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PHILOGENETIC CONTAINMENT OF BACTERIAL ROOTS INSULATED FROM PATIENTS WITH COLON CANCER COMPARED WITH OTHERS INSULATED FROM HEALTHY PATIENTS

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ABSTRACT

Given that in day-to-day surgery we are experiencing advanced colon cancer cases, this issue appearing amid a large and growing incidence, it is understandable why more and more researchers are being trained in the research of this type of neoplasm. The directions that inspire us and our interest are represented by the elucidation of the determinant and favoring factors in the appearance of colon cancer, of the molecular mechanisms of action of these factors in the normal cell and its transformation into a neoplastic cell. We analyzed the aerobic/ aerobic- facultative anaerobic species composition of intestinal microbiota in a group of colon cancer patients in an attempt to identify a pattern specific or more commonly associated with this disease, as well as the study of the antibiotic factors expressed by the isolated strains compared to those isolated from a group of healthy individuals, thus leading to several conclusions.

KEY WORDS: *colon cancer, colon microbiota, bacterial strains, colon bacteria, opportunistic microorganisms.*

INTRODUCTION

In recent years, cancer has become an important public health issue for Central and Eastern European countries, being the second leading cause of death right after cardiovascular disease deaths in Western and Eastern European countries.

Colon cancer (CC) is associated with an increased morbidity and mortality. In 2002, colon cancer accounted for 9.4% of all global neoplasms, causing about 1.4 million new cases annually.

In terms of global incidence, the CC was the fourth type of neoplasm in man (after lung, prostate and stomach cancer), and third in women (after breast and uterine cancer). Incidence rate is increased in North America, Australia and New Zealand, intermediate areas in Europe and low in Asia, South America and Africa (Ferlay *et al.,* 2013).

Colon cancer occupies the second place in all cancers of the digestive tract, after gastric cancer and on the same level as rectal cancer. The relatively high frequency of colon cancer is due to favorable affections such as: polyposis, chronic colitis, ulcerative hemorrhagic rectocolitis and colonic diverticulosis. The most frequent location is on the left colon and especially the sigmoid (Constantinescu, 1996).

Opportunistic microorganisms are components of normal or alohtone microbiota that produce infections but do not normally have effective means of overcoming host defense mechanisms. The opportunists produce infections in immunosuppressed individuals, which may have genetic predispositions (specific immune defects, cellular and humoral), disseminated lupus erythematosus, lymphoproliferative diseases, AIDS, chemo and radiotherapy, tissue lesions etc. (Chifiriuc *et al.,* 2011; Zarnea & Popescu, 2011).

To be integrated into the category of opportunistic microorganisms, bacteria must meet the following conditions:

- the microorganism must be isolated from clinical lesions several times from immunocompromised hosts;
- to be present and isolated more often in immunodepressed hosts than in the rest of the population;
- its presence in a pathological process is not due to indirect causes "(Lazar, 2007).

The opportunistic microorganisms belong to the following genera: *Enterobacteriaceae, Bacteroides, Bacillus, Clostridium, Shigella, Salmonella, Staphylococcus, Streptococcus, Lactobacillus* etc., which in some cases can become pathogenic and can influence the neoplastic evolution (Chifiriuc *et al.,* 2011; Zarnea & Popescu, 2011).

To date, a clear association of bacterial species with gastric or colorectal cancer has been demonstrated (Gao *et al.,* 2015; Bonnet *et al.,* 2016) (Figure 1).

