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Comparison of gut resistomes in healthy individuals and patients with severe alcoholic hepatitis

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Abstract: Human gut microbiota has been in the centre of scientific interest for a long period of time. Overall health status of an individual has a great impact on the composition of gut microbiota; however, gut microbiota can affect human health. Antibiotic resistance genes (ARGs) are often a part of human gut microbiome. In this paper, total genomic DNA was extracted from stool samples of 147 healthy individuals and of 45 patients with severe alcoholic hepatitis. The presence of six common ARGs (bla_{TEM}, bla_{SHV}, bla_{OXA}, *vanA*, *tet*(A), *tet*(E)) was analysed in the genomic DNA by end-point PCR. The results show significantly higher occurrence of ARGs in the DNA samples from patients ($p = 0.0001$) showing multiple ARGs significantly more often than in healthy individuals ($p = 0.00003$). Antibiotic treatment in patients strongly correlated with the occurrence of ARGs ($p = 0.0038$). Nutrition and sex of healthy individuals did not have significant effect on the occurrence of ARGs ($p = 0.156$; $p = 0.456$). ARGs' occurrence in healthy individuals was the highest in the oldest age group, but the age of individuals and ARGs' occurrence were not related ($p = 0.617$). In conclusion, the results underline the importance of health for normal functioning of gut microbiota. Antibiotic resistance represents a challenge in the treatment of patients with liver diseases.

Keywords: alcoholic hepatitis, antibiotic resistance, gut, microbiome, resistomer

Introduction

Gut microbiota is the set of all microorganisms colonising the human gastrointestinal (GI) tract. The set of genetic material of the gut microbiota is referred to as gut microbiome. Healthy gut microbiota has a health positive effect but changes in its composition and imbalance can also cause a change in health status (Doré and Corthier, 2010). However, pathological state can lead to changes in the microbiota through various mechanisms, such as change in eating habits, functioning of the intestinal tract, or the use of drugs, e.g. antibiotics (ATB) (Shreiner et al., 2015). Differences in the diversity, composition, and functionality of microbiota have been associated with Crohn's disease, ulcerative colitis, obesity, type 2 diabetes, depression, and colon cancer (Falony et al., 2019). Imbalance in the gut microbiota is also associated with a number of liver diseases. There is increasing evidence that the pathophysiology and treatment of a wide range of liver diseases is strongly influenced by the nature/behaviour of gut microbiota. Disruption of microbial and intestinal homeostasis is related to chronic liver diseases — non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), and alcoholic liver disease (ALD) (Xie et al., 2016; Tilg et al., 2016). Severe alcoholic hepatitis is the most serious form of ALD (Li et al., 2019; Hartmann et al., 2015). ALD includes several stages, such as fatty liver (steatosis), steatohepatitis, fibrosis, cirrhosis, and even liver cancer (Li et al., 2019). In general, alcohol abuse causes small intestinal bacterial overgrowth (Hartmann et al., 2015; Vassallo et al., 2015; Kwong and Puri, 2021). Cirrhotic patients usually have reduced abundance of beneficial microorganisms, such as families *Lachnospiraceae*, *Ruminococcaceae* and *Clostridiales* Family XIV *Incertae Sedis* (Hartmann et al., 2015) while families *Enterobacterales* (and *E. coli*), *Enterococcaceae* (genus *Enterococcus* spp.), *Fusobacteriaceae*, *Staphylococcaceae* (genus *Staphylococcus*) are often present in higher counts (Hartmann et al., 2015). Gut microbiome of patients with alcoholic hepatitis is often

linked with increased abundance of families *Enterobacterales* and *Streptococcaceae* (Bajaj, 2019), and genus *Veilonella* and *Enterococcus* (Lang et al., 2020). Genus *Akkermansia*, especially species *Akkermansia muciniphila* is reported in significantly lower abundance in patients with alcoholic hepatitis (Lang et al., 2020; Bajaj, 2019). The abundance of phylum *Bacteroidetes* in cirrhotic patients or patients with alcoholic hepatitis is decreased while abundance of *Actinobacteria* and *Proteobacteria* is increased in their gut microbiome (Bajaj, 2019; Kwong and Puri, 2021). Increased abundance of *Candida* species is reported in alcoholics and patients with alcoholic hepatitis, with species *Candida albicans* contributing the most to the alteration (Li et al., 2019; Kwong and Puri, 2021). Counts of *Epicoccum*, *Galactomyces* and *Debaryomyces* were also decreased in alcoholics (Li et al., 2019). Viruses are minor part of the gut microbiome and there are few studies describing their role in the gut microbiome of patients with alcoholic hepatitis. According to Jiang et al. (2000), patients with alcoholic hepatitis showed significantly higher occurrence of viruses in their faecal microbiota in comparison with healthy individuals. Patients with alcoholic hepatitis showed higher occurrence of *Escherichia-*, *Enterobacteria-*, and *Enterococcus* phages. From mammalian viruses, occurrence of *Parvoviridae* and *Herpesviridae* was significantly increased in these patients (Jiang et al., 2020).

Dysbiosis of gut microbiota caused by chronic alcohol abuse leads to increased levels of *Proteobacteria* and then to intestinal mucosal inflammation. Moreover, alcohol abuse causes bacterial overgrowth and increases gut permeability. These factors contribute to the transfer of bacteria from gut to portal blood and expose liver cells to lipopolysaccharides (LPS). LPS trigger immune response and cause release of pro-inflammatory mediators, which are responsible for liver damage and long term inflammation in alcoholics and patients with different stages of ALD (Hartmann et al., 2015; Minemura, 2015; Vassallo et al., 2015). Therefore, restoring the balance in gut microbiota of these patients is one of the possible treatment approaches. Administration of probiotics, prebiotics, and antibiotics, or faecal microbiota transplantation are commonly used in patients with ALD (Li et al., 2019; Bajaj, 2019).

There are differences in the intestinal microbiota between vegans/vegetarians and omnivores. Changes in its composition can be caused directly by the content of bacteria in the food, differences in the food consumed, the length of time the food passes through the GI tract, pH, host excretion dependent on the way of eating and regulation of the expression of the genes of host/its microbiota (Tomova et al., 2019).

Bacteria present in the gut microbiota can also be carriers of antibiotic resistance genes (ARGs) (Penders et al., 2013). In addition to the most dominant representatives of the human gut microbiota — phyla *Bacteroidetes* and *Firmicutes*, facultative anaerobic bacteria, such as family *Enterobacterales* or *Enterococcaceae* (about 100 times less) are a part of human gut microbiota. Gut microbiota can serve as a reservoir for opportunistic pathogens from these families, which can cause problems especially for immunocompromised individuals. ATB treatment can subsequently create selection pressure for the emergence of microbial resistance (van Schaik, 2015).

