

ORIGINAL ARTICLE

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Investigation of gut microbiota in suicide cases instead of forensic sciences

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Abstract

Suicidal cases are criminal cases that either should be prevented or be investigated forensically after occurring. One of the aims of our study is that arrange the bacterial flora - one of the biological factors- in the gut to reduce suicide cases. In our study, we compared the bacterias which are based in the gut flora of suicidal death with the control group among the 103 autopsy materials collected. In this study, besides the classic microbial and biochemical methods, we also used the Real-Time PCR and Droplet Digital PCR methods. According to the result of our study, it is determined that *Bacteroides sp.*, *Clostridium sp.*, *Lactobacillus sp.* and *Bifidobacterium sp.* which affect suicide are significantly different than control groups. It is thought that probiotic or prebiotic supplementation of individuals with intestinal flora imbalance may be beneficial about reduce the suicidal cases which are caused by depression and anxiety. Therefore arranging of the gut flora may decrease the suicidal cases besides increasing the academic studies shall have considerable importance and may gain favor in this sense.

Keywords: Suicide, gut microbiota, forensic sciences, droplet PCR

Introduction

Suicide crimes which are evaluated within the scope of crimes against living; are one of the crimes which must be prevented and need to be investigated forensically after occurring. Even if the victim and perpetrator of a suicide case are the same, the acts of “bringing a person to commit a suicide”, “abetment” and “helping” are crimes in our Turkish Punishment Act [1]. The biological, especially the metabolic aspect of the neglected sides of the crimes has been the subject of our study and the probability of the relationship between intestinal metabolism and suicide which are one of these biological factors becomes prominent. The most important aim of our article is to investigate the effect of intestinal flora, which is thought to affect psychiatric disorders, on suicidal cases. A psychological autopsy is collecting all available information about the deceased person. The method involves

collecting all available information on the deceased via structured interviews of family members, relatives, or friends as well as attending health care personnel [2]. It is so important to know the cause of death. In the study, four species of bacterias thought to affect behavior have been investigated [3,4] and these species have been found in intestinal samples of people who committed suicide in post-mortem suicide cases.

Studies on the metabolism of the intestine [5], which is also called as “second brain”, started with animal experiments. In the following studies, it was understood that the imbalance in the intestinal flora caused diseases in humans, and then the diseases were started to be treated by intervening in the intestinal microbiota by transferring bacteria to the diseased individuals. Bipolar disorder and anxiety disorder have been found in the majority of individuals who attempted suicide [6,7]. In the past ten years, research on whether there is a relationship or not between psychiatric disorders and gastrointestinal flora increased, studies began in our country, and the treatment of these disorders with probiotics come to the fore [8]. It was detected that the psychological stress levels of the subjects using probiotics regularly every day declined, the urinary free cortisol level diminished, and both anxiety and depression levels decreased in mice given *Lactobacillus rhamnosus* for 28 days [4]. In clinical studies, the *Bacteroides family* was

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found to be associated with depression, and some subspecies of the *Oscillibacter* class were found to carry valeic acid, which plays a similar role to gamma-aminobutyric acid (GABA), a neurotransmitter in humans [3].

Intestinal flora can produce some active metabolites which have a function in human metabolism. For instance; *Lactobacillus* sp. and *Bifidobacteria*; can synthesize the neurotransmitter GABA, which has a soothing effect from monosodium glutamate [3] to on the nervous system *Escherichia* sp., *Bacillus* sp. and *Sacromices* sp. norepinephrine; *Candida* sp., *Streptococcus* sp., *Escherichia* sp. and *Enterococcus* sp. species produce serotonin, while *Bacillus* sp. and *Serratia* sp. types produce dopamine [9].

Bifidobacterium infantis was peroral applicated to mice, and an increase in their plasma tryptophan levels was observed [10]. *Lactobacillus acidophilus* increases the expression of cannabinoid receptors in the brain stem [11]. Plasma serotonin levels were found to be high in mice raised under sterile conditions [12]. Messaoudi et al. (2011) found a decrease in psychological stress and urinary-free cortisol levels in the patients who are given a combination of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* probiotics [4]. Benton et al. (2007) found that the consciousness and psychiatric functions of patients who were given probiotic food over three weeks changed positively [13]. *Lactobacillus casei*, which is given as a probiotic supplement to people suffering from fatigue, improved anxiety [14].