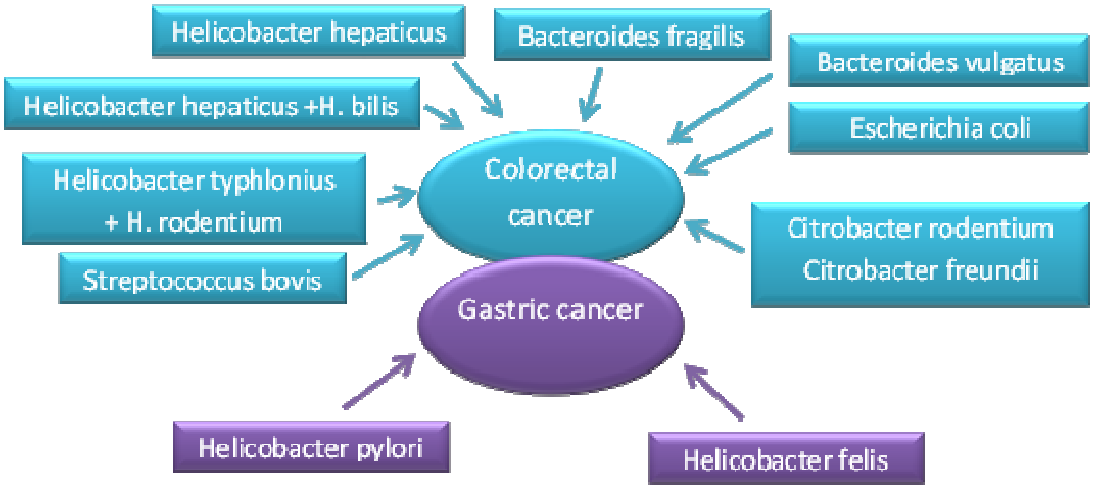


FIGURE 1. Synthetic representation of the main bacterial species cited in the literature as involved in the onset or evolution of digestive tract tumors

MATERIALS AND METHODS

A total of 41 samples (coprocultures) were collected, of which 32 from CC patients and 9 from healthy volunteers (control). Approximately 4 hours after collection, they were seeded with 10 µl calibrated strain on the Gelose / Muller-Hinton culture media supplemented with 5% ram blood, MacConkey / EMB / CLED, Selenite-Cysteine broth, and then in SS Agar medium. Incubation was performed at 37 ° C under aerobic conditions for 24 hours. Identification of microorganisms was performed on the basis of the examination of the culture and colony characters, Gram character, conventional biochemical tests (catalase, oxidase) and Triple Sugars Iron (MIU), MILF (Mobility, Indole, Lysine-decarboxylase, Phenyl-alanine-deaminase), Citrate-Simmons.

For the study, 205 bacterial strains were isolated, of which 42 microbial stems from the microbiota of CC patients and healthy volunteers were selected.

Subsequently, the genes encoding BLSE synthesis (Beta Spectrum Beta Lactamases by Polymerase Chain Reaction) were sequenced.

For strains identified as phenotypically resistant to BLSE or AmpC phenotypes, genotypic screening for the presence of resistance genes was performed for which oligonucleotide primer sequences corresponding to the following genes: bla_{CMY-2}, $bla_{CTX-M-I}$ and bla_{SHV} were selected.

For the phylogenetic framing of *E. coli* strains, a multiplex PCR targeting the *chuA, yjaA, TspE4.C2, arpA* (400bp), *arpA* (301bp) and *trpA* genes was performed. Initially, a quadruplex PCR was performed in which the first 4 genes were targeted; conformable:

- For fitting into the A filogroup, the profile is: (+---) respectively: (*arpA +, chuA-, yjaA-, TspE4.C2-*) or (+-+-) respectively: (*arpA+, chuA-, yjaA+, TspE4 .C2-*);
- For fitting into the B1 block, the profile is: (+ - +) respectively: (*arpA +, chuA-, yjaA-, TspE4.C2 +*);
- For fitting into the C-phylum, the profile is: (+ ---) respectively: (*arpA +, chuA-, yjaA-, TspE4.C2 -*);
- For fitting into the E filogroup, the profile is: (++ -) respectively: (*arpA +, chuA +, yjaA-, TspE4.C2-*) or (++ - +) respectively: (*arpA +, chuA +, yjaA-, TspE4. C2+*) or (+++ -) respectively: (*arpA +, chuA +, yjaA +, TspE4.C2-*);
- For the classification in phylogruppe D, the profile is: (++ -) respectively: (*arpA +, chuA +, yjaA-, TspE4.C2-*) or (++ - +) respectively: (*arpA +, chuA +, yjaA-, TspE4. C2 +*);
- For fitting into the F filogroup, the profile is: (- + -) respectively: (*arpA-, chuA +, yjaA-, TspE4.C2-*);
- For fitting into the filogroup B2 the profile is: (- ++ -) respectively: (*arpA-, chuA +, yjaA +, TspE4.C2-*) or (- + - +) respectively: (*arpA-, chuA +, yjaA-, TspE4 .C2 +*) or (- +++) respectively: (*arpA-, chuA +, yjaA +, TspE4.C2 +*);

If the profile was of the A or C/D or E phylogruppe, an additional multiplex PCR was performed in which the *arpA* (301pb) and *trpA* genes were targeted. If the *arpA* gene (301pb) was positive, then the phylogenetic framing is in group C, if negative, then the phylogenetic

framing is in group A. If the *trpA* gene was positive then the phylogenetic framing is in group E, if it is negative then Phylogenetic framing is in group D. (Clermont *et al.,* 2013).