Many representatives of *Enterobacterales* family belong to pathogenic microorganisms and are frequent carriers of antibiotic resistance genes. As beta-lactam ATBs are among the most important and widely used in human medicine, extended-spectrum beta-lactamases (ESBLs) represent a major problem, and it is highly likely that the genes encoding them are part of the intestinal resistome. These enzymes are capable to hydrolyze broad-spectrum beta-lactam antibiotics (oxyiminocephalosporins — 3rd and 4th generation, monobactams). They are divided into several groups according to the target substrate. Among the most widespread types are TEM, SHV and CTX-M. Another important representative of this group is the OXA type (Ur Rahman et al., 2018). Genus *Enterococcus* is an important part of human and animal intestinal microbiota. *E. faecalis* and *E. faecium* are most commonly found in the human GI tract. Enterococci are the most serious nosocomial pathogens because they are naturally resistant to several ATBs and easily become virulent and acquire multidrug resistance (even to the ATBs of last choice for glycopeptide resistance). The emergence of vancomycin-resistant enterococci (VRE), which cause infections and persistent colonisation, has major health and economic impacts (Ahmed and Baptiste, 2018). Resistance to vancomycin is caused by *Enterococcus* spp. Van operon, which can be part of a chromosome or a plasmid. It consists of *vanS/R* — response regulator, *vanH* — D-lactate dehydrogenase, *vanX* — D-Ala-D-Ala dehydrogenase and variable ligase, of which a total of nine have been identified — *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM* and *vanN*. These genes encode the variable ligase that determines the level of vancomycin resistance (low, medium or high). VanA phenotype is plasmid mediated and causes high resistance to glycopeptides. The emergence of resistance occurs due to the substitution of the terminal peptide D-Ala-D-Ala on the subunits of N-acetylmuramic acid with high affinity to vancomycin to the sequence D-Ala-D-Lac.

This leads to a 1000-fold reduction in the affinity of the pentapeptide to vancomycin. Due to the presence of the *vanZ* gene on the *vanA* operon, strains expressing VanA are also teicoplanin resistant. This phenotype is most often found in *E. faecalis* and *E. faecium* and is the most widespread worldwide (Faron et al., 2016).

Resistance genes labeled *tet* and *otr* are classified as genes for acquired resistance to tetracycline and oxytetracycline. Currently, 38 *tet* and *otr* genes encoding different mechanisms of tetracycline resistance are known (energy-dependent efflux pumps, ribosomal protection proteins, and inactivating enzymes). The most common mechanism of tetracycline resistance is efflux pump that transports tetracycline out of the cell; *tet*(A) and *tet*(E) genes are included in this group (Roberts, 2005).

ESBL-producing *Enterobacterales* and VRE are listed as serious threat in the AR Threats Report by Centers for Disease Control and Prevention in 2019 (CDC, 2019). Bacteria from family *Enterobacterales* and *Enterococcaceae* belong to frequent inhabitants of the GI tract, and high occurrence of genes encoding resistance to beta-lactams and vancomycin is expected. Tetracycline resistance genes are also frequently detected in the gut microbiome of humans.

The use of ATB has a significant effect on the formation of gut resistome. During the treatment, all bacteria in the human body are exposed to their selection pressure. The selection of antibiotic resistant strains can lead to horizontal gene transfer between other susceptible strains in the intestinal tract. Meat from farm animals contaminated with ARGs also represents a source of ARGs in human gut resistome (Schjørring and Krogfelt, 2011).

The main aim of this paper was to compare and analyse gut resistomes of healthy individuals and patients with severe alcoholic hepatitis. Samples of total DNA extracted from stools of subjects were analysed with end-point PCR for the detection of selected ARGs and their occurrence was compared in healthy individuals and patients based on the sex, nutrition, and age of the subjects.

Materials and Methods

Characteristics of stool samples

Healthy individuals

A group of 147 healthy individuals from Bratislava, Slovakia, and surrounding areas were randomly included into the study. According to diet, the group was divided into vegetarians and meat-eaters. Based on their sex, the group consisted of women and men. The main condition for the participation in the study was the subjective health of the individual (without cardiovascular disease, cancer, diabetes, kidney disease, gastrointestinal tract disease or thyroid disease). All the individuals were non-smokers. All volunteers were healthy and had not undergone antibiotic or other medical therapy for more than six months. All subjects signed informed consent. The main characteristics and numbers of individuals in each group are stated in Tab. 1 (Kudláčková (Krajčovičová) et al., 2012).

Tab. 1. Numbers and characteristics of healthy individuals (Kudláčková (Krajčovičová) et al., 2012).

	Vegetarians	Meat-eaters
n	79	68
Men	39	34
Women	40	34
Age span (y)	20–60	20–60
Average age (y)	40.33	40.87

The study was realised in spring (April, May) 2010. The samples of morning stool were immediately lyophilised and stored at –80 °C.

Patients with severe alcoholic hepatitis

A group of 35 patients with severe alcoholic hepatitis was randomly included into the study. All patients suffered from ALD, some in combination with NASH (n = 3) or hepatitis B virus (n = 1) and were treated at the 2nd SMU Department of Internal Medicine of F.D. Roosevelt General Hospital in Banská Bystrica (FDRH), Slovakia. One of the patients (male) suffered from clostridial colitis. A total number of 45 stool samples were collected from the patients. Thirteen patients underwent faecal microbiota transplant (FMT) as a form of treatment, therefore multiple stool samples before and after the procedure were sampled. Two of the individuals were donors of faecal microbiota for the procedure. Several patients were given non-specified antibiotic treatment during their hospitalisation. All patients were meat-eaters. The information about patients is provided in Table 2.

Tab. 2. Numbers and characteristics of patients.

	Patients	ATB treatment
n	35	20
Men	23	15
Women	12	5
Age span (y)	19–66	
Average age (y)	46.83	

Collection of morning stool samples was performed from September 2016 to January 2020. Samples were stored at –80 °C.

Extraction of total genomic DNA from stool samples
DNA extraction was performed with GenElute™ Stool DNA Isolation Kit (Sigma Aldrich, USA). Maximum of 200 mg of stool were weighed and processed according to the manufacturer's instructions. Concentration of genomic DNA was evaluated with a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). Samples of genomic DNA were stored at –40 °C until further analysis.

Detection of antibiotic resistance genes in total genomic DNA by PCR
The presence of antibiotic resistance genes in DNA samples was evaluated by simple or multiplex end-point PCR. Six common ARGs were chosen for the gut resistome analysis: ARGs responsible for extended spectrum betalactamases (ESBL) production in *Enterobacterales* (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$), vancomycin resistance gene in genus *Enterococcus vanA,* and two tetracycline resistance genes: *tet*(A) and *tet*(E). Sequences of the primers are listed in Tab. 3.
PCR mix for simple PCR (*bla*$_{TEM}$, *vanA*) of 50 µL total volume consisted of: PCR buffer, 1.5—2 mM $MgCl_2$, 200 µM dNTP Mix, 0.5—1 µM of both primers, 2 µL of total DNA, variable volume of DNase, RNase, and protease, free water, and 2.5 U of Hot Start Taq DNA Polymerase (Biotechrabbit, Germany). PCR mix for Multiplex PCR (Multiplex 1, *tet* Multiplex) of 50 µL total volume consisted of: 0.5—1 µM of both pairs of primers, 2 µL of total DNA, DNase, RNase, and protease, free water, and Multiplex PCR Master Mix (Biotechrabbit, Germany). PCRs were carried out in a Mastercycler gradient (Eppendorf, Germany) as follows: initial denaturation at 94 °C for 20 min, 30 cycles of 94 °C for 40 s, 60.5 °C for 1 min (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$), or 65 °C for 30 s (*vanA*), 72 °C for 1—1.5 min, and final elongation at 72 °C for 10 min. PCR program for *tet* Multiplex was: initial denaturation at 94 °C for 20 min, 72 °C for 30 s, 35 cycles of 94 °C for 1 min, 55.4 °C for 1 min, 72 °C for 1.5 min, and final elongation at 72 °C for 10 min. PCR products were separated by gel electrophoresis in 1.5 % agarose gel with TAE at 100 V for 1:40 h. Additional staining with Gel Red™ Nucleic Acid Gel Stain (Biotium, USA) was performed to visualise the products. For DNA bands identification, 100 bp DNA ladder (Biotechrabbit, Germany) was used as a size standard. For each ARG a sequenced PCR product was used as a positive control. Random PCR products of each ARG were sequenced for confirmation.

