Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.69321

Structure of intestinal microflora under different diets based on PCR-DGGE technology

Walid Kamal ABDELBASSET[1,2*], Tamer Emam ELNEGAMY[1], Mohamed Abdelaleim ABDELAZIZ[3,4], Shereen Hamed ELSAYED[5,6]

Abstract

This study aims to review the effects of conventional and organic diet on the diversity of rat intestinal microbiota and look at how intestinal microbiota composition changes the following moxibustion at the Piyu and Zusanli points. A total of twenty-four Sprague Dawley (SD) rats of the Specific-Pathogen-Free (SPF) grade were haphazardly assigned to one of 3 groups: conventional food + moxibustion, conventional food, and organic food. For 12 weeks, organic food was given to the organic category, and typical food was prepared for the conventional category of mices, and mices in the combination category received typical food for eight weeks and administered moxibustion at the Piyu and Zusanli points for another four weeks. Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) was used to assess alterations in intestinal flora. The content of intestinal microbiota differed significantly across the three groups, according to the similarity clustering analysis. In the combination group, the intestinal bacterial diversity index rose considerably ($P < 0.05$). Moxibustion at Zusanli and Piyu points enhanced Bacteroides stercoris and Barnesiella intestinihominis growth, according to DNA sequencing. It was concluded that moxibustion has a greater influence on intestinal bacterial diversity than an organic diet.

Keywords: organic food; Zusanli and Piyu; intestinal flora; PCR-DGGE.

Practical Application: The effects of conventional and organic diet on structure of intestinal microflora.

1 Introduction

Food spoilage can be caused by a variety of reactions, some of which are primarily physical or chemical in nature, while others are caused by enzymes or microorganisms. Microorganisms' dynamics of growth, survival, and biochemical activity in food are a result of stress reactions in response to changing physical and chemical conditions in the food microenvironment, the ability to colonize the food matrix and grow into spatial heterogeneity, and in situ cell-to-cell ecological interactions that frequently occur in the solid phase. Ecological approaches to studying the evolution of microbial flora in food would be beneficial for better understanding the microbiological processes involved in food processing and ripening, improving microbiological safety by monitoring in situ pathogenic bacteria, and evaluating the effective compositions of microbial populations (Jayan et al., 2020; Lorenzo et al., 2018; Qeshmi et al., 2018; Teneva et al., 2019; Devanthi & Gkatzionis, 2019). Microorganisms colonizing the human gastrointestinal tract are a large community, with ten times more intestinal microbes than human cells, and macroeconomic sequencing of the human microbiome has shown that there are 3.3 million non-redundant genes in the human microbiome, and more than 99% of them are of bacterial origin (Parker et al., 2018; Wiciński et al., 2020; Zielińska & Kołożyn-Krajewska, 2018). The intestinal flora genome is 150 times larger than the human genome and is known as the human "second genome." These intestinal floras play an important role in human health by participating in energy metabolism, maintaining energy balance, growth and aging processes, production of several enzymes, nutrient metabolism, cancer inhibition, and other physiological processes that are closely related to the health of the body. Research pointed out that intestinal flora is now considered one of the most important environmental factors affecting the physiology and metabolism of the host (Auchtung et al., 2018; Bowey et al., 2003; Clemente et al., 2012; Hirayama & Itoh, 2005; Kinross et al., 2008; Kong et al., 2020; Liu et al., 2018; Yang et al., 2018).

The high prevalence of suboptimal health and metabolic diseases due to environmental pollution and dietary changes has prompted research into the mechanisms of diet-mediated changes in intestinal function in the development of diseases and the search for more effective treatments and methods for their management. Therefore, this experiment was conducted to investigate the effects of consuming conventional foods with pesticides, antibiotics and hormone residues on the intestinal flora

Received 02 Aug., 2021
Accepted 09 Oct., 2021
[1] *Department of Health and Rehabilitation Sciences, College of Applied Medical Sciences, Prince Sattam bin Abdulaziz University, Al-Kharj, Saudi Arabia*
[2] *Department of Physical Therapy, Kasr Al-Aini Hospital, Cairo University, Giza, Egypt*
[3] *Department of Basic Medical Sciences, Collage of Medicine, Prince Sattam bin Abdulaziz University, Al-Kharj. Saudi Arabia*
[4] *Department of Medical Physiology, Faculty of Medicine, Al-Azhar University, Cairo, Egypt*
[5] *Department of Rehabilitation Sciences, Faculty of Health and Rehabilitation Sciences, Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia*
[6] *Department of Physical Therapy for Cardiovascular/Respiratory Disorders and Geriatrics, Faculty of Physical Therapy, Cairo University, Giza, Egypt*
*Corresponding author: walidkamal.wr@gmail.com

of rats by PCR-denaturing gradient gel electrophoresis (polymerase chain reaction-denaturing gradient electrophoresis, PCR-DGGE) and organic foods without pesticides, fertilizers, hormones and additives, and to observe whether wheat grain moxibustion has a benign effect on intestinal flora, and to investigate which types of intestinal flora it has a significant effect on. Theoretical reference for healthy diet and rehabilitation interventions targeting intestinal flora (Bigot et al., 2020; Dalmacio et al., 2011; Diaz et al., 2016; Wang et al., 2019; Xiong et al., 2014). The method was used to investigate the effect of organic food on the structure of intestinal flora in rats, and to observe whether Zusanli and Piyu has a benign effect on the intestinal flora, and to investigate which types of intestinal flora it has a significant effect on, in order to provide a theoretical reference for healthy diet and rehabilitation interventions targeting intestinal flora (Feng et al., 2018).