In order to determine the capsular type (K) in *K. pneumoniae* strains, the *wzi* gene coding for an extra-membrane protein that allows attachment of the capsule to the cell surface (Brisse *et al.,* 2013) was used, followed by sequencing Sanger with the same Primers used in the PCR reaction. To amplify the sequence of interest, the reagent mixture - KAPA Biosystems was used.

The agarose gels used to electrophoretic migration of fragments obtained from the PCR reaction had a concentration of 1.5% for amplicons of the antibiotic resistance genes and for the *wzi* gene amplicons; and 2% for amplificons of the multiplex PCR reaction of phylogenetic framing of E. coli strains. Identification of amplicons was performed on the basis of characteristic dimensions using specific molecular weight markers.

For spectral acquisition (with the help of FTIR – ATR <***Fourier Transform Infrared Spectroscopy with Attenueted Total Reflectance***>), the bacterial strains were grown on Muller-Hinton medium at 37 ° C for 18h. An isolated colony was transferred directly from the plate to the spectrophotometer crystal and allowed to dry for 3-4 minutes. For each bacterial strain there were 3 technical replicas made on the same day. Between each tested isolate, a background image was made. All chemometric models were performed using *Matlab* version 6.5-13 (*MathWorks, Natick, MS*) (Peixe *et al.,* 2014).

RESULTS AND DISCUSSIONS

The examination of the cultures obtained on the media referred to under "Materials and Methods" allowed the following characteristics of culture and colony to be observed: homogeneous, small and medium size, round, regular appearance colonies. Also, on the environment of blood gelosis, it was observed that in some colonies the environment became transparent, which indicated that the environment had been consumed and some strains were haemolytic (Figure 2).

A B C

FIGURE 2. Coprocultures obtained on different media: A) - Blood gelosis; B) - EMB; C) – XLD

In order to reveal the microorganisms, Gram stained smears were made. Their examination requires the use of the Optical Microscope with the immersion lens, thus

highlighting the morphological characteristics of the cells as well as the Gram character (Figure 3).

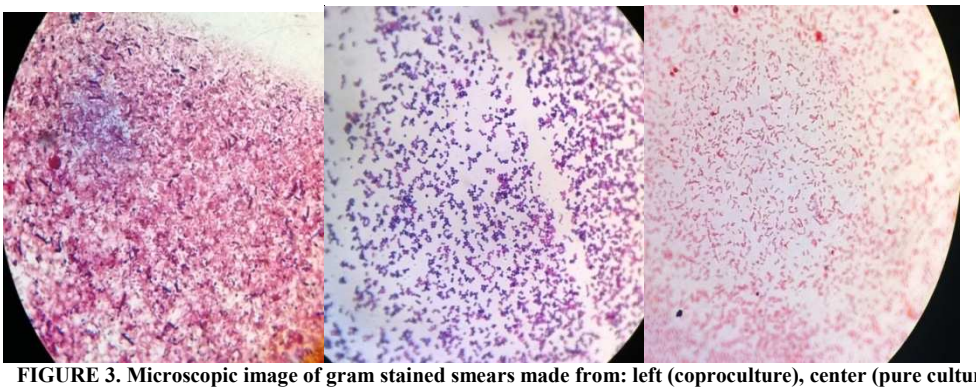
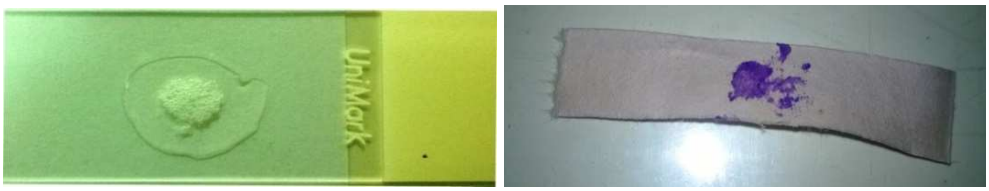


FIGURE 3. Microscopic image of gram stained smears made from: left (coproculture), center (pure culture of Gram prozitive bacteria), right (pure culture of Gram negative bacteria) (Enlargement: 2500x).



