Original Article

Microflora in Rats Metagenomic Analysis of Intestinal Microflora in Rats Treated with Ellagic Acid and Sinapic Acid Using 16 S rDNA Gene Region

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ABSTRACT

Background: The correct functioning of the intestinal mechanism is essential for human health. In particular, the micro ecosystem of the gut has a vital role in the development of the host and the continuity of metabolism. For this reason, many studies have been conducted on the intestinal microbiota. Today, the use of phenolic compounds in the production of complementary and alternative medicines is quite common. **Purpose:** In our study, changes in intestinal microflora at the species level were evaluated in Wistar Albino rats treated with Ellagic Acid and Sinapic Acid. **Materials and Methods:** The first group was formed as the control group. Ellagic Acid (EA) was given to the second group of rats (50 mg/kg/d). The third group of rats was given Sinapic Acid (SA) (20 mg/kg/d). The fourth group of rats was given Ellagic Acid EA (50 mg/kg/d) and Sinapic Acid SA (20 mg/kg/d). At the end of the study, intestinal tissues were taken under appropriate sterile conditions. The V3-V4 points located in the 16 S rDNA gene regions of the bacteria in the microflora were replicated by the specific primers developed in accordance with these regions. **Results and Conclusion:** The data obtained from of sequencing analyzes were evaluated comparatively for four groups. As a result, it was determined that Ellagic Acid and Sinapic Acid have a positive effect on probiotic microorganisms (Lactobacillus) in the intestine. It was also determined that while it caused a decrease in the rate of some pathogenic microorganisms such as Streptococcus, it caused an increase in the number of some pathogens.

Keywords: Gut microbiota, Metagenome, Ellagic acid, Sinapic acid, 16 S rDNA.

INTRODUCTION

Intestine; It is a metabolic organ with very important roles such as digestion, absorption of nutrients, energy need, regulation of natural and adaptive immune functions, vitamin synthesis and resistance to diseases and hosts active micro-organisms.[1-3] Symbiotic bacteria that live in our intestine are essential for the intestinal system. There is a continuous metabolic mechanism of interaction between the host and the microbiota.[4] It is well known that the colonization of microorganisms in the host gut is affected by factors related to host and non-host interactions.[5] The composition of the gut microbiota is affected by diet, antibiotic therapy, maternal microbiota, and genotype.[6] In the human gastrointestinal tract, the vast majority of the microbiota plays a critical role in maintaining health throughout life.[7] Intestinal flora varies significantly between individuals. And it is thought to play a key role in diagnosing many diseases.[8]

Dysfunction of the microbiome-brain-gut axis plays a role in stress-related disorders such as irritable bowel syndrome (IBS), depression and anxiety, and neurodevelopmental disorders such as autism.[9] Many phytochemicals are used in the protection of human health. Among them, the use of phenolic compounds is quite common. In recent years, phenolic structures such as Ellagic Acid and Sinapic Acid as material in scientific studies have increased. Ellagic

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acid (EA), also known as trihydroxy acid ($C_{14}H_6O_8$), is generally found in red and purple fruits like raspberry, strawberry, pomegranate, grape, and blackcurrant.[10-11] It has focused on the contribution of EA consumption to a healthy life, especially on studies investigating cancer prevention and its anti-inflammatory and antidiabetic effects.[12-14] Another phenolic compound, Sinapic Acid (3,5-dimethoxy-4-hydroxycinnamic acid), is a derivative of hydroxycinnamic acid. Found in fruits, vegetables, grains, plant seeds and some spices, SA has antioxidant, antimicrobial, anti-inflammatory, anticancer, chemopreventive and antiapoptotic activity.[15-17]

Molecular diagnostic tests and next-generation metagenomic approaches analyze all the Nucleic Acids present in a sample, enabling the microbiome analysis with a wide range of pathogens such as parasites, bacteria and viruses.[18-19] Studying the gut microbiota has become more accessible due to rapid advances in microarray technology and statistical analysis of data. For this reason, it is known that genomic analyzes make essential contributions to phylogenetic studies. With the emergence of next-generation sequencing technologies, sequence analysis of the 16S rDNA gene region has become a culture-independent approach to diagnosing bacterial communities.[20-21]

Our study aimed to reveal positive or negative results that may occur in bacterial species in the intestinal composition in rats treated with Ellagic Acid and Sinapic Acid. The first group was formed as the control group. The second is the group administered Ellagic Acid. The third group was determined as the group that was administered Sinapic Acid and the fourth group was given Ellagic Acid and Sinapic Acid. Sequence analyzes of the V3-V4 points of the 16 S rDNA gene regions were performed to evaluate the metagenomic data comparatively.