2 Material and methods

2.1 Material

24 SD rats weighing 200 g ± 20 g SPF grade ♂ were purchased from Beijing Weitong Lihua Laboratory Animal Technology Co., Ltd (Zhang et al., 2019). The feed was provided by Jiangsu Synergy Pharmaceutical and Biological Engineering Co (Li et al., 2019). The organic diet was the formula provided by Synergy (38% corn, 22% wheat, 15% soybean, 12% fish meal, 10% bran, 2% yeast, and the remaining minerals and trace elements), which was replaced by organic corn, organic oats and organic soybean in equal amounts. The organic corn and organic soybeans were purchased from Dewei Organic Series produced by Songwon Honest Industry Co (Cascone & Lamberti, 2020). The purchased products have the organic trademark logo on the packaging. Shielding environment experimental facilities: Nanjing University of Traditional Chinese Medicine Animal Experiment Center. Moxa velvet was 1:50 refined moxa velvet and Scutellaria baicalensis oil paste (20 g/box, lot no. 1401020), purchased from Jiangsu Provincial Hospital of Traditional Chinese Medicine (Fu et al., 2013; Hesketh & Zhu, 1997; Jia et al., 2008; Su et al., 2018; Sun et al., 2007). The bacterial universal primers GC-338F, 518R and 338F/518R, lysozyme, protease, high-efficiency centrifugal column agarose gel recovery kit, and restriction endonuclease were purchased from Beijing Yiming Revival Biotechnology Co.

2.2 Methods

Animal grouping and treatment

After 24 SD rats were acclimatized for three days, they were randomly divided into three groups using SPSS17.0, namely, the organic group, the conventional group, and the conventional group + Zusanli and Piyu intervention group. During the experiment, the organic group was fed organic food, and the conventional and conventional groups + Zusanli and Piyu intervention groups were fed conventional food. After eight weeks, Zusanli and Piyu intervention was started in the conventional group + Zusanli and Piyu intervention group for four weeks, and the remaining two groups were left untreated.

Zusanli and Piyu intervention

The rats were artificially immobilized, and the back and hind limbs were exposed (1 cm × 1 cm shaved at the treatment acupuncture points). The acupuncture points were positioned with reference to the Basic Skills of Basic Medical Laboratory Animal Operation. The acupuncture points (Zusanli (housanli) Acupoints: below the knee joint of the hind limbs, about 5 mm inside the head of the fibula; Piyu: the intercostals of the twelfth thoracic vertebrae, on the left and right sides) were applied with astragalus oil and 5 mg/strength of moxibustion floss was placed on the acupuncture points, and the floss was ignited with incense and removed when the rat showed a contraction of the moxibustion area. Repeat for five strokes per point, one time/d alternating sides. 6 d is a course of treatment, a total of 4 courses of intervention, rest 1 d between courses.

Extraction of rat intestinal feces

After the intervention, the rats were put to death by cervical dislocation at the 13th week, and they were immediately placed on the ultra-clean workbench. The intestinal feces of each group were collected aseptically, and the intestinal feces of every two rats in the same group were collected together and then frozen in liquid nitrogen for storage.

Extraction of DNA from fecal specimens

After the fecal specimens were thawed, bacterial DNA was extracted according to the operation manual of the Fecal Genomic DNA Rapid Extraction Kit.

Amplification of bacterial 16S rDNA fragments

The extracted DNA was tested for its integrity by 0.8% agarose gel electrophoresis. The 16S rDNA variable region V3 was used as the target, and PCR amplification was performed using universal bacterial primers GC-338F and 518R; the primer sequences are shown in Table 1. The PCR amplification system (50 μL) was as follows: ddH_2O, template DNA 50 ng, 518R (20 mmol/L) 1 μL, GC-338F (20 mmol/L) 1 μL, rTaq (5 U/μL) 0.4 μL, dNTP (2.5 mmol/L) 3.2 μL, and 10 × PCR buffer 5 μL was added to 50 μL. The PCR amplification procedure was as follows: pre-denaturation at 94 °C for 5 min; denaturation at 94 °C for 1 min, denaturation at 55 °C for 45 s, extension at 72 °C for 1 min, 30 cycles; final extension at 72 °C for 10 min.

Table 1. Synthesis report.