A B

FIGURE 4. A) - Positive reaction for the presence of catalase (O2 release); B) - positive oxidase reaction (indicated by the blue color of the filter paper impregnated with the reagent)

Biochemical tests to highlight: catalase and oxidase (Figure 4).

The negative oxidase test and the Gram character have led to a presumptive identification of bacterial strains, these belonging to the *Enterobacteriaceae* Family. If the oxidase test is positive, the catalase assay will continue to be done to discriminate between *Streptococcus sp.* and *Staphylococcus sp.*, *Bacillus sp.* and *Clostridium sp.*

Identification of bacterial species was performed using the multitest TSI, MIU, MILF and Citrate biochemical tests.

The results indicate that the highest incidence of all strains isolated from these patients is *E. coli* strains, followed by *Hafnia alvei, Klebsiella pneumoniae* and *Citrobacter freundii*, and the remaining isolates in approximately equal proportions (Figure 5).

Unlike these patients, in the control individuals, the incidence of *E. coli* strains was the highest. In contrast, they were followed by *Staphylococcus sp., Candida sp.* and *Enterococcus faecium*, and the remaining isolates were in approximately equal proportions. These individuals have noted that there is a fairly large diversity and some balance between Gram positive and Gram negative species, as well as species of microfungi (Figure 6).

Therefore, it can be argued that in patients with CC the results indicate a state of dysbiosis, compared to healthy individuals, whose microbiota are interspecific equilibrium or

the so-called eubios condition. Regardin the fact that no Gram-positive strains have been isolated in any of the CC patients, we can assume that this result was not accidental and that these Gram negative bacteria have not been eliminated from the antibiotic treatment of a recent infection.

Results obtained indicate that 148 Gram negative strains (100%) - 19 different species were identified in the CC patients, 38 Gram negative strains were identified in the control patients - 4 different species, 19 Gram positive strains - 9 different species and 5 yeast strains.

Of the 31 strains of E. coli tested, 21 strains were isolated from CC patients and 10 strains from healthy volunteers (Table 1).