MATERIALS AND METHODS

Ethical Statement

The experimental protocols of this study were approved by the Van Yüzüncü Yıl Animal Experiments Local Ethics Committee (Permit Number: 2019/03).

Test Animals

Twenty-eight female Wistar Albino rats used in this study were obtained from Van Yüzüncü Yıl University Experimental Medicine Application and Research Center. Rats were housed under normal light and dark cycles (21 ± 2°C). The feed and water of the rats kept in suitable cages were given per the standards (*ad libitum*). A total of 28 rats were used in this study. Rats were randomly divided into four groups (n = 7).

1. Control (C) group: They were fed with standard rat food and water until the end of the experiment. No further action was taken.
2. Ellagic Acid (EA) group: Rats were given EA (50 mg/kg/d).
3. Sinapic Acid (SA) group: Rats were given SA (20 mg/kg/d).
4. Ellagic Acid + Sinapic Acid (SA + EA) group: Rats were given SA (20 mg/kg/d) and EA (50 mg/kg/d).[22-24]

At the end of the study period, the rats, which were deprived of feed for 12 hr, were rendered dead by direct cannulation of the heart after the administration of 50 mg/kg ketamine. Intestinal tissues of the rats were taken and stored at -20°C until the study.

Isolation of Bacteria and Genetic Analysis

Isolation of bacteria in the intestinal flora, V3, V4 region analyzes of the 16S rDNA gene were performed by BM Labosis using the QIIME2 procedure. DNA isolation and quality control were performed from the samples to create a library.

The V3-V4 region of the 16S rDNA gene from the isolated target DNAs was amplified using specific primers F (5'-TCGTCGGCAGCGTCAGATGTGTATA AGAGACAGCCTACGGG NGGCWGC AG-3') and R (5'-GTCT CGTGGGCTCGGAGAT GTGTATAA GAGAGAGGA CTACHVGGGTAT CTAATC C-3'). Then purification was done. Indexes and adapters were added using the index kit (Nextera XT) during the PCR step. After purification, the concentration of the libraries created by Real-time PCR was measured. It was then diluted to 4nM and normalized. Normalized samples were brought together by the pooling method. After sieving particles, primers and barcodes smaller than twenty nucleotides; Species identification was performed for taxonomic analyses. The analysis steps including the taxonomy classification step of the 16 S rRNA gene regions, are given in Figure 1.

RESULTS

Species Level Comparative Analysis Results

Taxonomic analysis of bacteria in the intestinal microflora of all groups was performed. The data generated as a result of the metagenomic analysis of the V3, V4 region of the 16S rDNA gene point were examined comparatively. The taxonomic column chart created for the species level is given in Figure 2. The comparative analysis of bacteria and their ratios obtained

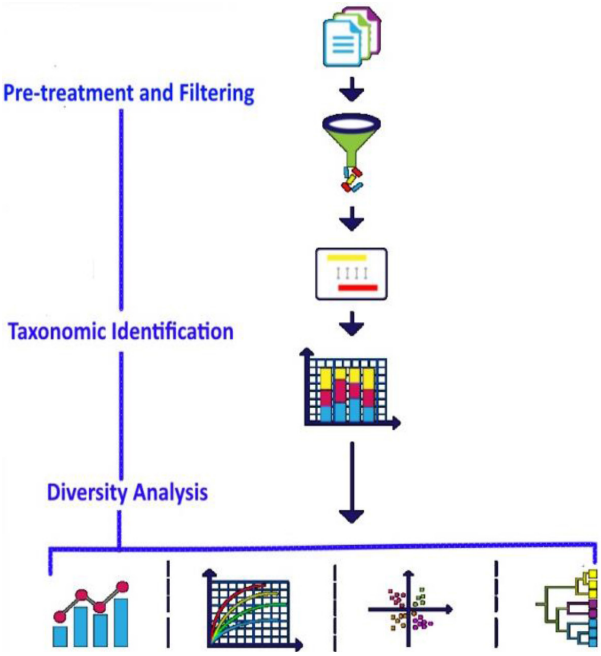

Figure 1: Genetic Analysis steps.