Oligo Name	Sequence
GC338F	5'- CGCCCGGGGCGCGCCCCGGGGCGGGGCGGGGGCGCGGGGGGCCTACGGGAGGCAGCAG- 3'
518R	5'- ATTACCGCGGCTGCTGG- 3'
338F	5'- CCTACGGGAGGCAGCAG- 3'

The PCR products were purified and recovered, then imaged under a UV gel imaging system, and the profiles were stored.

DGGE analysis of PCR products

Ten μL of PCR products were taken for DGGE analysis. A polyacrylamide gel (100% chemical denaturant containing seven mol/L urea and 40% (v/v) acrylamide) with a deformation gradient of 35%-55% and a concentration of 8% was used for electrophoresis at 150 V for five h at 60 °C in 1 × TAE buffer. After DGGE was completed (Ercolini, 2004; Han et al., 2010; Rantsiou et al., 2004), silver staining was used as follows: (1) fixation solution (ethanol 50 mL, glacial acetic acid 2.5 mL, constant volume 500 mL) for 15 min; (2) Milli-Q pure water washing, 20 s and 2 min each; (3) silver staining solution (silver nitrate 1 g, 37% formaldehyde 0.75 mL, volume 500 mL) for 15 min; (4) Milli-Q pure water washing, 20 s and 2 min each; (5) silver staining solution (silver nitrate 1 g, 37% formaldehyde 0.75 mL, constant volume 500 mL) for 15 min. (4) Milli-Q pure water washing, 20 s and 2 min each; (5) color development solution (sodium hydroxide 7.5 g, 37% formaldehyde 2.5 mL, fixed volume 500 mL) for 5-7 min. Finally, the reaction was terminated with the termination solution (50 mL of ethanol, 2.5 mL of glacial acetic acid, 500 mL of fixed volume).

Recovery and sequencing of dominant bands in DGGE profiles

Cut off the DGGE bands to be recovered with a sterilized scalpel and re-amplify the 16S rDNA V3 region using the same method as above. The re-amplified DNA fragments were recovered, purified, ligated into Pmd18-T vector, and transformed into DH5α receptor cells to screen positive clones for sequence determination. SPSS17.0 software was used for data processing. The experimental data were expressed as mean ± SD, and one-way ANOVA with SNK test was used for the comparison between groups, and $P < 0.05$ was considered statistically significant.

3 Results

3.1 DNA extraction

The DNA samples extracted with the kit could obtain clear bands. From Figure 1, it can be seen that there is a faint trailing phenomenon, low protein amount, and obvious brightness, which indicates that the amount of DNA is sufficient to perform PCR.

3.2 PCR results

518R and GC-338F amplified the gene of the 16S rDNA V3 region of rat intestinal bacteria as primers, and a DNA fragment of about 200 bp was obtained. After 2% agarose gel electrophoresis and EB staining, a bright and clear band at 200 bp could be seen under UV light (Figure 2), which was used for DGGE analysis.

3.3 DGGE mapping analysis

The DGGE profiles of the 16S rDNA V3 region of each group of rat fecal bacteria (Figure 3) showed that the number and intensity of the bands varied in the lanes of each group of samples. The bands in different positions represent different

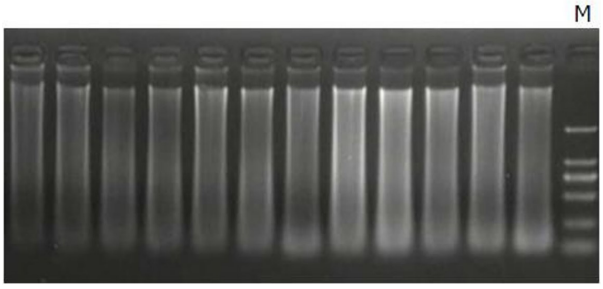

Figure 1. DNA extraction results. M: DL2000 DNA Marker bands.

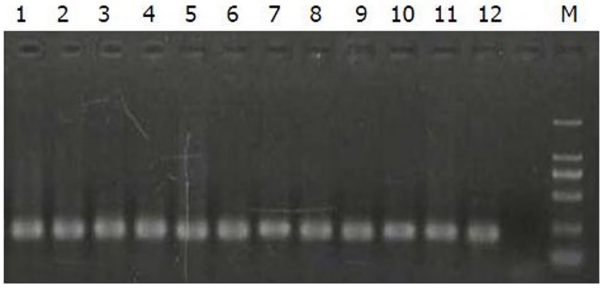

Figure 2. Amplification of 16S rRNA gene in V3 region. 1-4: organic group; 5-8: conventional group; 9-12: conventional group + Zusanli and Piyu intervention group; M: DL2000 DNA Marker bands.

