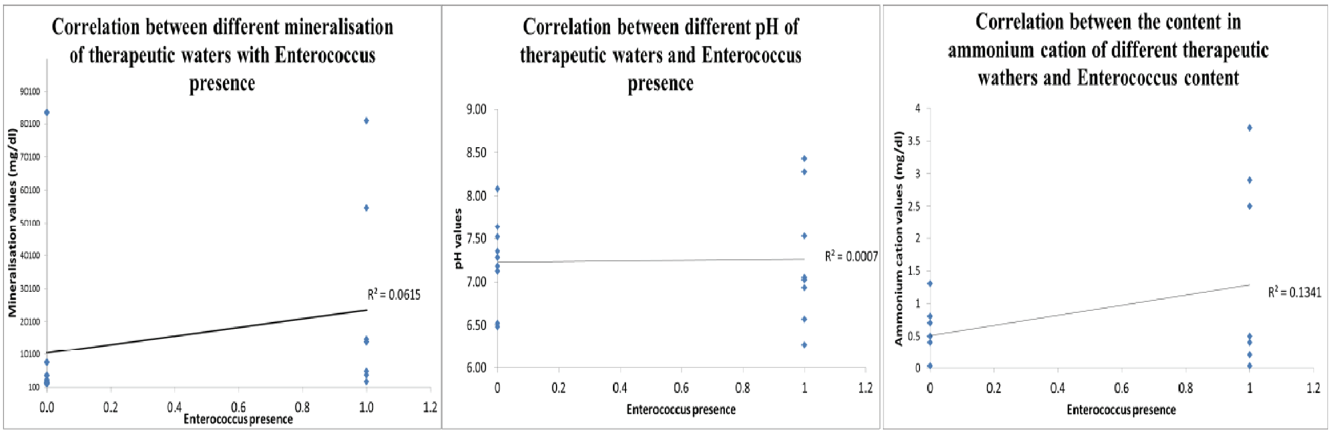


Figure 2. The percentage presence of the microbial associations from mineral therapeutic waters contaminated.

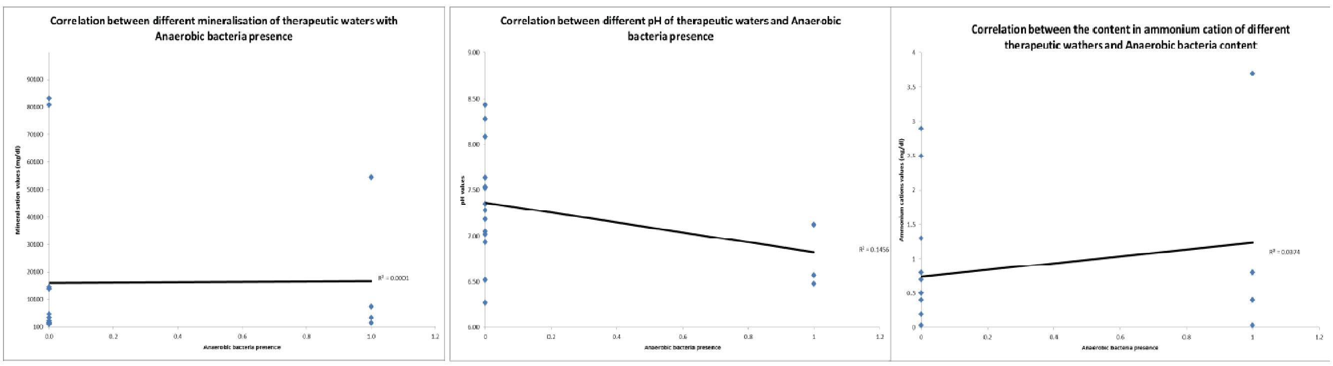
DISCUSSIONS

Intestinal enterococci were identified in mineral therapeutic waters regardless of their pH (R = 0.0264) but their numerical densities varied directly in proportion to their mineralization (R=0.248) and also to the ammonium ion concentration (R=0.3662) (Figs. 3a; b; c).