Statistical analysis
The occurrence of ARGs was expressed as total numbers of detected genes or as a ratio of detected ARGs to number of samples in the particular group in percent (%). The occurrence of ARGs in different groups was visualised in bar plots as percentage. To determine whether there is a significant difference in ARGs' occurrence between different groups of individuals, the Chi-squared (χ^2) test was performed with the significance set to $p < 0.05$.

Results

Presence of ARGs in gut microbiota of healthy people
A set of 147 samples of total genomic DNA from stools of healthy individuals was tested for the presence of six ARGs. Together, 94 ARGs were detected in the samples. The most common ARG was *bla*$_{TEM}$ gene, which was present in 28.6 % of the

Tab. 3. Names and sequences of used primers.

ARG	Primers	Primer sequence (5´ — 3´)	Product size
vanA **(Depardieu et al., 2004)**	EA1 (+)	GGGAAAACGACAATTGC	732 bp
	EA2 (–)	GTACAATGCGGCCGTTA	
Multiplex 1 (*bla*$_{SHV}$, *bla*$_{OXA}$) (Dallenne et al., 2010)	MultiTSO-S_for	AGCCGCTTGAGCAAATTAAAC	713 bp
	MultiTSO-S_rev	ATCCCGCAGATAAATCACCAC	
	MultiTSO-O_for	GGCACCAGATTCAACTTTCAAG	564 bp
	MultiTSO-O_rev	GACCCCAAGTTTCCTGTAAGTG	
***bla*$_{TEM}$** **(Dallenne et al., 2010)**	MultiTSO-T_for	CATTTCCGTGTCGCCCTTATTC	800 bp
	MultiTSO-T_rev	CGTTCATCCATAGTTGCCTGAC	
***tet* Multiplex (*tet*(A, E)) (Ng et al., 2001)**	*tet*(A)	GCTACATCCTGCTTGCCTTC	210 bp
		CATAGATCGCCGTGAAGAGG	
	tet(E)	AAACCACATCCTCCATACGC	278 bp
		AAATAGGCCACAACCGTCAG	

samples, followed by *vanA* (23.1 %), bla_{OXA} (10.2 %), *tet*(A) (1.4 %) and bla_{SHV} (0.7 %). Gene *tet*(E) was not detected in any of the samples. The most common resistance genes were the group of ESBL encoding genes bla_{TEM}, bla_{SHV}, and bla_{OXA}. Total number of positive individuals (with at least 1 ARG) was 72 (49.0 %) which means that some of the individuals were carriers of multiple resistance genes. The majority of positive samples carried only single resistance gene (75.0 %). In 19.4 % of individuals, two ARGs and in 5.6 % of samples, three ARGs were detected simultaneously.

Comparison of gut resistomes based on the sex of individuals

The group of healthy individuals consisted of 74 women and 73 men. In the female part of the group, the presence of 49 ARGs was detected while 45 ARGs were detected in the male group.

The occurrence of ARGs in the two groups is shown in Fig. 1. Based on the overall results, the most common ARGs in both sexes are bla_{TEM} and *vanA* with slight dominance in women. Gene bla_{OXA} was more prevalent in men. Genes *tet*(A) and bla_{SHV} were detected only in women and *tet*(E) gene was not identified in healthy individuals. The differences in ARGs' occurrence between both sexes are minor with exception of the bla_{OXA} gene (Fig. 1). The occurrence of ARGs and the sex of healthy individuals did not show significant relationship ($p = 0.456$). The presence of multiple ARGs in both sexes was detected. The total numbers of positive women and men was 38 and 34, respectively. Majority (76.3 % of positive women, 73.5 % of positive men) carried single resistance gene, 18.4 % of women and 20.6 % of men carried two ARGs, and in the total DNA of 5.3 % of women and 5.9 % of men, three ARGs were detected. The detected numbers of ARGs did not depend on the sex of individuals ($p = 0.938$).

Comparison of gut resistomes based on the nutrition of individuals

Based on the preferred diet habits, healthy individuals were divided into two groups: vegetarians and meat-eaters. The vegetarian group consisted of 79 individuals and meat-eater group of 68 individuals. As shown in Fig. 2, the most prevalent resistance gene in the vegetarians group was *vanA* gene (29.1 %), followed by bla_{TEM} and bla_{OXA} gene. Genes *tet*(A) and bla_{SHV} were detected only in a couple of cases in vegetarian total genomic DNA. On the contrary, the most prevalent resistance gene in meat-eaters was bla_{TEM}, followed by *vanA* and bla_{OXA}. With the exception of bla_{TEM} genes, all resistance genes were more prevalent in the vegetarian group. Differences between vegetarians and meat-eaters were clear mainly with *vanA* and bla_{OXA} genes. Although differences were detected in the ARGs' occurrence between vegetarians and meat-eaters, statistical analysis did not prove significant relationship of nutrition style and ARGs' occurrence in healthy individuals ($p = 0.156$).

In addition, vegetarians showed higher occurrence of multiple ARGs in one sample. Together, ARGs were detected in 40 vegetarians and 32 meat-eaters. In both groups, the majority of samples showed the presence of only one resistance gene (65.0 % positive vegetarians, 87.5 % positive meat-eaters). On the contrary, two ARGs were present in 27.5 % of vegetarian total DNA compared to 9.4 % of

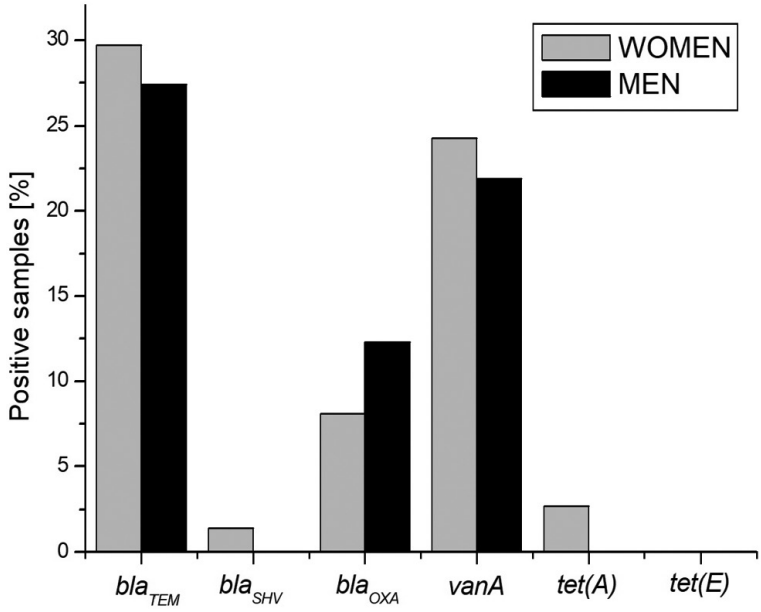

Fig. 1. Occurrence of ARGs in healthy men and women.