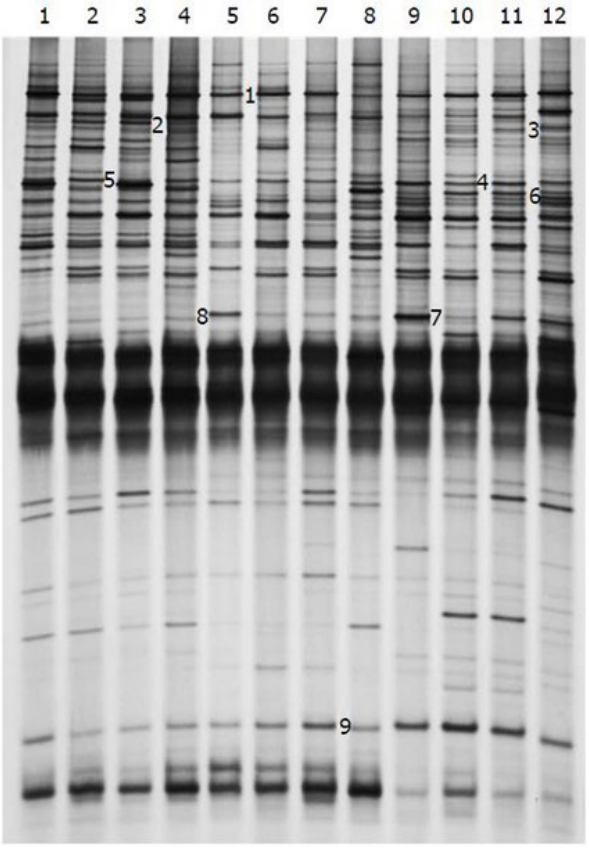

Figure 3. DGGE mapping. 1-4: organic group; 5-8: conventional group; 9-12: conventional group + Zusanli and Piyu intervention group. Each number corresponds to a different sample, and each band with different brightness represents a different amount of flora. The black numbers marked in the plots correspond to those in Table 3.

dominant bacteria, and the brightness reflects the relative number of bacteria. The number and brightness of the bands in lanes 1, 2, 3, and 4 of the organic group were higher than those in lanes 5, 6, 7, and 8 of the conventional group; the number and brightness of the bands in lanes 9, 10, 11 and 12 increased after Zusanli and Piyu intervention, indicating that the diversity of the intestinal flora of the rats in the organic group was higher than that in the conventional group, and the diversity of the intestinal flora of the rats in the conventional group increased after Zusanli and Piyu intervention.

For quantitative analysis, the number of electrophoretic bands and band density of each sample were digitally analyzed by Quantity one software, and abundance (S), Shannon index (H'), and equilibrium index (E) were used to compare the diversity of different samples. Abundance (S) was expressed as the number of bands per lane in the DGGE profile; Shannon index (H') was calculated as follows: (Equation 1); $P_i = N_i/N$; where P_i is the ratio of the intensity of a single band to the total intensity of all bands in the sample, N is the abundance of all bands in a single lane of the DGGE profile, and Ni is the abundance of the i^{th} band; S is the sum of all bands in a sample; Eq. S is the sum of all bands in a sample; the equilibrium index (E) indicates the consistency of the distribution of the genus of the population, calculated as E = H'/lnS. The results are shown in Table 2. One-way ANOVA showed that: (1) the homogeneity between groups was > 0.05, which was not statistically significant; (2) the Shannon index between groups was P > 0.05, which was not statistically significant. However, P = 0.06 between the conventional group and the conventional + Zusanli and Piyu group, considering that the sample size of each group was 4, if the sample size was increased, the diversity of intestinal flora after Zusanli and Piyu intervention might be different; (3) the richness of P_1> 0.05 between the conventional group and the organic group was not statistically significant. Compared with the conventional group, the richness of the conventional + Zusanli and Piyu group was significantly different (P_2 = 0.02 < 0.05); compared with the organic group, the richness of the conventional + Zusanli and Piyu group was different (P_3 = 0.04 < 0.05), and $P_2<P_3$, indicating that the richness of the intestinal flora of the rats in the organic group was slightly higher than that of the conventional group, and the richness of the intestinal flora could be significantly increased by moxibustion at the Zusanli and Piyu points.

The unweighted pair-group method with arithmetic means (UPGMA) was used for similarity cluster analysis on the electropherograms (Figure 4), and it was found that organic groups 1, 2, 3, and 4 were clustered separately. Also, conventional groups 5, 6, 7, and 8 were clustered separately too, indicating that the structure of the intestinal flora of the organic groups and the conventional groups had more obvious differences. The intestinal flora structure of the rats in the conventional group was significantly different from that of the conventional + Zusanli and Piyu intervention group by removing 12, 9, 10, and 12 clusters. It can be seen that long-term consumption of organic food and moxibustion at the Zusanli and Piyu points can affect the structure of intestinal flora in rats.

3.4 DNA sequence analysis of the differential intestinal bands corresponding to DGGE profiles

The differences of the bands in the DGGE map can well reflect the differences and similarities of the intestinal bacterial composition of rats during the experiment. The differential bands were sequenced and compared with the Blast tool's GenBank database, and the results are shown in Table 3. The bacteria

Table 2. Analysis of bacterial diversity.