Materials and Methods

Samples were collected from 58 hanging and 45 traffic accident cases among those who came to the Morgue Specialization Department of The Council of Forensic Medicine for autopsy between 07.07.2017 and 22.05.2018. Among the 103 autopsy materials collected, 77 (74.8 %) of them belonged to male bodies and 26 (25.2 %) of them belonged to female corpses. There is no sample not included in the study. Approval was obtained from the ethics committee of the Istanbul University Cerrahpasa Medical Faculty. The youngest one whose stool sample was taken is the person who died at the age of 10 as a result of a traffic accident; the oldest one is also the example of a person who died as a result of a traffic accident at the age of 87. It was determined that the youngest person who committed suicide was a 13-year-old teenage girl, and the oldest person was a 77-year-old woman.

Samples were collected between the sigmoid colon and rectum by opening the abdominal cavity during the autopsy, and two samples were taken for each case. If the samples are directly for aerobic culture, they can be transferred to Tryptic Soy Broth (TSB) broth; for the preservation of anaerobic microorganisms, the samples were taken into Fluid Thioglycollate Medium (THIO) liquid medium, considering similar studies, the samples were brought to the laboratory within two hours in the cold chain box [3,4,13]. It was cultivated on media such as Agar, Chocolate Agar, Kanamycin-Vancomycin-Laked Blood Agar, Paromycin agar, Endo Agar, Mac Conkey Agar, Malt Agar. Although molecular-based typing was performed in our study, analysis was also performed by using cultural methods to extract a general microbial flora of the intestine. The cultivated species were first examined macroscopically then microscopically, for phenotypic examination, and gram coloration properties were tried to be understood by staining methods. Species

reproduced by culture methods were also typed with the API 20A, 20E identification kit belonging to the Biomeriux company. On the score of there is no sequence analysis and cell count measuring device in our institute's student laboratory; stool samples were defined in the genetic laboratory of the company by landing a service contract with the genetic diagnosis center krosngen.

A commercial AMBRD kit based on silica spin column technology was used For bacterial isolation. Pure DNA was obtained by treating 5 different solutions in this kit with samples that are taken from stool samples in certain amounts and times. The isolated bacterial DNAs were tested with Qubit 2.0 and NanoDrop brand measuring device, and the DNA amounts were measured before PCR. The essential mixtures for PCR were added to the pure DNA and operationalise for the Real-Time PCR device. SYBR Green method, based on detecting the elongated DNA strand with the help of fluorescent dyes used in conjunction with Real-Time PCR with the SYBR Green method, was the most suitable method for our study. 16S rRNA sequencing process is also a method that can be used in our study. However, the application of the SYBR Green method was found more cost-effective. The number of bacteria detected in the stool sample after PCR was measured by the droplet digital PCR method and compared with the control group. The identification and quantitative measurement of the microorganisms in the intestinal microbiota of the suicide cases and the control group were statistically interpreted with the help of the SPSS (Statistical Package for the Social Sciences) version 22 package program released by IBM.

Results

As a result of classical microbiological analyzes using the culture method, colonies frequently encountered in the stool were identified with API 20A and API 20E kits, and the identified bacteria are listed in the table below (Table 1).

The average of the DNA strand and bacterial cell numbers of the relevant bacteria included in the table in 1 microliter of mixed DNA calculated according to the results of the Real-Time DNA analysis of the pure DNAs of the samples are shown below. (Table 2).