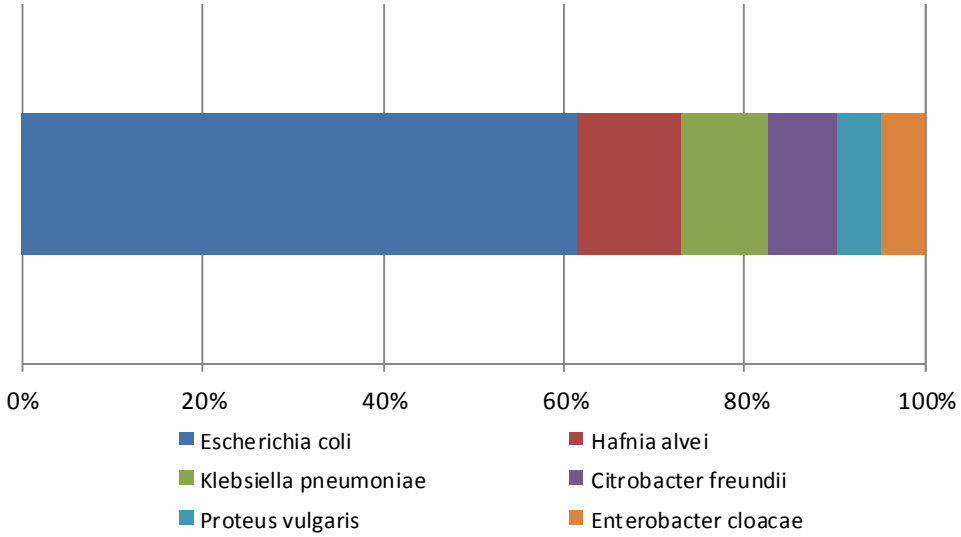


FIGURE 5. The isolation frequency of isolated bacterial strains from CC patients

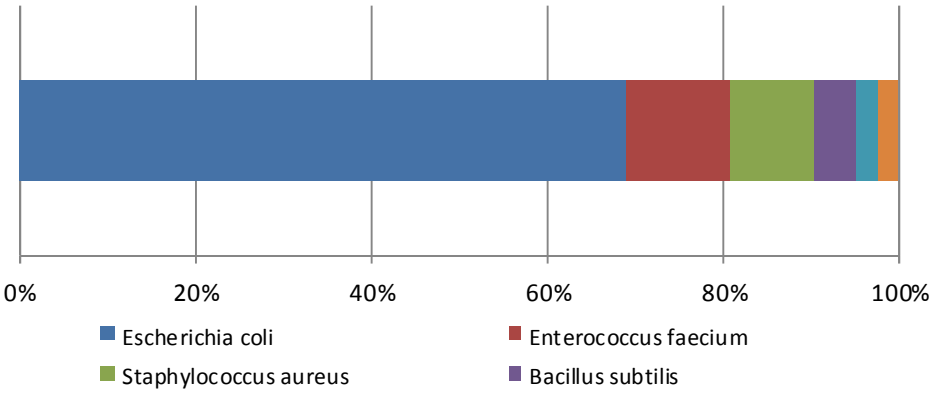


FIGURE 6. Frequency of isolation of isolated bacterial strains from healthy volunteers

TABLE 1. Phylogenetic framing of isolated E. coli strains from CC patients and healthy volunteers

Nr. Crt.	Strain	Patient/Volunteer	Sex	*E. coli* PhG
1	62	Patient 1	M	A
2	70	Patient 2	F	A
3	112	Patient 3	F	A
4	1014	Patient 4	F	A
5	1065	Patient 5	F	A
6	OP-McC*	Patient 17*	M	A
7	MD-McC	Patient 6	M	A
8	McC1	Patient 19	M	A
9	1	Patient 7	F	B1
10	1071	Patient 8	M	B1
11	1021	Patient 9	M	B1
12	BN-McC	Patient 10	M	B2
13	1001	Patient 11	F	B2
14	BE-MH1*	Patient 18*	M	C
15	BE-SS2*	Patient 18*	M	C
16	8	Patient 12	F	D
17	1017	Patient 13	F	D
18	SP-SS1*	Patient 17*	M	D
19	1023	Patient 14	M	E
20	1041	Patient 15	F	E
21	1027	Patient 16	M	F
22	L-Sg	Volunteer 3	F	A
23	M2	Volunteer 6	M	A
24	FM-EMB*	Volunteer 5	M	A
25	M14*	Volunteer 5’	M	A
26	FMIV – sg*	Volunteer 5	M	A
27	M4	Volunteer 7	F	B1
28	M-EMB	Volunteer 1	F	B1
29	M12	Volunteer 9	F	B2
30	M5	Volunteer 8	M	C
31	CC-EMB	Volunteer 4	F	D

The analysis of the PCR reaction, both for CC patients and for healthy volunteers, showed that the highest incidence had been attributed to the phylogroup A. According to a study by Străuţ and collaborators in 2015 on isolated blood strains, *E. coli* phylogruppe A is most common in Romania.

In patients with CC, after phylogroup A, the most common is filogroup D, followed by B1 (Figure 7).

Unlike these patients, the highest incidence for the phylogroup A in the control individuals was B1, followed by B2, C and D in equal proportions (Figure 8).

These data confirm other studies (Kohoutova *et al.,* 2014), according to which the A, D, B1 and B2 phylogroups are most effective in CC patients.

In the controlling individuals, even if the number of samples was relatively small (10), the results obtained attest the data from the literature.