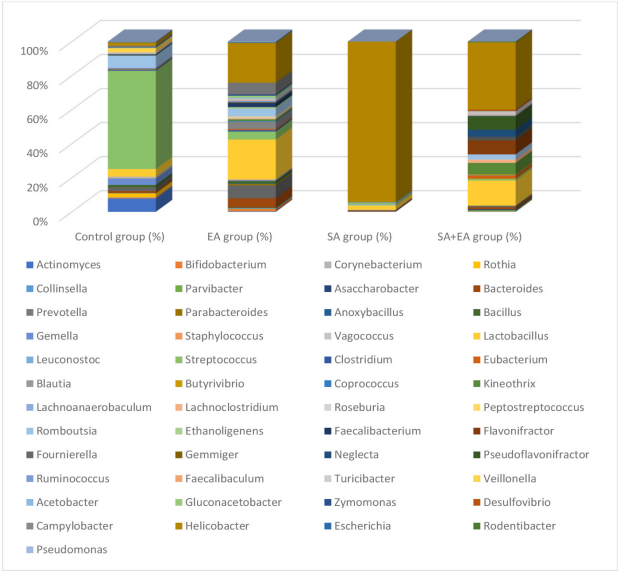

Figure 2: Taxonomic Column Chart for Species Level.

from metagenome analysis of all groups are given in Table 1.

Statistical Analysis

Statistical analyzes were performed using the SPSS (20.0) package program. Statistical significance level was taken as $p<0.05$.

DISCUSSION

The intestinal area, which contains many species, accounts for about 2kg of body weight. It is estimated that number of microorganisms it contains is ten times higher than the number of cells in the human body.[25] Comprehensive analysis of the genetic material of microorganisms results rapidly using next-generation sequencing (mNGS), and metagenomic research. Metagenomic analyz facilitate the diagnosis and treatment of many infectious diseases because it has a broad scope such as gene expression, antimicrobial resistance, microbiome, and oncology.[26] Metagenomic studies contribute to the diagnosis of beneficial and harmful bacteria in the body with the help of 16S rDNA gene sequencing technology. In our research, we determined the change in the diversity of microorganisms due to the use of Ellagic Acid and Sinapic Acid in the intestinal microbiota due to metagenome analysis.

Metagenome analysis of body microbiota is usually done with stool samples or small intestinal fluid.[27] In our study, rat intestine was used for metagenome analysis. Bifidobacterium, Lactobacillus, Bacteroides spp. Compounds produced by commensal bacteria, such as commensal bacteria, inhibit the growth of some intestinal pathogens.[28] It is observed that Lactobacillus species (*Lactobacillus delbrueckii, Lactobacillus faecis, Lactobacillus intestinalis, Lactobacillus johnsonii, Lactobacillus reuteri, Lactobacillus satsumensis*), which is one of the most important bacterial groups in the intestinal ecosystem, showed a significant increase in the group using Ellagic acid compared to the control group. On the other hand, there was a decrease in the ratio of Lactobacillus species in the group in which Sinapic acid was used. The rate of Vagococcus (*Vagococcus fessus* and *Vagococcus lutrae*) species that cause disease in humans in some cases decreased in the groups using Sinapic acid and Ellagic acid compared to the control group.[29] In rats with ethanol-induced ulcers, pretreatment with Sinapic Acid was found to decrease the acidity rate and reduce the stiffness of injuries in the gastric mucosa.[30] Ellagic Acid taken into the body through food or dietary supplements has been shown to have the potential to be used in reducing intestinal inflammation.[31]

In rats treated with Gallic and Ellagic acid, diltiazem permeability was significantly increased in the ileum.[32] The stomach is the principal place where Helicobacter species are located. However, they can cause infection in any part of the gastro-intestinal system where gastric epithelial cells are present.[33] In our study, it was observed that Helicobacter species (*Helicobacter cholecystus, Helicobacter ganmani, Helicobacter pylori, Helicobacter rodentium)* increased in groups using Ellagic Acid and Sinapic Acid.