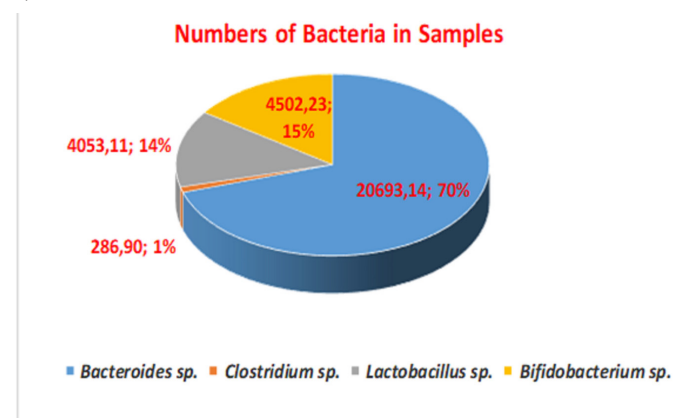


Figure 1. Numerical distribution of bacteria in the flora within the context of all the samples

Multiplied bacteria by using real-time PCR device and measured ones by Droplet Digital PCR device, bacterial values per microliter of bacteria with significant growth values are shown in the table below (Table 3).

Table 1. The bacteria which are frequently encountered in cultured samples

Name of microorganism	Frequency	Characteristic
<i>Actinomyces israelii</i>	++++	Anaerobic, Gram-positive, bacillus,
<i>Bacteroides caccae</i>	+	Anaerobic, Gram-negative, bacillus,
<i>Bacteroides ovatus</i>	++++	Anaerobic, Gram-negative, bacillus,
<i>Bacteroides thetaiotaomicron</i>	++++	Anaerobic, Gram-negative, bacillus,
<i>Bacteroides ureolyticus</i>	+	Anaerobic, Gram-negative, bacillus,
<i>Bacteroides vulgatus</i>	+	Anaerobic, Gram-negative, bacillus,
<i>Bifidobacterium adolescentis</i>	+	Anaerobic, Gram-positive, branched bacillus
<i>Bifidobacterium breve</i>	+	Anaerobic, Gram-positive, branched bacillus
<i>Bifidobacterium ssp.</i>	++++	Anaerobic, Gram-positive, bacillus,
<i>Bifidobacterium dentium</i>	+	Anaerobic, Gram-positive, branched bacillus
<i>Clostridium butyricum</i>	++	Anaerobic, Gram-positive, bacillus,
<i>Clostridium beijerinckii</i>	+++	Anaerobic, Gram-positive, bacillus,
<i>Escherichia coli</i>	+	Anaerobic, Gram-negative, bacillus,
<i>Fusobacterium varium</i>	++	Anaerobic, Gram neg., non-sporeous bacillus,
<i>Fusobacterium mortiferum</i>	+++	Anaerobic, Gram neg., non-sporeous bacillus
<i>Parabacteroides distasonis</i>	+	Anaerobic, Gram-negative, bacillus,
<i>Porphyromonas asaccharolytica</i>	+	Anaerobic, Gram-negative, bacillus
<i>Prevotella bivia</i>	+	Anaerobic, Gram-negative, bacillus,
<i>Prevotella disiens</i>	++	Anaerobic, Gram-negative, bacillus,
<i>Prevotella intermedia</i>	++	Anaerobic, Gram-negative, bacillus,
<i>Propionibacterium avidum</i>	++	Facultative Aerobic, Gram positive, cocci,

Note: + (less) ++ (middle) +++ (many) ++++ (full plate)

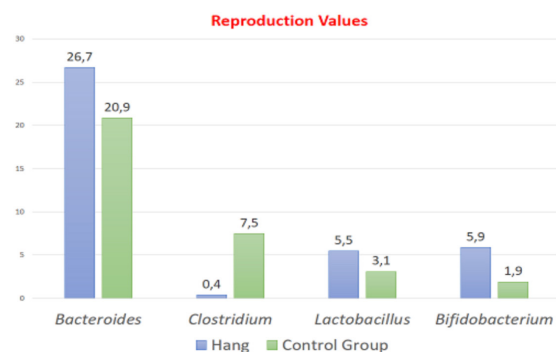
Table 2. The number of bacteria per nanoliter of microorganisms grown in stool samples belonging to the vaccine and control groups