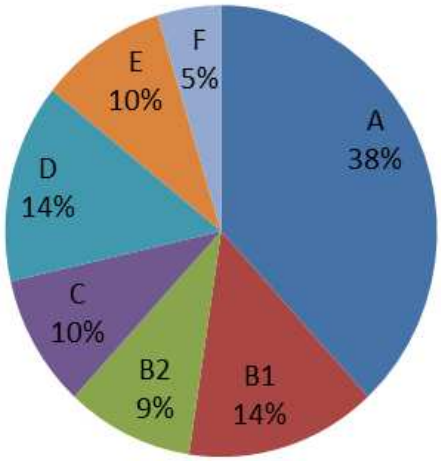


FIGURE 7. Frequency of *E. coli* folate groups in CC patients

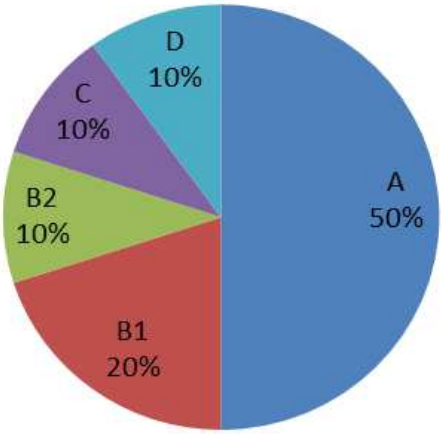


FIGURE 8. Frequency of *E. coli* phylogroups in healthy volunteers

Determination of the capsular K type in *K. penumoniae* strains was achieved by amplifying the *wzi* gene and sequencing it. These two methods were correlated and complemented by the FTIR-ATR spectral technique.

FTIR-ATR is an inexpensive method of spectroscopy, used as a screening method, preceded by molecular techniques, which makes clonal differentiation between bacterial strains belonging to the same species based on capsular polysaccharides.

In the present study, the FTIR-ATR results indicate that each of the 9 strains tested is a separate clone distinct from the rest. In addition to this, strains were grouped into three distinct groups (Figure 9).

Within each group, *K. pneumoniae* strains are more similar to each other, compared to the similarity of these strains across groups.

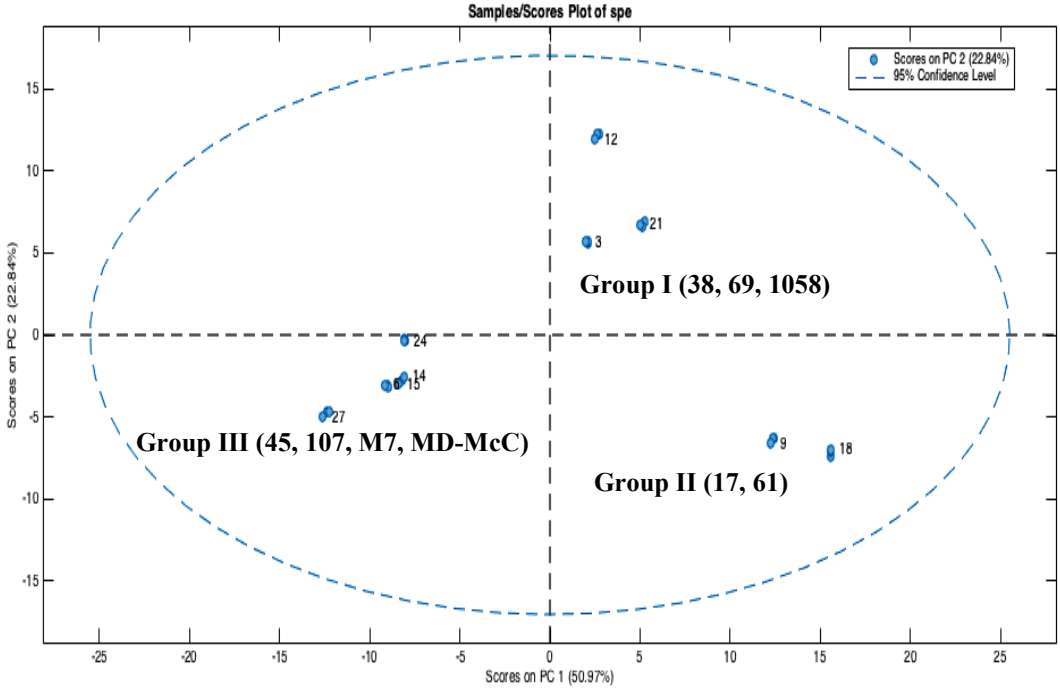


FIGURE 9. The result of FTIR-ATR spectral analysis dividing *K. pneumoniae* strains into 3 distinct groups: Group I with isolates 38, 69 and 1058, Group II with isolates 17, 61 and Group III with isolates 45, 107, M7, MD-McC

The results obtained with the FTIR-ATR spectral technique were completed using molecular biology methods (Table 2).