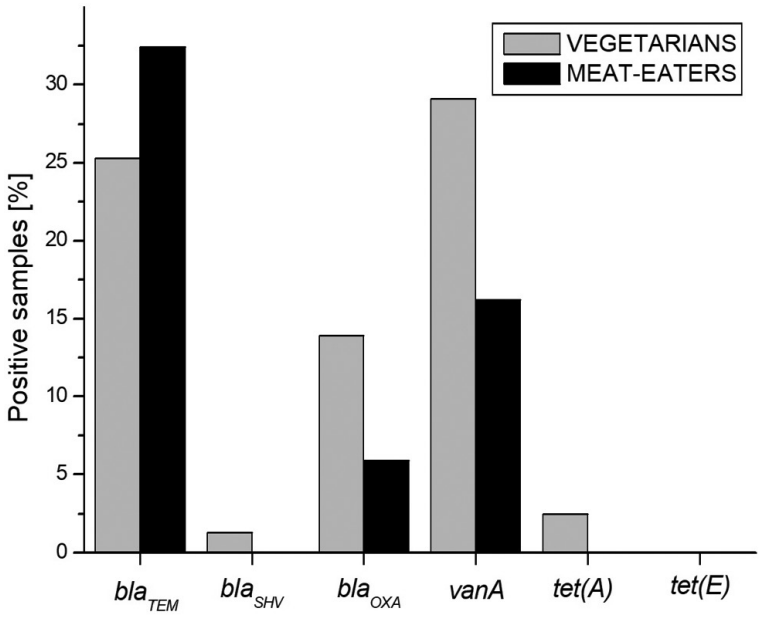

Fig. 2. Occurrence of ARGs in healthy vegetarians and meat-eaters.

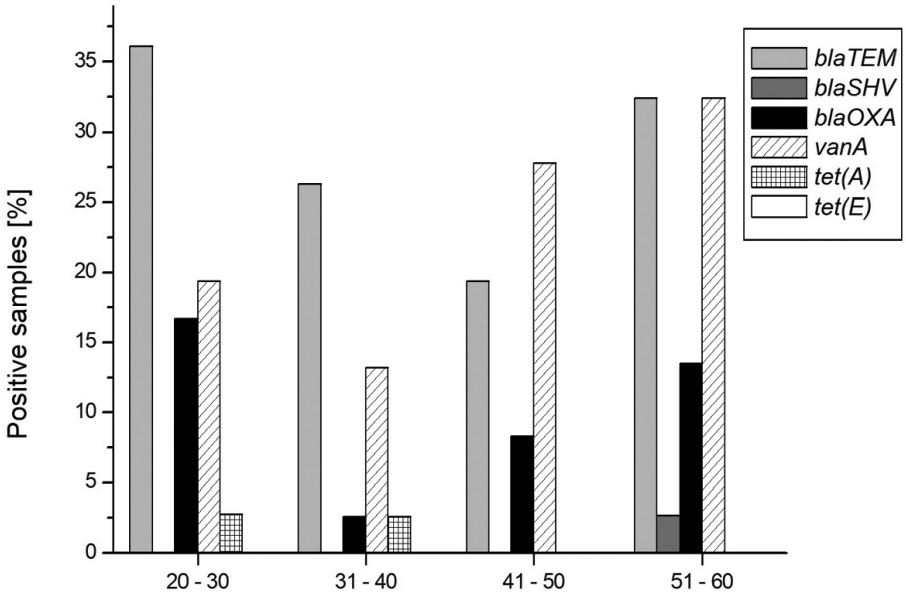

Fig. 3. Occurrence of ARGs in different age groups of healthy individuals.

meat-eaters. Three ARGs were detected in 7.5 % of vegetarians and in 3.1 % of meat-eaters. The numbers of ARGs in one individual did not relate to the nutrition type of the individual ($p = 0.174$).

Comparison of gut resistomes based on the age of individuals

The age span of the healthy individuals ranged from 20 to 60 years. Therefore, the group was divided into four groups according to age: 20–30 (n = 36), 31–40 (n = 38), 41–50 (n = 36), and 51–60 years old (n = 37). Based on the overall results shown in Fig. 3, the most prevalent ARGs in each age group were bla_{TEM} or *vanA* genes, bla_{TEM}, bla_{OXA} and *vanA* were present in all age groups. One case of bla_{SHV} gene was detected only in the eldest individuals, and two cases of *tet*(A) genes only in 20–30 and 31–40 years old individuals. Absolute numbers show that the highest number of ARGs (30) was detected in 51–60 years old (81.1 %), 27 ARGs in 20–30 years old (75.0 %), 20 ARGs in 41–50 years old (55.6 %) and the lowest number of ARGs was present in 31–40 years old (17; 44.7 %) individuals. The Chi-squared test did not confirm any significant relationship between the ARGs' occurrence and the age of the individuals ($p = 0.617$).

From the positive samples of all age groups, multiple ARGs (9.5 % 3 ARGs, 23.8 % 2 ARGs, 66.7 %

1 ARG) were most often found in 51—60 years old individuals. The best results were obtained for the group of 31—40 years old individuals, where no triple occurrence of ARGs was detected, 13.3 % of positive individuals carried two ARGs, and 86.7 % carried one ARG. Distribution of ARGs was not affected by the age of individuals ($p = 0.674$).

Presence of ARGs in gut microbiota of patients

A total number of 45 DNA samples was recovered from the stool samples of patients with severe alcoholic hepatitis. After PCR detection, 59 ARGs were detected in the DNA samples with *vanA* gene as the most prevalent in 46.7 % of the samples, followed by bla_{TEM} and bla_{OXA} (24.4 % and 22.2 %). Interestingly, *tet(A)* occurrence reached 20.0 % and bla_{SHV} only 13.3 %. Also, the *tet*(E) gene was detected in 4.4 % of the samples. Percentage of positive samples (with min. one ARG) was 77.8 %. In 65.7 % of positive samples only a single ARG was detected. Two ARGs were detected in 31.4 %, three ARGs in 14.3 %, and four ARGs in 5.7 % of the positive samples. In addition, there was a sample with five ARGs detected. Since all patients belonged to the group of meat-eaters, ARGs' occurrence was compared between groups divided according to age and sex.

Comparison of gut resistome based on the sex of patients

Thirty-one samples of total genomic DNA originated from men and 14 samples from women. Fig. 4 shows a comparison of ARGs' occurrence in both sexes. In total, 23 ARGs were present in female samples and 36 ARGs in male samples. The most evident difference was detected in the occurrence of *vanA* and bla_{TEM} genes as *vanA* genes were more prevalent in men and bla_{TEM} in women. Also, *tet*(E) genes were present only in the DNA samples of women. Statistical analysis did not confirm any significant relationship between sex of the patients and ARGs occurrence ($p = 0.130$).