Subgroup	Uniformity (E)	Shannon index (H')	Richness (S)
Available groups	0.98 ± 0.00	3.62 ± 0.03	40.00 ± 1.15
Conventional group	0.98 ± 0.01	3.59 ± 0.12	39.50 ± 3.87
Conventional + Zusanli and Piyu group	0.98 ± 0.00	3.78 ± 0.04	47.25 ± 2.06

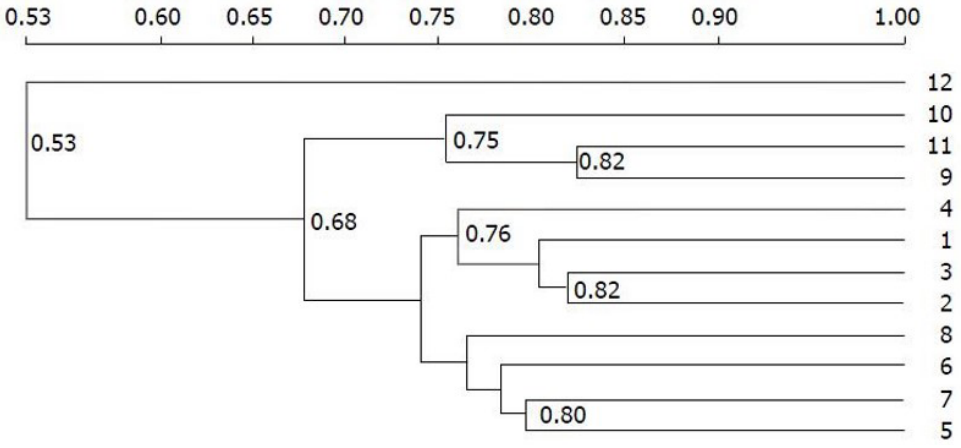

Figure 4. Similarity clustering analysis graph. 1-4: organic group; 5-8: conventional group; 9-12: conventional group + Zusanli and Piyu intervention group.

Table 3. Identification results of representative strip colonies.

Band	Most similar strains	Similarity percentage	Similar strain registration number	Phylum
Band9	Barnesiella intestinihominis	90	NR_041668	Bacteroidetes
Band2	Bacteroides stercoris	95	NR_027196	Bacteroidetes
Band8	Clostridium methylpentosum	95	NR_029355	Firmicutes
Band3	Barnesiella intestinihominis	96	NR_041668	Bacteroidetes
Band7	Flavonifractor plautii	97	NR_043142	Firmicutes
Band4	Bacteroides stercoris	99	NR_027196	Bacteroidetes
Band5	Coprococcus comes	99	NR_044048	Firmicutes
Band6	Novosphingobiumnitrogenifigens	99	NR_043857	Proteobacteria
Band1	Clostridium populeti	100	NR_026103	Firmicutes

in rat feces showed high similarity with the known bacterial sequences in the database, all above 90%. The sequencing results indicated that the possible bacteria in bands 1, 5, 8, and 9 were Clostridium populeti, Barnesiella intestinihominis, Clostridium methylpentosum, Coprococcus comes with similarities of 100%, 90%, 95%, and 99%, respectively, suggesting that Firmicutes and Bacteroidetes are the normal dominant flora in the conventional rat intestine and that Zusanli and Piyu can increase. The sequencing results indicated that band 2 was probably Bacteroides stercoris, with 95% similarity, suggesting that long-term consumption of organic food materials could promote the reproduction of this bacterium in the rat intestine; the bands 3 and 4 were specific to the conventional + Zusanli and Piyu group. The sequencing results indicated that bands 3 and 4 were probably Barnesiella intestinihominis and Bacillus faecalis, with similarities of 96% and 99%, respectively, suggesting that Zusanli and Piyu could promote the reproduction of these two bacteria in the rat intestine.

4 Conclusions

In this research, after 12 weeks, the three groups of intestinal flora were individually clustered according to similarity clustering analysis, suggesting that there is a relationship between the consumption of organic food and moxibustion at the Zusanli and Piyu points and the change in the diversity of intestinal flora. In the conventional + Zusanli and Piyu group, No. 12 was a separate branch, which may be due to individual differences. The DGGE overall pattern suggested that the diversity of intestinal flora was higher in the 12 weeks organic diet-fed rats than in the conventional group, and the diversity of intestinal flora increased in the conventional group after moxibustion of the Zusanli and Piyu points with wheat grain.

However, digital analysis of the intestinal flora diversity in these three groups showed that the differences were not significant except for the abundance in the conventional + Zusanli and Piyu group ($P_2 = 0.02 < 0.05$, $P_3 = 0.04 < 0.05$). This may be due to the fact that this experiment is not a large sample (n = 4), as the Shannon P = 0.06 between the conventional group and the conventional + wheat grain moxibustion group, suggesting that increasing the sample size, wheat grain moxibustion at the Zusanli and Piyu points can increase the Shannon index of rat intestinal flora. The higher the diversity index of intestinal flora, the more stable the structure of intestinal flora and the more difficult to break the balance. It is suggested that moxibustion of Zusanli and Piyu points can increase the stability of intestinal flora. The results of sequencing the differential bands on the DGGE map showed that:

(1) Firmicutes and Bacteroidetes are the normal dominant flora in the conventional rat intestine. Eckburg et al. (2005) found that intestinal microorganisms could be classified into six major categories: Firmicutes, Bacteroides, Proteobacteria, Actinobacteria, Verrucomicrobia, and Fusobacteria, of which Bacteroides and Firmicutes are the main ones. The results of this experiment are consistent with Eckburg's report;

(2) the abundance of fecal Bacteroides increased in the intestine of rats in the organic group, suggesting that this kind of intestinal bacteria is more competitive in the organic feed environment;

(3) The abundance of Barnesiella intestinihominis and Bacillus faecalis in the intestine of rats in the conventional + Zusanli and Piyu group increased, suggesting that Zusanli and Piyu points could promote the reproduction of these two bacteria in the intestine of rats. Barnesiella intestinihominis and fecal mycobacterium belong to the phylum Bacteroides, suggesting that the consumption of organic feed and moxibustion on the foot and spleen acupuncture points with the wheat grain can increase the abundance of Bacteroides.

Jumpertz et al. (2011) reported that the proportion of calorie intake was positively correlated with the abundance of Bacteroides in feces and negatively correlated with the abundance of Firmicutes. The results of this experiment are consistent with the fact that rats with wheat grain moxibustion intervention had a high abundance of Bacillus phylum compared to conventional rats. Organic food is an international term for uncontaminated natural food, which is truly natural, nutritious, high quality, safe, and environmentally friendly ecological food. Diet is one of the most important factors in determining the bacterial composition of the intestinal flora, and Zhang et al. found that changes in the structure of the diet can cause changes in the structure of the intestinal flora (Zhang et al., 2010).

Other studies found that a high-fat diet could lead to changes in the structure of the intestinal flora and a decrease in the number of probiotics (Lactobacillus and Bifidobacterium) and

conventional commensal bacteria (Bacteroides and Enterococcus) (Li et al., 2017; Sui'e et al., 2006). In this experiment, we found that the long-term feeding of organic food increased the diversity of intestinal flora in rats compared with those fed conventional food. Wheat grain moxibustion is one of the earliest treatment methods in China, and it has been reported in the literature that Zusanli and Piyu have certain anti-inflammatory, lipid regulating, digestive system improving, immune system improving, and can improve the quality of life of unhealthy people. In recent years, it has been found that the intestinal flora, as the largest micro-ecosystem in the human body, can, directly and indirectly, influence the growth and development of the host organism, as well as the immune activity and the homeostasis of the internal environment, and it is closely related to metabolic diseases.

In conclusion, the results of this study showed that the intestinal flora of rats was differentially altered by organic food and conventional food, and the diversity of intestinal flora could be improved by eating organic food and moxibustion at the Zusanli and Piyu points. It suggests that a long-term organic diet and grain moxibustion are beneficial to the stability of intestinal flora and health.

Acknowledgements

This project was supported by the Deanship of Scientific Research at Prince Sattam Bin Abdulaziz University under the research project #2020/03/17131.

References

Auchtung, T. A., Fofanova, T. Y., Stewart, C. J., Nash, A. K., Wong, M. C., Gesell, J. R., Auchtung, J. M., Ajami, N. J., & Petrosino, J. F. (2018). Investigating colonization of the healthy adult gastrointestinal tract by fungi. *mSphere*, 3(2), e00092-18. http://dx.doi.org/10.1128/mSphere.00092-18. PMid:29600282.

Bigot, C., Bugaud, C., Camilo, J., Kapitan, A., Montet, D., & Meile, J.-C. (2020). Impact of farming type, variety and geographical origin on bananas bacterial community. *Food Control*, 109, 106925. http://dx.doi.org/10.1016/j.foodcont.2019.106925.

Bowey, E., Adlercreutz, H., & Rowland, I. (2003). Metabolism of isoflavones and lignans by the gut microflora: a study in germ-free and human flora associated rats. *Food and Chemical Toxicology*, 41(5), 631-636. http://dx.doi.org/10.1016/S0278-6915(02)00324-1. PMid:12659715.

Cascone, S., & Lamberti, G. (2020). Hydrogel-based commercial products for biomedical applications: a review. *International Journal of Pharmaceutics*, 573, 118803. http://dx.doi.org/10.1016/j.ijpharm.2019.118803. PMid:31682963.

Clemente, J. C., Ursell, L. K., Parfrey, L. W., & Knight, R. (2012). The impact of the gut microbiota on human health: an integrative view. *Cell*, 148(6), 1258-1270. http://dx.doi.org/10.1016/j.cell.2012.01.035. PMid:22424233.

Dalmacio, L. M., Angeles, A. K., Larcia, L. L., Balolong, M., & Estacio, R. (2011). Assessment of bacterial diversity in selected Philippine fermented food products through PCR-DGGE. *Beneficial Microbes*, 2(4), 273-281. http://dx.doi.org/10.3920/BM2011.0017. PMid:22146687.