The *wzi* gene is a highly conserved gene, being one of the few genes present in the *K. pneumoniae* capsular polysaccharide group.

Types of *K. pneumoniae* strains are required to identify clonal groups to help epidemiological investigations and to link genetic diversity and site-specific strains. The

variation of the capsular K type was correlated with the presence or absence of specific genes from the locus cps (locus with mosaic structure, specifically polysaccharides, consisting of a group of six conserved genes (*galF, orf2, wzi, wza, wzb, wzc*) at the 5' end).

TABLE 2. Allele variants of the *wzi* genes and the capsular K type of *K. pneumoniae* strains

Nr.	Strain code	Patient/ Volunteer	Strain name	Allele variants - Capsular K type	FTIR-ATR
1	38	P. 21	*K. pneumoniae*	wzi-367 - like (11 nt. ≠)	Group I
2	69	P. 23	*K. pneumoniae*	wzi-30 - K30	
3	1058	P. 15	*K. pneumoniae*	wzi-101 - K24	
4	17	P. 20	*K. pneumoniae*	wzi-62 *like* (1 nt. A ≠ T – 315) - K63	Group II
5	61	P. 12	*K. pneumoniae*	wzi-324 *like* (1 nt. A ≠ G - 238)	
6	45	P. 22	*K. pneumoniae*	wzi-37 - K22.37	Group III
7	107	P. 3	*K. pneumoniae*	wzi-8 - K8	
8	MD-McC’	P. 6	*K. pneumoniae*	wzi-8 - K8	
9	M7	V. 5	*K. pneumoniae*	wzi-37 - K22.37 (1 nt. ≠)	

Following the PCR reaction and Sanger sequencing, out of the total of 9 strains of *K. penumoniae*, seven alleles of the *wzi* gene corresponding to 7 different K-types were identified. Since the selected group of patients has been low (9), the allelic variability of the *wzi* gene is increased.

For each allelic variant and capsular type, clonal groups that vary according to their virulence are described as follows:

- allelic variant wzi-30 and capsular type K30, corresponds to clonal groups ST43 and ST135.
- allelic variant wzi-101 and capsular type K24 corresponds to clonal group ST45 that is associated with BLSE resistance and more specific CTX-M-15.
- the wzi-37 allele variant and the K22.37 capsular type corresponds to BLSE, CTX-M clonal ST35 cluster (Brise et al., 2013); And the ST43 clonal group is associated with carbapenem resistance (OXA-48-like) (Lascolas et al., 2013).
- the wzi-8 allele variant and the K8 capsular type, corresponds to clonal groups ST9 and ST29.

In addition to the five types of allelic variants (*wzi-30, wzi-101, wzi-62 like, wzi-37, wzi-8*) described in the literature, new allelic variants (*wzi 324- Wzi 367-like*), which have not been described so far.

CONCLUSIONS

Analysis of the aerobic / aerobic- facultative anaerobic species composition of intestinal microbiota in a group of colon cancer patients in an attempt to identify a pattern specific or more commonly associated with this disease, as well as the study of the antibiotic factors expressed by the isolated strains compared to those isolated from a group of healthy individuals led to the following conclusions:

- In colonic individuals, colonic microflora is varied and balanced, both in terms of bacterial prokaryotic microorganisms, the *E. coli* species being dominant, as

well as eukaryotic - yew microorganisms. This leads to the conclusion that the microbiota of healthy individuals is in a state of eubiosis;

- In colon cancer patients due to cancer treatments (radio and chemotherapy) but possibly due to changes in intestinal cell metabolism, the diversity of species in the Gram-positive bacterial population is lower, which results in the colony microflora of these patients In a state of discord;
- In both control and colon cancer patients, the variability of *E. coli* phylogroups was high, which demonstrates the great versatility of these strains.
- Determination of the K-type K in strains of *K. pneumoniae* needed to identify clonal groups could help to model a pattern that could be considered in the future as a possible early marker, a potential indicator of the condition predisposing to tumor progression.

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