Comparison of gut resistomes based on the age of patients

Patients' age ranged from 19 to 66 years. The group was divided similarly as the group of healthy individuals — 19—30 (n = 4), 31—40 (n = 11), 41—50 (n = 11), 51—66 (n = 19). The results are shown in Fig. 5. Gene *vanA* was the most prevalent ARG in the groups of 51—66 and 31—40 years old patients. The highest number of ARGs to the number of samples was detected in the group of 31—40 years old patients (20 ARGs). This means that patients in this group were also the most frequent carriers of multiple ARGs with 182 % occurrence of ARGs (ratio of detected genes to the number of patients in the age group). Statistical analysis did not show any significant relationship between age of the patients and the ARGs' occurrence ($p = 0.755$).

Effect of antibiotic treatment on the gut resistomes of patients

From 45 samples, 28 were gathered from patients on antibiotic treatment. ARGs' presence was confirmed in 78.6 % of these samples. The occurrence of ARGs in samples from patients without antibiotic treatment was 76.5 %. In one case, five ARGs were detected in the sample from a 36-year-old woman

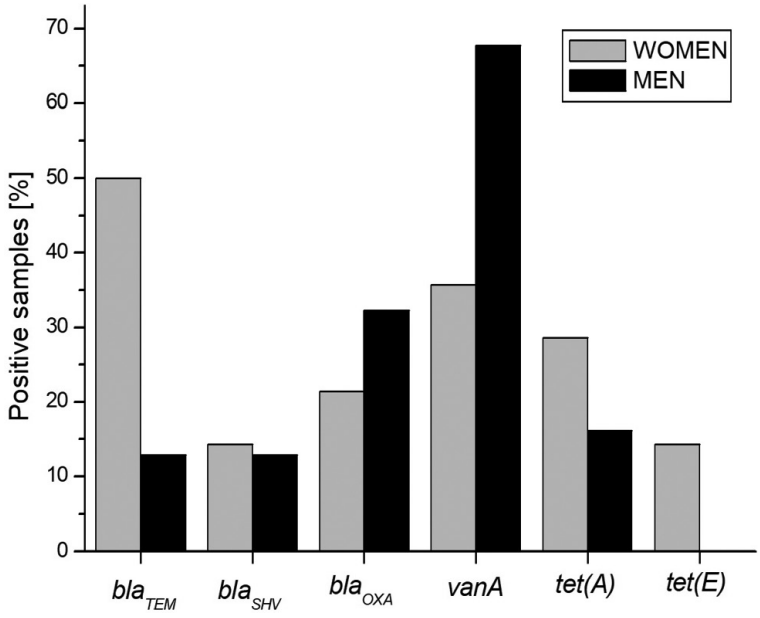

Fig. 4. Occurrence of ARGs in patients based on their sex.

who did not receive antibiotic treatment. Although relative occurrence of ARGs was similar in both groups of patients, results of the Chi-squared test show significant relationship between the antibiotic treatment and ARGs' occurrence in patients ($p = 0.0038$).

Comparison of gut resistomes of healthy individuals and patients with severe alcoholic hepatitis

One of the most important characteristics is the comparison between the two groups of subjects. In general, the positivity for ARGs in healthy individuals was 49.0 %, while in patients it was 77.8 %. The distribution of ARG types is shown in Fig. 6. Gene *vanA* was the most prevalent gene in patients, with double occurrence in comparison with healthy individuals. Except for bla_{TEM}, all monitored ARGs were more prevalent in patients. Gene *tet*(E) was detected only in the genomic DNA of patients, *tet*(A) and bla_{SHV} genes occurred also in some healthy individuals.

The occurrence of ARGs in healthy individuals and patients strongly correlates with the health status of the tested individuals (p = 0.0001).

The comparison of patients and healthy individuals based on sex is shown in Fig. 7. The variety of ARGs in patients is wider than in the healthy individuals. Also, higher percentage of ARGs was detected in

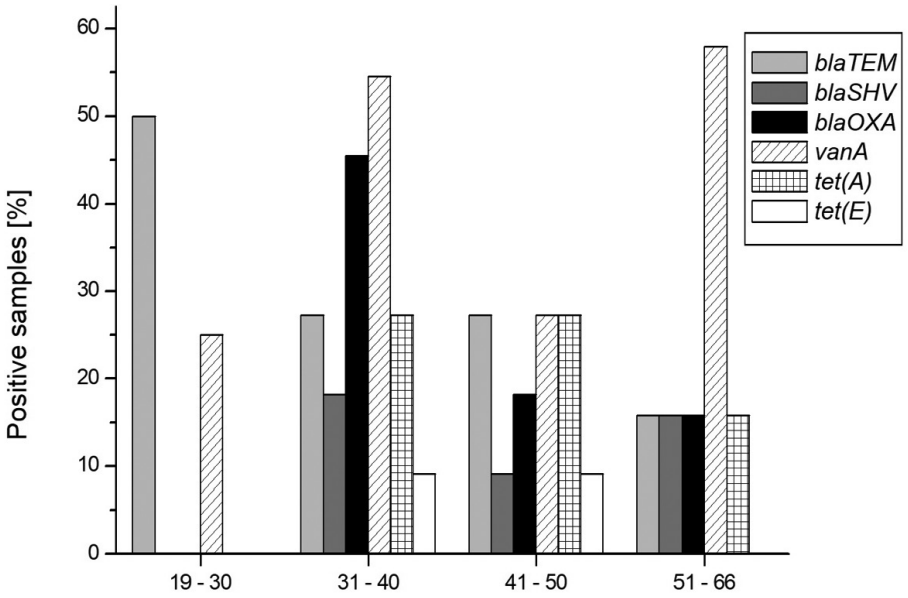

Fig. 5. Occurrence of ARGs in patients based on their age.

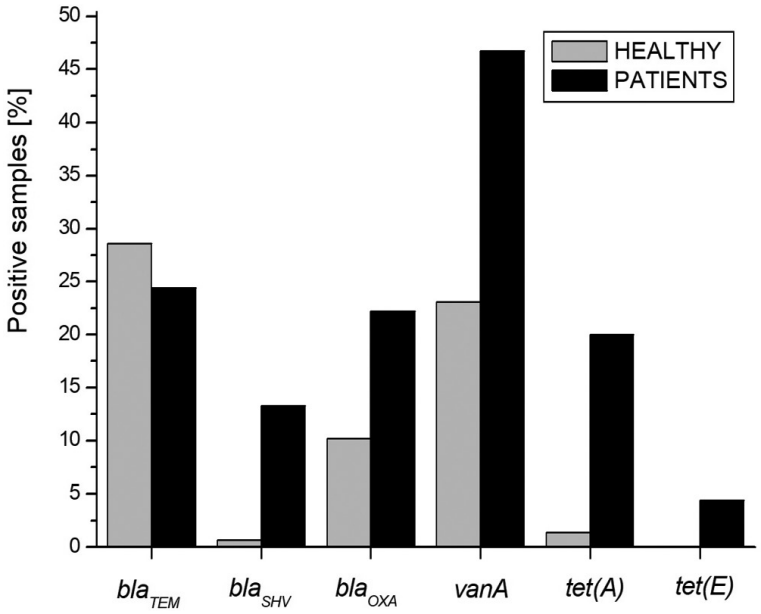

Fig. 6. Occurrence of ARGs in healthy individuals and patients.