Devanthi, P. V. P., & Gkatzionis, K. (2019). Soy sauce fermentation: microorganisms, aroma formation, and process modification. *Food Research International*, 120, 364-374. http://dx.doi.org/10.1016/j.foodres.2019.03.010. PMid:31000250.

Diaz, M., Ladero, V., Redruello, B., Sanchez-Llana, E., del Rio, B., Fernandez, M., Martin, M. C., & Alvarez, M. A. (2016). A PCR-DGGE method for the identification of histamine-producing bacteria in cheese. *Food Control*, 63, 216-223. http://dx.doi.org/10.1016/j.foodcont.2015.11.035.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E., & Relman, D. A. (2005). Diversity of the human intestinal microbial flora. *Science*, 308(5728), 1635-1638. http://dx.doi.org/10.1126/science.1110591. PMid:15831718.

Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods*, 56(3), 297-314. http://dx.doi.org/10.1016/j.mimet.2003.11.006. PMid:14967221.

Feng, Y., Fang, Y., Wang, Y., & Hao, Y. (2018). Acupoint therapy on diabetes mellitus and its common chronic complications: a review of its mechanisms. *BioMed Research International*, 2018, 3128378. http://dx.doi.org/10.1155/2018/3128378. PMid:30426006.

Fu, H., Qiu, Z., & Zhang, X. (2013). Analysis of perioperative use of prophylactic antibiotics in patients undergoing typeiincision operation in Jiangsu Provincial Hospital of traditional Chinese medicine: analysis of 319 cases. *Evaluation and Analysis of Drug-Use in Hospitals of China*, 3, 227-230.

Han, Y., Xu, X., Jiang, Y., Zhou, G., Sun, X., & Xu, B. (2010). Inactivation of food spoilage bacteria by high pressure processing: evaluation with conventional media and PCR–DGGE analysis. *Food Research International*, 43(6), 1719-1724. http://dx.doi.org/10.1016/j.foodres.2010.05.012.

Hesketh, T., & Zhu, W. X. (1997). Health in China: traditional Chinese medicine: one country, two systems. *BMJ*, 315(7100), 115-117. http://dx.doi.org/10.1136/bmj.315.7100.115. PMid:9240055.

Hirayama, K., & Itoh, K. (2005). Human flora-associated (HFA) animals as a model for studying the role of intestinal flora in human health and disease. *Current Issues in Intestinal Microbiology*, 6(2), 69-75. PMid:16107039.

Jayan, H., Pu, H., & Sun, D.-W. (2020). Recent development in rapid detection techniques for microorganism activities in food matrices using bio-recognition: a review. *Trends in Food Science & Technology*, 95, 233-246. http://dx.doi.org/10.1016/j.tifs.2019.11.007.

Jia, X., Chen, Y., Li, X., Tan, X., Fan, C., & Li, L. (2008). New thoughts and methods of studying material base of traditional Chinese herbal formula. *China Journal of Traditional Chinese Medicine and Pharmacy*, 23(5), 420.

Jumpertz, R., Le, D. S., Turnbaugh, P. J., Trinidad, C., Bogardus, C., Gordon, J. I., & Krakoff, J. (2011). Energy-balance studies reveal associations between gut microbes, caloric load, and nutrient absorption in humans. *The American Journal of Clinical Nutrition*, 94(1), 58-65. http://dx.doi.org/10.3945/ajcn.110.010132. PMid:21543530.

Kinross, J. M., Von Roon, A. C., Holmes, E., Darzi, A., & Nicholson, J. K. (2008). The human gut microbiome: implications for future health care. *Current Gastroenterology Reports*, 10(4), 396-403. http://dx.doi.org/10.1007/s11894-008-0075-y. PMid:18627653.

Kong, Y., Olejar, K. J., On, S. L., & Chelikani, V. (2020). The potential of Lactobacillus spp. for modulating oxidative stress in the gastrointestinal tract. *Antioxidants*, 9(7), 610. http://dx.doi.org/10.3390/antiox9070610. PMid:32664392.

Li, J., Guo, Y., Li, Q., Miao, K., Wang, C., Zhang, D., Tian, C., & Zhang, S. (2019). Presence of white matter lesions associated with diabetes-associated cognitive decline in male rat models of pre-type 2 diabetes.

Medical Science Monitor, 25, 9679-9689. http://dx.doi.org/10.12659/MSM.918557. PMid:31848329.

Li, L. I., Zu Wang, H., Rong, Y. I., Luo, M., Bao, Y. X., & Yan, L. I. (2017). Clinical observation of ling gui ba fa time-based points selection in treating diarrhea-predominant irritable bowel syndrome. *Shanghai Journal of Acupuncture and Moxibustion*, 36(10), 1181-1185.

Liu, J., An, N., Ma, C., Li, X., Zhang, J., Zhu, W., Zhang, Y., & Li, J. (2018). Correlation analysis of intestinal flora with hypertension. *Experimental and Therapeutic Medicine*, 16(3), 2325-2330. http://dx.doi.org/10.3892/etm.2018.6500. PMid:30210587.