Sample Group	<i>Bacteroides</i>	<i>Clostridium</i>	<i>Lactobacillus</i>	<i>Bifidobacterium</i>
Hang	20.7	0.3	4.0	4.5
Control Group	16.8	4.0	2.6	1.6
General Avarage	18.7	2.2	3.3	3.0

Table 3. The number of bacteria per nanoliter of the hanging and control group samples with significant growth graphic

Sample Group	<i>Bacteroides</i>	<i>Clostridium</i>	<i>Lactobacillus</i>	<i>Bifidobacterium</i>
Hang	26.7	0.4	5.5	5.9
Control Group	20.9	7.5	3.1	1.9
General Avarage	23.8	3.9	4.3	3.9

As the summary of our article study, the quantitative number of species that are thought to affect behaviors in the intestinal flora of the people who died as a result of suicide and the control group is shown in the graphic below (Figure 2).

**Figure 2.** Data on biogenic bacteria in the samples of the hanging and control groups

The identification and quantitative measurement of the microorganisms in the intestinal microbiota of the suicide cases and the control group were statistically interpreted with the help of the SPSS (Statistical Package for the Social Sciences) version 22 package program released by IBM. Pearson Ki-Kare value is founded 6.0 and the standard deviation is founded 4, Likelihood value is founded 6,6 and Relationship between rows is founded 2.0 (Table 4).

Table 4. Some values for statistical analysis

Data	Value	Standard deviation
Pearson Ki-Kare value	6.0 ^a	4
Likelihood rate	6.6	4
Relationship between rows	2.0	1

Discussion

In our study, the economic, cultural, and social causes of suicide were excluded from the scope of our study, since the psychological autopsy of the stool samples was not performed and only the intestinal samples were compared with the control group. Our study, unlike many studies on this subject, is a post-mortem study in which human-based study rather than animals and suicide attempts are studied. Resulting of the psychological autopsy of those who committed suicide was not performed and the obtained data were limited, any information couldn't be accessed that would affect the psychiatric history, drug utilization, substance usage habits, antibiotic use cases, other treatment conditions, or flora of the specimen deceased persons.

Microbiological methods used in our study start with the macroscopic examination which is defined as classical microbiological analysis microscopy, staining methods, culture methods and continues until biochemical and molecular methods, which are advanced. It is hoped that obtaining a healthy and sufficient amount of DNA belonging to the intestinal flora after the collection, transportation, preservation, and storage of the collected samples can provide a methodological path for researchers who want to do a study similar to our research later on.

It cannot be said that the intestinal contents or the mucosal flora are the same as the data obtained by the microbiological analysis of the stool. In our study, the samples were collected from the inner part of the intestine using a spatula without disturbing the tissue integrity. The samples taken during the autopsy were taken directly with a sterile swab and placed in a liquid medium (Fluid Thioglycollate Medium-THIO). Successful results were obtained in our anaerobic culture studies and it was determined that the use of this method and the medium was beneficial. Our samples taken for aerobic culture were placed in Tryptic Soy Broth (TSB) broth and transported to the laboratory in a cold chain box for approximately 1 hour. Although a miserable amount of samples was taken at the end of the study, results were obtained from all samples and when the amount of DNA was measured in the pre-PCR process, it was seen that the amount of DNA was quite good.

In stool analysis; many criteria such as physical appearance, color, odor, chemical content, pH, microbiology, parasitology, hematology are evaluated. Before our analyses, a general evaluation of the sample taken from the autopsy was carried out. However, no significant difference was specified between stool color and bacterial rates. Between 10-50% of the intestinal flora is culturable and the selection of the appropriate medium is difficult [15]. In our study, *Bacteroides*, *Bifidobacterium*, and *Clostridium* species were able to be reproduced among the bacterial colonies reproduced by culture methods before molecular analysis, but no colonies of *Lactobacillus* species could be detected. However, *Lactobacillus* species have also been detected in molecular studies. Therefore, it has been confirmed in our study that molecular studies are more sensitive than culture methods.