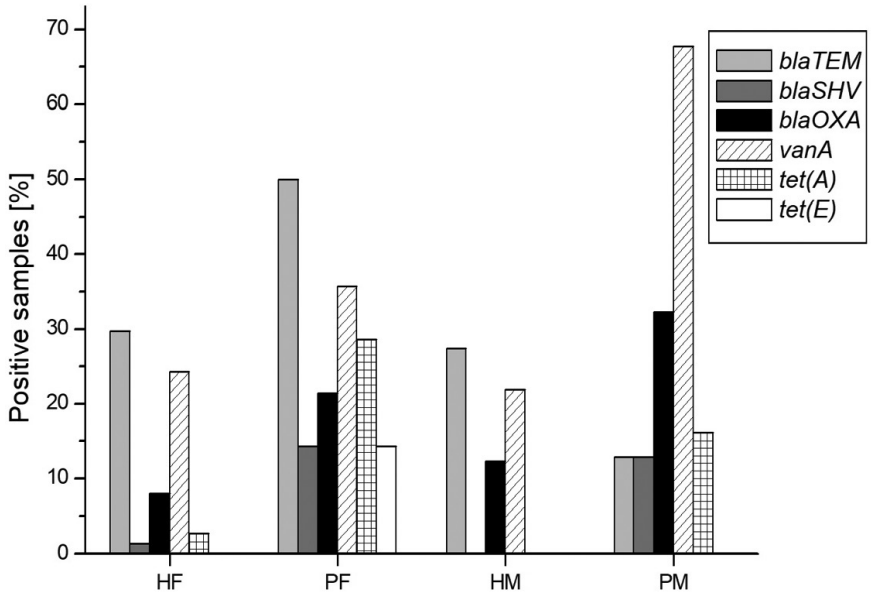

Fig. 7. Occurrence of ARGs in healthy individuals and patients based on the sex (HF — healthy female; PF — patient female; HM — healthy male; PM — patient male).

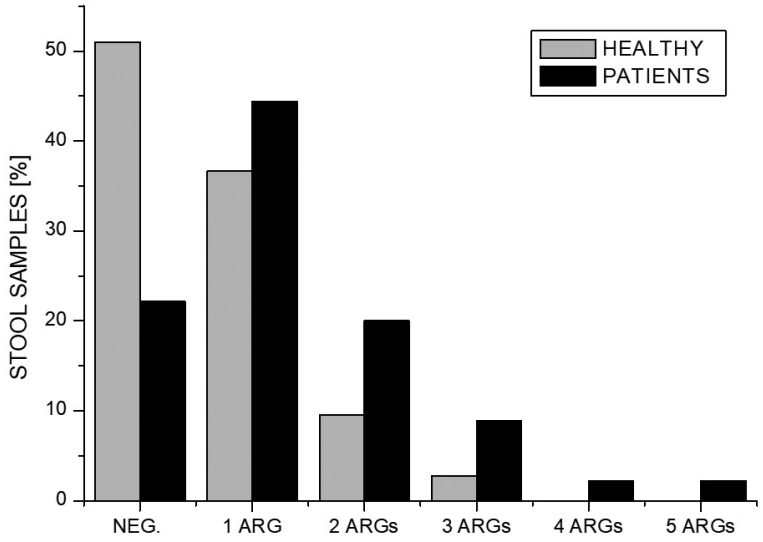

Fig. 8. Numbers of ARGs [%] detected in healthy individuals and patients.

patients. The occurrence of the *vanA* gene was the highest in male patients. In female patients, bla_{TEM} was the most frequently observed ARG. The distribution of ARGs in healthy individuals is more balanced and on a very similar level in both sexes. The statistical analysis showed significant relationship between the sex, health status, and the occurrence of ARGs ($p = 0.0005$).

The last comparison is the number of ARGs detected in samples (Fig. 8). Genomic DNA from patients' stools contained multiple ARGs more often than that from healthy individuals. The number of ARGs detected in one sample strongly correlated with the health status of tested individuals ($p = 0.00003$).

Discussion

Resistome analysis of healthy individuals showed the presence of maximum three ESBL-encoding genes in total. Similar results were observed in a study performed at a California university campus. Authors detected bla_{TEM} in 55 % of bacterial isolates from 102 stool samples of healthy individuals. They also registered bla_{SHV} in 18 % and bla_{OXA} type in 1 % of isolates (Rubin et al., 2020).

Sinha et al. (2019) analysed the gut microbiota composition of 1135 individuals based on sex and resistome profiling in a cohort study concluding that the resistomes of women and men differ in their richness. Females showed higher prevalence

of resistance genes than males and a higher number of gene families. The biggest difference between women and men was manifested in the family of genes encoding lincosamide nucleotidyltransferases: 85.98 % in women and 79.07 % in men. The gene cluster for glycopeptide resistance was detected in 39.45 % of women and 31.29 % of men. The gene family encoding ESBLs was detected in 7.70 % of women and 4.44 % of men (Sinha et al., 2019). The results of this study point to a similar trend as our results, a higher number of resistance genes in women. The difference was evident mainly in case of female patients with multiple ARGs' occurrence in one sample. On the contrary, no significant differences were found in the ARGs' occurrence between healthy men and women ($p = 0.456$).

An important period for a person is their early age when the foundations of the gut microbiota are formed. However, the emergence of resistance to ATB in this period has not yet been mapped. We assumed the highest number of resistance genes in the oldest age group. Similarly Lu et al. (2014) reported the accumulation of resistance genes from childhood to adulthood. This phenomenon may be due to older people being usually exposed to higher impact of ATB during their entire lives and them being exposed to several types of antibiotics. Tavella et al. (2021) reported accumulation of ARGs with increasing age with some ARGs present in all age groups and creating a core gut resistome. These assumptions were confirmed in case of healthy individuals where the eldest individuals were the most frequent ARGs carriers. In patients, ARGs occurred the most in the age group of 31—40 years old individuals. An interesting finding, however, is the second highest number of genes detected in the youngest age group of healthy individuals — 21 to 30 years. However, such a result is no exception. Ohashi and Fujisawa (2017) studied the prevalence of ARGs in the total DNA extracted from the stools of young Japanese women aged from 21 to 22 years monitoring ARGs to tetracyclines, macrolides, chloramphenicol, betalactams (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$, *bla*$_{CTX\text{-}M}$), fluoroquinolone, ampicillin, meticillin, streptomycin, sulfonamide, and vancomycin (*vanA*, *vanB* and *vanC1*). Gene *bla*$_{TEM}$ was detected in 62.5 %, *bla*$_{SHV}$ in 21.9 %, *bla*$_{CTX\text{-}M}$ in 6.3 % and *bla*$_{OXA}$ in 3.1 % of subjects. From vancomycin resistance genes, only *vanC*1 gene was detected in 6.3 % of cases (Ohashi and Fujisawa, 2017). The genes of beta-lactam resistance were most commonly represented by the *bla*$_{TEM}$ gene in the group of 21—30 years old, but also in the groups of 31—40 years old, and 51—60 years old healthy individuals (in this case at the same level with *vanA*). This study did not find any significant differences in ARGs' occurrence between the groups with different age ($p = 0.617$).