Lorenzo, J. M., Munekata, P. E., Dominguez, R., Pateiro, M., Saraiva, J. A., & Franco, D. (2018). Main groups of microorganisms of relevance for food safety and stability: general aspects and overall description. In F. J. Barba, A. S. Sant'Ana, V. Orlien & M. Koubaa (Eds.), *Innovative technologies for food preservation* (pp. 53–107). London: Elsevier. http://dx.doi.org/10.1016/B978-0-12-811031-7.00003-0.

Parker, A., Lawson, M. A., Vaux, L., & Pin, C. (2018). Host-microbe interaction in the gastrointestinal tract. *Environmental Microbiology*, 20(7), 2337-2353. http://dx.doi.org/10.1111/1462-2920.13926. PMid:28892253.

Qeshmi, F. I., Homaei, A., Fernandes, P., & Javadpour, S. (2018). Marine microbial L-asparaginase: biochemistry, molecular approaches and applications in tumor therapy and in food industry. *Microbiological Research*, 208, 99-112. http://dx.doi.org/10.1016/j.micres.2018.01.011. PMid:29551216.

Rantsiou, K., Comi, G., & Cocolin, L. (2004). The rpoB gene as a target for PCR-DGGE analysis to follow lactic acid bacterial population dynamics during food fermentations. *Food Microbiology*, 21(4), 481-487. http://dx.doi.org/10.1016/j.fm.2003.10.002.

Su, J., Zhu, Q., Yuan, L., Zhang, Y., Zhang, Q., & Wei, Y. (2018). Transumbilical laparoendoscopic single-site radical prostatectomy and cystectomy with the aid of a transurethral port: a feasibility study. *BJU International*, 121(1), 111-118. http://dx.doi.org/10.1111/bju.13965. PMid:28734080.

Sui'e, Z., Yanmin, D., & Jiaping, T. A. O. (2006). Clinical observation on medicated diet with spleen-invigorating and phlegm-removing herbs for simple obesity. *Journal of Guangzhou University of Traditional Chinese Medicine*, 23, 209-211.

Sun, G., Luo, C., Ren, P., & Shi, C. (2007). Construction of fingerprintological system of traditional Chinese medicine. *Central South Pharmacy*, 15, 69-73.

Teneva, D., Denkova-Kostova, R., Goranov, B., Hristova-Ivanova, Y., Slavchev, A., Denkova, Z., & Kostov, G. (2019). Chemical composition, antioxidant activity and antimicrobial activity of essential oil from Citrus aurantium L zest against some pathogenic microorganisms. *Zeitschrift Für Naturforschung C*, 74(5–6), 105-111. http://dx.doi.org/10.1515/znc-2018-0062. PMid:30685748.

Wang, X., Tang, J., Wu, Y., Yao, X., Sun, H., Shen, Q., & Li, Y. (2019). Construction of compound bacteria for polluted water purification based on mixed culture and PCR-denaturing gradient gel electrophoresis. *Acta Agriculturae Zhejiangensis*, 31(11), 1896-1902.

Wiciński, M., Gębalski, J., Mazurek, E., Podhorecka, M., Śniegocki, M., Szychta, P., Sawicka, E., & Malinowski, B. (2020). The influence of polyphenol compounds on human gastrointestinal tract microbiota. *Nutrients*, 12(2), 350. http://dx.doi.org/10.3390/nu12020350. PMid:32013109.

Xiong, X., Hu, Y., Yan, N., Huang, Y., Peng, N., Liang, Y., & Zhao, S. (2014). PCR-DGGE analysis of the microbial communities in three different Chinese "Baiyunbian" liquor fermentation starters.. *Journal of Microbiology and Biotechnology*, 24(8), 1088-1095. http://dx.doi.org/10.4014/jmb.1401.01043. PMid:24809292.

Yang, R., Xu, Y., Dai, Z., Lin, X., & Wang, H. (2018). The immunologic role of gut microbiota in patients with chronic HBV infection. *Journal of Immunology Research*, 2018, 2361963. http://dx.doi.org/10.1155/2018/2361963. PMid:30148173.

Zhang, C., Zhang, M., Wang, S., Han, R., Cao, Y., Hua, W., Mao, Y., Zhang, X., Pang, X., Wei, C., Zhao, G., Chen, Y., & Zhao, L. (2010). Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. *The ISME Journal*, 4(2), 232-241. http://dx.doi.org/10.1038/ismej.2009.112. PMid:19865183.

Zhang, L., Liu, J., Zhao, Y., Liu, Y., & Lin, J. (2019). N-butylphthalide affects cognitive function of APP/PS1 transgenic mice (Alzheimer's disease model). *Chinese Journal of Tissue Engineering Research*, 23(19), 3025.

Zielińska, D., & Kołożyn-Krajewska, D. (2018). Food-origin lactic acid bacteria may exhibit probiotic properties. *BioMed Research International*, 2018, 5063185. http://dx.doi.org/10.1155/2018/5063185. PMid:30402482.