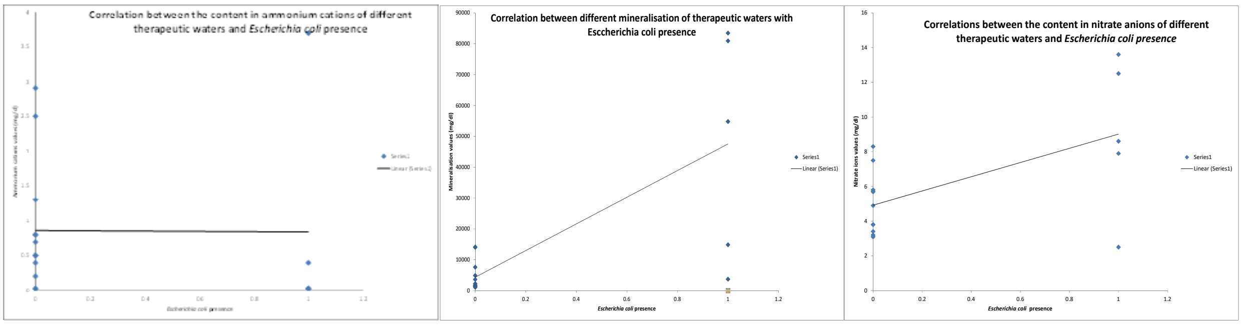
Figures 3a; b; c. Presence of intestinal enterococci in mineral therapeutic waters with variable biogenic profile (NH_4^+, pH, mineralization).

Anaerobic sulphite-reducing bacteria (*Clostridium* sp.) showed a tendency to increase the UFC/100mL when ammonium concentrations in MTW increased (R=0.1934). For pH values, the results indicated an inverse correlation with the number of anaerobic bacteria (R=-0.3816), and the degree of mineralization of therapeutic waters was indifferent to the development of this type of bacteria in mineral therapeutic waters (R = 0.01) (Figs. 4a; b; c).



Figures 4a; b; c. Presence of anaerobic sulphite-reducing bacteria (Clostridia) with variable biogenic profile (NH_4^+, pH, mineralization).

The number of *Escherichia coli* showed a tendency to increase (CFU/100ml) when the mineralization of therapeutic waters increased (R=0.7355) as well the concentration of NO_3^- ions (R= 0.5924). For NH_4^+ values, the results indicated an inverse correlation with the number of *E.coli* (R=-0.0087) (Figs. 5a; b; c).



Figures 5a; b; c. Presence of *Escherichia coli* with variable biogenic profile (NH_4^+, NO_3^-, mineralization)

Coliform bacteria were identified in all samples of the analysed mineral therapeutic waters except the one taken from the drilling F4573bis from Băile Herculane. *Pseudomonas aeruginosa* was only present in the F3 drilling in Geoagiu Băi – Hunedoara.

CONCLUSIONS

From the bacteriological analysis of 148 mineral therapeutic waters samples, within 24 months, 32 samples (21.62%) were evaluated as microbiologically contaminated; in 37.5% of cases, the unique microbiological pollutant was coliform bacteria or enterococci, the maximum incidence being represented by coliform bacteria identified in 12 samples.

In the case of microbial associations that constituted the contaminating microbiota of the analysed mineral therapeutic waters, coliform bacteria and intestinal enterococci were the most frequently identified group (16.67%);

The following microbial clusters were identified as pollution agents for mineral therapeutic water with the lowest frequency (2.78%): BC + *E. coli*, BC + *E. coli* + E, *E. coli* + E, BC + *E .coli* + E + P.a + BASR, BC + *E. coli* + Pa, E + P.a + BASR.

Intestinal enterococci were identified in mineral therapeutic waters regardless of their pH (R=0.0264) but their numerical densities varied directly in proportion to their mineralization (R=0.248) and also with the ammonium ion concentration (R=0.3662).

Anaerobic sulphite-reducing bacteria (Clostridia) showed a tendency to increase the UFC/100ml when ammonium concentrations in mineral therapeutic waters increased (R = 0.1934). For pH values, the results indicated an inverse correlation with the number of anaerobic bacteria (R=-0.3816). The mineralization degree of therapeutic waters was indifferent to the development of this type of bacteria in mineral therapeutic waters (R = 0.01).

The total cells of *Escherichia coli* showed a tendency to increase (CFU/100ml) when the mineralization of therapeutic waters increased (R=0.7355) as well the concentration of NO_3^- ions (R=0.5924). For NH_4^+ values, the results indicated an inverse correlation with the number of *E.coli* (R=-0.0087).

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