Streptococci, Enterococci, *Mycoplasma* and *Staphylococci* species in Firmicutes phylum are generally pathogenic.[34]

Table 1: Taxonomy Table for Species Level.				
Bacteria species	**Control group (%)**	**EA group (%)**	**SA group (%)**	**SA+EA group (%)**
Actinomyces bovis	7,658±0,032	0,157±0,002[a]	0,111±0,001[a]	0,104±0,001[a]
Bifidobacterium adolescentis	0,229±0,001	0,714±0,026[b]	0,019±0,011[a]	0,083±0,009[a]
Corynebacterium ulcerans	0,244±0,013	0,036±0,002[a]	0	0
Rothia terrae	2,087±0,010	0	0	0,069±0,009[a]
Parvibacter caecicola	0,070±0,001	0,235±0,009[b]	0	0,586±0,003[b]
Bacteroides dorei	0,298±0,008	2,084±0,012[b]	0,184±0,001[a]	0,223±0,002[a]
Bacteroides vulgatus	0,394±0,007	0,790±0,015[b]	0,044±0,001[a]	0
Prevotella copri	1,759±0,021	6,256±0,034[b]	0,206±0,003[a]	0,565±0,006[a]
Parabacteroides distasonis	0,036±0,001	0,243±0,011[b]	0	0
Parabacteroides merdae	0,088±0,004	0,481±0,010[b]	0,025±0,001[a]	0,146±0,001[b]
Bacillus vallismortis	1,313±0,030	0,911±0,015[a]	0	0,041±0,002[a]
Gemella parahaemolysans	3,703±0,045	0,364±0,013[a]	0,098±0,008[a]	0,076±0,006[a]
Staphylococcus succinus	0	0,251±0,009[b]	0	0
Vagococcus fessus	0,331±0,003	0,031±0,001[a]	0	0
Vagococcus lutrae	0,560±0,025	0,120±0,003[a]	0	0,048±0,005[a]
Lactobacillus delbrueckii	2,652±0,055	14,655±0,085[b]	1,063±0,024[a]	2,449±0,024[a]
Lactobacillus faecis	0,291±0,002	0,364±0,013[b]	0,111±0,004[a]	0,362±0,013
Lactobacillus intestinalis	0,225±0,003	0,678±0,024[b]	0,050±0,001[a]	1,611±0,016[b]
Lactobacillus johnsonii	0,442±0,006	3,053±0,036[b]	0,387±0,010[a]	8,651±0,062[b]
Lactobacillus murinus	0,265±0,001	0,141±0,002[a]	0,082±0,003[a]	0,495±0,014[b]
Lactobacillus reuteri	0,106±0,001	0,481±0,015[b]	0	0,327±0,010[b]
Lactobacillus satsumensis	0,202±0,003	0,625±0,026[b]	0,038±0,001[a]	0
Streptococcus danieliae	25,729±0,070	8,053±0,092[a]	0	0,341±0,011[a]
Streptococcus hyointestinalis	0,811±0,004	0	0	0,118±0,003[a]
Streptococcus sanguinis	29,005±0,085	1,005±0,030[a]	0,279±0,008[a]	0,334±0,010[a]
Streptococcus thermophilus	0,317±0,002	0,948±0,015[b]	0,215±0,007[a]	0,244±0,009[a]
Clostridium disporicum	0,162±0,001	0,225±0,007[b]	0	0
Clostridium sporogenes	0,280±0,002	0,054±0,001[a]	0	0
Eubacterium coprostanoligenes	0,147±0,002	0,219±0,003[b]	0	1,179±0,018[b]
Blautia luti	0,121±0,001	3,077±0,040[b]	0,060±0,002[a]	0,216±0,008[b]
Kineothrix alysoides	0,066±0,001	0,817±0,016[b]	0,047±0,001[a]	6,398±0,055[b]
Romboutsia timonensis	6,647±0,068	3,949±0,050[a]	0,133±0,001[a]	2,721±0,020[a]
Ethanoligenens harbinense	0,284±0,005	0,893±0,014[b]	0,028±0,002[a]	0
Faecalibacterium prausnitzii	0,608±0,015	2,142±0,028[b]	0,079±0,003[a]	0
Pseudoflavonifractor phocaeensis	0,025±0,001	0	0	5,253±0,080[b]
Faecalibaculum rodentium	0,118±0,002	0,261±0,006[b]	0	0,348±0,012[b]
Veillonella atypica	1,184±0,022	0,052±0,001[a]	0,015±0,001[a]	0
Veillonella rogosae	1,161±0,018	0,049±0,001[a]	0	0
Mesorhizobium shangrilense	0,036±0,001	0,217±0,007[b]	0	0
Acetobacter indonesiensis	0,081±0,002	0,411±0,010[b]	0	0
Gluconacetobacter liquefaciens	0,309±0,008	1,031±0,025[b]	0,098±0,008[a]	0,055±0,001[a]
Zymomonas mobilis	0,188±0,022	0,720±0,009[b]	0,034±0,002[a]	0,083±0,002[a]
Campylobacter jejuni	0,844±0,035	5,965±0,076[b]	0	0
Helicobacter cholecystus	0,044±0,001	2,613±0,048[b]	0	0
Helicobacter ganmani	1,730±0,040	17,620±0,093[b]	24,031±0,080[b]	22,125±0,075[b]
Helicobacter pylori	0	0,246±0,002[b]	0,031±0,001[b]	0
Helicobacter rodentium	0	0,075±0,003[b]	46,342±0,090[b]	25,140±0,078[b]
Escherichia coli	0,284±0,003	0,403±0,015[b]	0,025±0,001[a]	0
Mycoplasma muris	0	0,026±0,001[b]	23,828±0,078[b]	0,907±0,015[b]
Other species	6,866±0,045	16,259±0,088[b]	2,337±0,022[a]	18,751±0,065[b]