Differences in the occurrence of resistance genes between vegetarians, vegans, and meat-eaters were discussed by Losasso et al. (2018). In fecal DNA samples from 101 people divided according to the type of diet, the presence of four resistance genes: *sul2*, *tet*(A), *bla*$_{TEM}$ and *strB* was determined using qPCR. Vegans had the lowest number of resistance genes, however, when comparing the relative representation of individual genes between the three groups, no major differences were found. The results suggest that conventional and vegetarian diets (intake of animal products) promote the accumulation of resistance (Losasso et al., 2018). Milanović et al. (2017) observed the effects of long-term general, ovo-lacto vegetarian, and vegan diet on the occurrence of 12 resistance genes in 144 people who came from the Italian cities of Turin, Bari, Bologna, and Parma. Between the analysed genes were *erm(A), erm(B), erm(C), blaZ, tet(M), tet(O), tet(K), tet(S), tet(W), mecA, vanA* and *vanB*. The most commonly found genes were *erm* and *tet* genes, which occurred in almost all samples. The *vanA* gene occurred in 17 % of omnivores, 25 % of vegans, and 21 % ovolactovegetarians. The *blaZ* gene, which encodes resistance to beta-lactam antibiotics, was detected in 65 % of omnivores, 48 % of vegans, and 58 % ovolactovegetarians (Milanović et al., 2017). When comparing the results of their study with our results, it can be stated that the numbers of ESBL genes are almost balanced between the two groups of healthy individuals, the only noticeable difference is in case of *bla*$_{OXA}$. With the *vanA* gene, the trend is similar as in the aforementioned study, higher occurrence of this gene in vegetarians was observed compared to omnivores. Although diet has a major influence on the gut microbiota, very little is known about its impact on the gut resistome and this area requires further research (Milanović et al., 2019). Food chain represents a route of transmission for antibiotic resistant bacteria from food to people. Therefore, the spread of antibiotic resistance is greatly influenced by food safety and food hygiene (World Health Organization, 2011). Stege et al. (2022) compared ARGs' abundance between four different nutrition types (omnivores, pescatarians, vegetarians, and vegans) in Dutch population using metagenomic shotgun sequencing but found no significant differences in abundance of ARGs between the groups. Based on their data, ARGs represent a very small part of all genetic information found in gut microbiota and long-term dietary habits do not influence resistomes in a specific way (Stege et al., 2022). This study confirms our results that there is no correlation between the

diet of healthy individuals and ARGs' occurrence ($p = 0.174$).

Several liver diseases are linked with changes and dysbiosis of gut microbiota (Minemura, 2015). Antibiotic treatment causes profound changes in the gut microbiota, it can selectively increase the spectrum of resistant microorganisms leading to higher prevalence of resistance genes and increased likelihood of their spread through horizontal gene transfer (Sommer and Dantas, 2011). The significant difference observed in the occurrence of *vanA* genes in patients is most likely caused by the disruption of their gut microbiota. According to non-published data from the sequencing analysis of total DNA samples from patients and controls (healthy individuals), the composition of gut microbiota of patients and controls significantly differed in the presence of genus *Enterococcus*, with major occurrence in patients (Supplementary material). This shift in the gut microbiota composition and combination with ATB treatment can lead to high occurrence of the *vanA* gene. Higher occurrence of genus *Enterococcus* (besides *Clostridium* spp.) in the gut microbiome of cirrhotic patients was reported in literature (Hartmann et al., 2015; Lang et al., 2020). The results also show significant differences in ARGs' occurrence between patients with and without ATB treatment ($p = 0.0038$). Antibiotic treatment is important for patients with ALD, mainly for those in the end stage of the disease. However, antibiotics cause antimicrobial resistance to some enteric pathogens and change the microbial community in the gut and its function (Yan, 2012). The appearance of stool samples from patients was unusual in colour, shape, and consistency which also supports the assumption of gut microbiome disruption. Shamsaddini et al. (2021) reported higher abundance of ARGs from beta-lactamases, vancomycin, and quinolone resistance groups in patients with cirrhosis. These patients also had higher abundance of bacteria from families *Enterobacterales*, *Streptococcus* spp., and *Enterococcus* spp. (Shamsaddini et al., 2021; Pan et al., 2022). Patients with liver disease are also prone to bacterial infections, for example Merli et al. (2010) describe hospital acquired infections in cirrhotic patients, of which 64 % are caused by antibiotic resistant pathogens. Cirrhotic patients frequently suffer from multidrug resistant infections (Vergis et al., 2020). Acute-on-chronic liver failure (ACLF) describes health condition of patients with liver cirrhosis and is defined by organ failure. This syndrome is characterised by high mortality (15 %) within 28 days. Based on the number of organ failures, the severity of the syndrome is divided into three grades: ACLF-1(a/b), ACLF-2, and ACLF-3(a/b). Grade 1 is characterised by one organ failure (kidney or another organ). In grade 2, two organ failures occur, and in grade 3, three to six organs fail (Kumar et al., 2020). In our group of patients, ten patients did not have any symptoms of ACLF (28.6 %), seven patients were in grade 1 (20.0 %), 12 patients in grade 2 (34.3 %), and three patients suffered grade 3 ACLF (8.6 %). ACLF disease was not present in three patients (two were controls and one patient had clostridial colitis). Due to the high mortality of this disease, health condition of the patients was very serious. Although 28.6 % of patients did not show the ACLF syndrome, Kumar et al. (2020) reported 28-day mortality of 4.4 % without organ failure and 6.3 % in case of failure of one organ (except for kidneys) in the absence of ACLF.

The analysis of ARGs occurrence based on age and sex of patients showed similar results as in case of healthy individuals. There was no correlation in ARGs occurrence between female and male patients ($p = 0.130$) or between the age groups of patients ($p = 0.755$). The effect of nutrition in patients was not assessed since all of the patients were meat-eaters.

The most significant results were obtained by comparing the numbers of detected ARGs in both groups: healthy individuals and patients. There was a strong correlation between the ARGs' occurrence and health status of the individuals ($p = 0.0001$). Furthermore, statistical analysis of healthy individuals and patients divided by the sex showed a relationship between the health status and the ARGs' occurrence ($p = 0.0005$). The detected numbers of ARGs in the stool samples from healthy individuals and patients were significantly different ($p = 0.00003$). In the group of healthy individuals, a higher number of negative individuals (75 vs. 65 expected) as well as of individuals with two resistance genes (4 vs. 10 expected) were present compared to the expected values. On the other hand, in the group of patients, the number of negative samples was lower than expected (10 vs. 20 expected) and that of samples with one ARG (20 vs. 17 expected) and two ARGs was higher (9 vs. 3 expected).