In recent years, 16S rRNA analysis has attracted attention, especially in microbiota studies. The most important advantage of 16S rRNA analysis is that it can estimate the quantity, unlike the DNA analysis method [16,17]. Microorganisms that cannot be generally cultured are identified by 16S rRNA analysis and

identification is made at the species and subspecies level. After the replication process, the RNA profile is amplified by using databases such as ribosomal gene bank Greengenes, and the identification is completed by introducing Silva to the system [18]. In our study, instead of the 16S rRNA method or multiple-locus DNA replication systems such as Multichip, Microarray, the cost-effective SYBR Green method which is approximately 1/10 according to this analysis was used since the identification remained at the genus level, and successful results were obtained.

Microorganisms that make up the human microbiota; bacteria, fungi, viruses, archaea, and parasites, and most of the microorganisms in the intestinal contents are bacteria and almost all of the bacteria are anaerobic bacteria in the literature [19]. As a result of the analyzes made in our article study, it was verified that the information is correct as a result of the performed test. (Table I). Gram-positive cocci and bacillus - Gram-negative cocci and bacillus, these species may also have different conformational and metabolic effects. Since 4 previously determined species were investigated in our study, all the flora in our samples were not detected. However, when we survey the distribution of the species reproduced by culture method and identified by biochemical methods; It has been determined that most of the species are anaerobic bacillus-shaped bacteria. It has been determined that this information is compatible with literature studies [20,21].

Although it has been reported in literature studies that mice with sterile guts called Germ Free (GF) are more carefree and peaceful than normal mice and have lower stress levels [22,23], no results were found to support this information since there was no sterile sample in our study. The knowledge that *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* families live predominantly in the human intestines [16,22,24] was tested in our study, and it was found that the most common family among the searched species was *Bacteroidetes*. It was determined that the *Bacteroides* species was significantly more surplus than the other three species. If we peruse on the samples we studied, 69 % of *Bacteroides* sp., 7 % of *Clostridium* sp., 12.3 % of *Lactobacillus* sp., 11.7 % of *Bifidobacterium* sp consisted of species belonging to the genus.

Beneficial species of *Enterococcus*, *Lactobacillus*, *Escherichia*, *Bifidobacterium* genera; *Citrobacter brachiasis*, *Candida albicans* species have been reported to be harmful [25-27]. Since we collected samples that recently died among our species that were generally researched by culture method, a result close to the percentage of living things was found. In particular, the obtained *Bifidobacterium* sp. and *Lactobacillus* sp. are beneficial microorganisms used as probiotics for food supplementation. In some studies, it was evaluated that the quantitative ratio of species belonging to the *Bacteroides* and *Firmicutes* families could be significant in depression and anxiety, in addition, in literary studies; the quantity and quality of the species of *Bacteroides* sp., *Lactobacillus* sp., *Clostridium* sp. and *Bifidobacterium* sp., in the intestinal flora of a normal human, were investigated [28,29]. The presence of these species, *Bacteroides* sp., *Lactobacillus* sp., and *Bifidobacterium* sp., in the content of probiotics, helped us to determine the species sought in our study.

It was also determined in our study that the most common family in the human intestine was *Bacteroidetes*, and a significant relationship was found between the rate of *Bacteroides* sp. and suicidal cases.

It was determined that 59.34 % of the detected *Bacteroides sp.* were present in suicidal individuals and 40.66 % were present in the control group. These statistics show that *Bacteroides* species have an increasing effect on depression and suicide cases. This data was found to be compatible with literature studies [30,31]. One of the most popular bacteria thought to affect behavior is *Bifidobacterium lognum*. This bacterium has been measured to reduce anxiety in mice. It has been reported that *Bifidobacterium infantis* plays an antidepressant role [32]. However, in our study, it was found that the percentage of *Bifidobacterium sp.* was less than the control group. This information could not be confirmed in our study, on the contrary, the opposite result was obtained.