Other species (The total of species below 0.2% in four groups). In the same line; It shows ([a]) statistical decrease, ([b]) statistical increase (p<0,05).

Streptococcus species (*Streptococcus danieliae, Streptococcus hyointestinalis, Streptococcus sanguinis*), which had a significant proportion in the control group, were significantly reduced in the groups using Ellagic Acid and Sinapic Acid. *Mycoplasma muris*, is a pathogenic bacterium in the mouse genital tract.[35] (It was observed that the rate of *Mycoplasma muris* (23,828%) increased significantly in rats treated with sinapic acid. The amount of *Escherichia coli* bacteria, the source of infection in the intestinal system, increased approximately two times in the group using ellagic acid compared to the control group. *Campylobacter jejuni,*[36] bacteria, one of the most common causes of gastroenteritis, showed a high increase (5.965%) in rats treated with Ellagic Acid. Veillonella strains are symbiotic bacteria found in mammals' intestines and oral mucosa.[37-38] While the rate of Veillonella species was over 1% in the control group created in the study, the rate of these bacterial species in the groups using Sinapic Acid and Ellagic Acid decreased below 0.1%. It was observed that the *Kineothrix alysoides* species,[39] in the Fimicutes phylum showed a significant increase in rats where Ellagic Acid and Sinapic Acid were used together. While the rate of *Pseudoflavonifractor phocaeensis* bacteria diagnosed by Ricaboni *et al*., (2017) was 0.02% in the bacteria in the control group, the rate of these bacteria was determined as 5.25% in the group where Ellagic Acid and Sinapic Acid were used together.[40] *Faecalibacterium prausnitzii,*[41] which lives as a commenseal bacterium in the intestinal system, increased in the group given Ellagic Acid. At the same time, it decreased in rats given Sinapic Acid compared to the control group. *Romboutsia timonensis*,[42] bacteria decreased in rats other than the control group. Bacteria of the genus Blautia are microorganisms found in the intestines of mammals and have probiotic properties.[43] It was determined that *Blautia luti* type bacteria increased primarily in the group using Ellagic Acid. When stool samples of patients with the onset of rheumatoid arthritis were examined metagenomicly, it was observed that the number of *Prevotella copri* bacteria showed a large increase.[44] In our study, when compared to the control group, *Prevotella copri* bacteria showed a significant increase in rats using Ellagic Acid, while this rate tended to decrease in rats using Sinapic Acid. In addition, it was determined that *Rothia terrae* species showed a significant decrease in rats treated with Ellagic Acid and Sinapic Acid.