Gut microbiome is a reservoir of ARGs, even in healthy individuals. Therefore, the impact of antibiotic resistant microorganisms on immunocompromised individuals, such as patients with severe alcoholic hepatitis, is more serious. The diversity of gut microbiota in patients is disrupted by dysbiosis caused by alcohol abuse and antibiotic treatment. The presence of antibiotic resistant pathogens can lead to pathogen invasion and colonisation. Some promising solutions are faecal microbiota transplantation, and the use of probiotics (Lamberte and van Schaik, 2022; Anthony et al., 2021).

Conclusion

The results of our study show differences in the ARGs' occurrence in healthy individuals and patients with severe alcoholic hepatitis. The analysis of the healthy individuals based on their diet style and sex did not show significant differences between the groups. The main differences in ARGs' occurrence were observed between groups of healthy individuals and patients. These results show that the overall health and healthy gut microbiome play an important role in the occurrence of ARGs. Actual trends in research of resistomes show the importance of metagenomics and sequencing and allow detection of many ARGs.

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Supplementary Materials

Tab. S1. Significant differences in composition of gut microbiome on genus level in patients with severe alcoholic hepatitis and selected healthy individuals as controls.

Fig. S1. Distribution of genus *Enterococcus* in patients with severe alcoholic hepatitis and healthy individuals.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of F. D. Roosevelt Hospital, Banská Bystrica, Slovakia (9/2018, 17th April 2018).

References

Ahmed MO, Baptiste KE (2018) Microb Drug Resist. 24(5): 590–606.

Anthony WE, Burnham CD, Dantas G, Kwon JH (2021) J. Infect. Dis. 223: 209–213.

Bajaj JS (2019) Nat. Rev. Gastroenterol. Hepatol. 16(4): 235–246.

CDC (2019) Atlanta, GA: U.S. Department of Health and Human Services, CDC.

Dallenne C, Da Costa A, Decré D, Favier C, Arlet G (2010) J. Antimicrob. Chemother. 65(3): 490–495.

Depardieu F, Perichon B, Courvalin P (2004) J. Clin. Microbiol. 42(12): 5857–5860.

Doré J, Corthier G (2010) Gastroentérologie Clin. Biol. 34: 7–15.

Falony G, Vandeputte D, Caenepeel C, Vieira-Silva S, Daryoush T, Vermeire S, Raes J (2019) Acta Clin. Belg. 74(2): 53–64.

Faron ML, Ledeboer NA, Buchan BW (2016) J Clin Microbiol. 54(10): 2436–2447.

Hartmann P, Seebauer CT, Schnabl B (2015) Alcohol. Clin. Exp. Res. 39(5): 763–775.

Lu J, Lang S, Duan Y, Zhang X, Gao B, Chopyk J, Schwanemann LK et al. (2020) Hepatology 72(6): 2182–2196.

Kudláčková (Krajčovičová) M, Valachovičová M, Bírošová L (2012) Bratislava, Slovakia: Slovenská zdravotnícka univerzita.

Kumar R, Mehta G, Jalan R (2020) Clin Med (Lond). 20(5): 501–504.

Kwong EK, Puri P (2021) Transl. Gastroenterol. Hepatol. 6: 3.

Lamberte LE, van Schaik W (2022) Curr. Opin. Microbiol. 68: 102150.

Lang S, Fairfied B, Gao B, Duan Y, Zhang X, Fouts De, Schnabl B (2020) Gut Microbes 12(1): 1785251.

Li F, McClain CJ, Feng W (2019) Liver Res. 3(3–4): 218–226.

Losasso C, Di Cesare A, Mastrorilli E, Patuzzi I, Cibin V, Eckert EM, Fontaneto D, Vanzo A, Ricci A, Corno G (2018) Int. J. Antimicrob. Agents 52(5): 702–705.

Lu N, Hu Y, Zhu L, Yang X, Yin Y, Lei F, Zhu Y (2014) Sci. Rep. 4(1): 4302.

Merli M, Lucidi C, Giannelli V, Giusto M, Riggio O, Falcone M, Ridola L, Attili AF, Venditti M (2010) Clin. Gastroenterol. Hepatol. 8(11): 979–985.

Milanović V, Osimani A, Aquilanti L, Tavoletti S, Garofalo C, Polverigiani S, Litta-Mulondo A et al. (2017) Mol. Nutr. Food Res. 61(9): 1601098.

Milanović V, Osimani A, Cardinali F, Litta-Mulondo A, Vignaroli C, Citterio B, Mangiaterra G et al. (2019) PLoS One 14(8): e0220549.

Minemura M (2015) World J. Gastroenterol. 21(6): 1691.

Ng LK, Martin I, Alfa M, Mulvey M (2001) 15(4): 209–215.

Ohashi Y, Fujisawa T (2017) Biosci. Microbiota, Food Heal. 36(4): 151–154.

Pan X, Zhou Z, Liu B, Wu Z (2022) Front. Microbiol. 13.

Penders J, Stobberingh EE, Savelkoul PHM, Wolffs PFG (2013) Front. Microbiol. 4.

Roberts MC (2005) FEMS Microbiol Lett 245(2): 195–203.

Rubin J, Mussio K, Xu Y, Suh J, Riley LW (2020) Microb. Drug Resist. 26(10): 1227–1235.

Van Schaik W (2015) Philos. Trans. R. Soc. B Biol. Sci. 370(1670): 20140087.

Schjørring S, Krogfelt KA (2011) Int. J. Microbiol. 2011: 1—10.
Shamsaddini A, Gillevet PM, Acharya C, Fagan A, Gavis E, Sikaroodi M, McGeorge S et al. (2021) Gastroenterology 161(2): 508—521.e7.
Shreiner AB, Kao JY, Young VB (2015) Curr. Opin. Microbiol. 31(1): 69—75.
Sinha T, Vich Vila A, Garmaeva S, Jankipersadsing SA, Imhann F, Collij V, Bonder MJ et al. (2019) Gut Microbes 10(3): 358—366.
Sommer MOA, Dantas G (2011) Curr. Opin. Microbiol. 14(5): 556—563.
Stege PB, Hordijk J, Shetty SA, Visser M, Viveen MC, Rogers MRC, Gijsbers E et al. (2022) Sci. Rep. 12(1): 1892.
Tavella T, Turroni S, Brigidi P, Candela M, Rampelli S (2021) mSphere 6(5): e00691-21.
Tilg H, Cani PD, Mayer EA (2016) Gut 65(12): 2035—2044.
Tomova A, Bukovsky I, Rembert E, Yonas W, Alwarith J, Barnard ND, Kahleova H (2019) Front. Nutr. 6.
Ur Rahman S, Ali T, Ali I, Khan NA, Han B, Gao J (2018) Biomed Res Int. 26: 9519718.
Vassallo G, Mirijello A, Ferrulli A, Antonelli M, Landolfi R, Gasbarrini A, Addolorato G (2015) Aliment. Pharmacol. Ther. 41(10): 917—927.
Vergis N, Atkinson SR, Thursz MR (2020) Semin. Liver Dis. 40(1): 11—19.
World Health Organization (2011) Copenhagen: World Health Organization.
Xie G, Wang X, Liu P, Wei R, Chen W, Rajani C, Hernandez BY et al. (2016) Oncotarget 7(15): 19355—19366.
Yan AW (2012) World J. Hepatol. 4(4): 110.