It has been reported that some bacteria which cause colitis are associated with anxiety, infections in the intestines change the central nervous system and behaviors, affect the tryptophan level, *Clostridium difficile* infection and inflammation increase anxiety, and this effect is eliminated with *Lactobacillus casei* and *Lactobacillus helveticus* [32]. In our study, *Clostridium sp.* type was found less in the samples taken from suicide cases. It was determined that the percentage of *Clostridium sp.* was less than the control group. Therewithal 5.11 % of the detected *Clostridium sp.* were present in suicidal patients and 94.8 % were present in the control group (Figure 1). This statistic does not support the information that species belonging to the genus *Clostridium sp.* affect increasing depression and suicide cases. The significant difference in the rate is *Clostridium sp.* indicates a lack of species. The rate of *Lactobacillus sp.* of people who committed suicide was 19.45 % lower than the control group. Therefore, it could not be determined whether *Lactobacillus sp.* species has a preventive effect on depression and suicide. In a study by Foster et al. (2013), *Lactobacillus rhamnosus* was reported to reduce anxiety and depression in mice [32] and human [33]. However, our results do not support the literature sources. In the present study, no relationship was found between gender and intestinal flora. There is no consensus on this matter. Our result is compatible with some studies but not others [34,35]. In some studies, it has been reported that the *Bacteroides/Firmicutes* ratio is effective in depression and anxiety. In our study, the ratio of *Bacteroides* and *Firmicutes* families to each other was 4.77 in suicide cases; 2.56 in the control group. It is seen that the ratio of *Bacteroides / Firmicutes* in people who commit suicide is twice as high as in the control group. This information was found to be compatible with literature studies [8,28]. *Lactobacillus sp.* and *Bifidobacterium sp.* species are considered to be beneficial in literature studies, and *Clostridium* which is reported to be harmful considering the ratio of the species; it was determined that the number of beneficial microorganisms was higher in the suicide group and less in the control group. However, there is no information about the positive effects of bacteria, which are beneficial for metabolism and health, on behavior.

In addition to research on whether there is a relationship or not between psychiatric disorders and gastrointestinal flora, we will contribute to the literature in this direction with the publication of our study. Our contribution is that this study aims to determine whether the intestinal flora of the people who committed suicide is different from the control group. One reason why the article process is long is; molecular-based methods should be used in intestinal analyses and these studies are costly. Having more

opportunities while conducting similar studies to our article study will enable more comprehensive and detailed studies to be carried out. Although our article study requires more comprehensive and detailed research, material and methods have been created within the possibilities provided by our university. In the future, it will be useful to carry out additional studies on the current article, as well as to conduct different studies on a similar subject.

Conclusion

In our study, while measuring the effect of intestinal bacteria on behavior, *Bacteroides sp.*, *Clostridium sp.*, *Lactobacillus sp.* and *Bifidobacterium sp.* species which can be found in the intestines of suicide cases and control group were investigated. Microorganisms thought to affect behavior were analyzed in the study. According to the difference in flora here, it has become possible to change the intestinal flora with probiotic supplements or other methods. Therefore, the treatment of the psychiatric disorder is expected and the number of suicides caused by these disorders can be reduced.

Since it has been determined that the intestinal flora of people who committed suicide and those who died as a result of traffic accidents are different, it will be beneficial to regulate the intestinal flora with probiotic supplementation of people with intestinal flora imbalance or prebiotic intake according to the nutritional preferences of the deficient species. In addition, in acute situations, bacteria can be transplanted to sick people through the colon. However, further studies are needed for this treatment method to be applied routinely in this area. It is possible to analyze the intestinal flora commercially in private laboratories. It is also possible to analyze the entire intestinal flora by providing a certain amount of stool samples for people who have a disease or want to develop a general profile of their flora. Although there are many books, scientific publications, and researches on crime theories, intestinal microorganisms, which are among the biological factors of crimes; it is considered that it can be taken into account in criminal events and bio crimes and will gain even more importance. It is thought that this research, which has recently been in the field of brain, neurology, and intestine, which are popular microbiota study areas, will be a basic step for similar studies to be done in the future, and it will be beneficial for this field to conduct similar studies.

Conflict of interests

The authors declare that they have no competing interests.

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Ethical approval

Ethics approval was obtained from the ethics committee of the Istanbul University Cerrahpaşa Medical Faculty (Number: 59491012-604.01.02-).

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