Intestinal microbiota; It affects immune development and homeostasis, host cell proliferation, neurological signaling, intestinal endocrine functions, bone density and energy mechanism.[45]

While *Actinomyces bovis*,[46] which rarely causes disease in humans, had a significant rate in the control group, it decreased in other rat groups. The genus Bacteroides is one of the dominant bacterial groups in the human gut and has a vital role in maintaining a healthy intestinal ecosystem.[47] There seems to be supporting data that commensal *Parabacteroides distasonis* found in the gut can be used as a probiotic to treat functional metabolic disorders and regulate host metabolism.[48] In our study, it was determined that Bacteroides and Parabacteroides species increased in rat groups using Ellagic Acid. The intestinal ecosystem is affected by many factors such as antibiotic use and diet. As a result of the deterioration of healthy intestinal flora, there is a decrease in colonization resistance and an increase in the rate of pathogens.[49] In recent years, the number of studies on intestinal metabolism has been increasing. It is especially important to investigate metabolic disorders that may occur due to inflammation of the intestinal microbiota because studies on the diagnosis and treatment of these health problems are essential to increasing the quality of life.

As a result of the metagenomic analyzes of the V3-V4 points of the 16S rDNA gene regions of the bacteria isolated from the intestine, it was observed that there were significant differences in the species diversity and microorganism ratio of the intestinal flora in the rat groups treated with Ellagic Acid and Sinapic Acid. It was observed that the number of some Lactobacillus species increased in the group in which Ellagic Acid and Sinapic Acid were given together. As a result of this change, it was determined that there was an increase in the number of probiotic bacteria, especially in rats using Ellagic Acid, and a decrease in the number of some pathogens. On the other hand, the number of Lactobacillus species decreased in rats using Sinapic Acid compared to the control group, causing an abnormal increase in some Helicobacter species.

As a results, one of the most critical elements of living life is nutrition. While some living things carry out this work with their adaptive mechanisms, many living things have to obtain food. Producer and consumer organisms need phenolic compounds such as Ellagic Acid and Sinapic Acid and their derivatives to perform some metabolic activities. In case of excess or deficiency of these compounds, troublesome situations may arise in homeostasis. It can cause significant changes, especially in the species diversity of the microflora in our body. As a result of the study we have done, it has been determined that the Ellagic Acid and Sinapic Acid we use positively affect the probiotic (Lactobacillus) microorganisms in the intestinal system. In addition, it has been determined that while it causes a decrease in the rate of some pathogenic microorganisms such

as Streptococcus, it causes an increase in the number of Helicobacter. As a result, it shows that the phenolic compounds we use may have an effect that increases the mechanism of action of beneficial microorganisms and therefore facilitates digestion. It reveals that further studies should be done on the increase in the Helicobacter rate.

CONCLUSION

Intestinal microflora is a very important factor for the continuity of a healthy metabolism. For this reason, it is necessary to investigate the effects of phenolic compounds taken with food on intestinal bacteria. In this respect, metagenomic analyzes have gained importance. It is seen that Ellagic Acid and Sinapic Acid, which we used in the study, increase the effect of beneficial microorganisms in the intestinal system. More comprehensive studies are needed for the increase in Helicobacter rate.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

ABBREVIATIONS

IBS: Irritable Bowel Syndrome; **EA:** Ellagic Acid; **SA:** Sinapic Acid.

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