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Rhythms and Microbiomes: The Impact of Circadian Rhythms on Gut Microbiota Via The Vagus Nerve

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Abstract. The gut microbiota, which is as the "second largest gene" of human, is the microbial community within the gastrointestinal tract, The circadian rhythm is a 24-hour internal clock in nearly all organisms, associated with a wide range of physiological and psychological activities. It is found that there is a complex interaction and balance relationship between gut microbiota and circadian rhythm. The circadian disruption caused by unhealthy lifestyles is becoming increasingly prevalent in modern society, influencing the composition and diversity of the gut microbiota via the gut-brain axis. The vagus nerve, a pathway in the parasympathetic nervous system, is an important information regulation pathway for the crosstalk between the gut and the brain. This study investigated the impact of circadian rhythms on gut microbiota via the vagus nerve and set 3 specific aims. Firstly, the rhythmicity of the gut microbiota will be tested in normal and vagotomized mice, examining the vagus nerve in maintaining the impact of circadian rhythms on the rhythmicity of gut microbiota. Furthermore, the fecal samples from vagotomized mice will be transplanted into normal germ-free mice and vagotomized germ-free mice, thus testing the role of the vagus nerve in initiating the microbial rhythmicity regulated by the circadian cycle. Last but not least, mice will be divided into five groups of different light/dark cycles, each containing normal and vagotomized mice, measuring the specific changes in the composition and diversity of the gut microbiota caused by the circadian disturbances, and the role of gut microbiota in mediating these alterations.

Keywords: Circadian rhythms; gut microbiota; vagus nerve.

1. Introduction

Microorganisms, including bacteria, fungi, and viruses, colonize the human body. 97% of human microbiota resides in the gastrointestinal (GI) tract, both in the small intestine and colon and is known as gut microbiota [1, 2]. As the most well-studied microbial collection in the human body, the gut microbiota is known for its capability in gut homeostasis, regulating the metabolism of nutrients, and defending against the colonization of pathogens [3].Gut microbiota, usually described as the “forgotten organ”, is a dynamic ecosystem that undergoes natural selection at both the microbial and host levels [4]. It is known that the composition and diversity of the gut microbiota are both indicators and mediators of one’s health. Microbial dysbiosis, changes in gut microbial composition, can relate to inflammation and psychiatric disorders [5]. Understanding the gut microbiota makes it possible to identify new potential therapeutic targets for a wide range of conditions, ranging from metabolic syndromes to neurodegenerative disorders [6,7].

Current studies have shown the bidirectional relationship between gut microbiota and brain activity. Through the well-developed theory of the gut-brain axis (GBA), researchers can understand the mechanisms of communication between the GI tract and the brain (Figure 1), including signaling via the vagus nerve, alteration in intestinal permeability, bacterial metabolites, changes in the permeability of the blood-brain barrier (BBB), etc [3, 8]. Particularly,the vagus nerve, as one of the multiple direct and indirect pathways within the GBA (Figure 2), is known to take part in the effects of gut microbiota on circadian rhythm [9].

One of the most important functions of the brain is to regulate the diurnal cycle of the host body, known as the circadian rhythm.Circadian rhythm, as the central clock in mammals, can control the diurnal oscillation of the gut microbiota. With the rapid development of modern technology,

unhealthy lifestyles, such as working in shifts, jet lagging, and anxiety, often result in circadian disturbance, causing sleep-related syndromes to become common, not to mention a series of health problems [10]. According to 2021 statistics from the Centers for Disease Control and Prevention (CDC), about 77.3% of high school students in the US had insufficient sleep, given that the recommended amount of sleep for high school students is 8 hours each day. If the rhythmicity of gut microbiota and the changes in composition and diversity caused by host circadian rhythm are known, the gut microbiota samples would be better comprehended, giving a more accurate reflection on the host health status. As circadian disorder affects a large portion of the population, the impact of circadian rhythm on gut microbiota can raise awareness of this unusual global epidemic, provide innovative approaches to modulate these pathways for therapeutic benefit, fully harnessing the power of the GBA in the interplay between circadian rhythm and gut microbiota [11].As part of the gut-brain axis, the efferent pathways of the vagus nerve may potentially take part in the diurnal oscillation and alterations induced by light in the gut microbiota.Investigating the role of the vagus nerve in the impact of circadian rhythm on gut microbiota has the potential to reveal new insights into human health and disease.

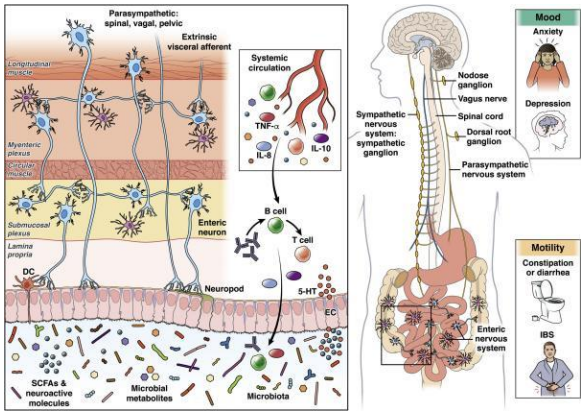

Fig. 1 Various pathways of microbiota-gut-brain axis [12].

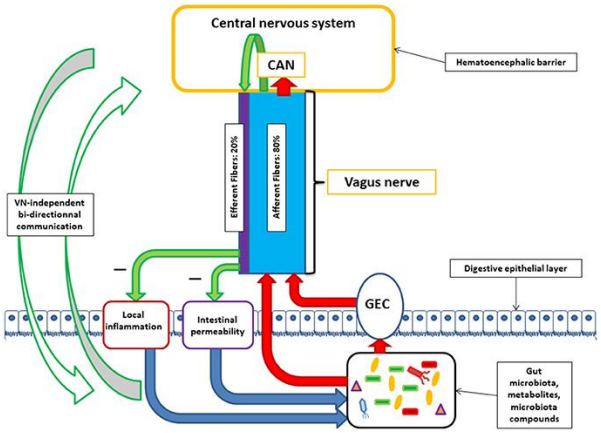

Fig. 2 Role of the vagus nerve in the microbiota-gut-brain axis [13].

2. The Current Stiuation of Circadian Rhythms on Gut Microbiota Via the Vagus Nerve

The gut microbiota is shaped by intertwined factors (Figure 3), such as age, gender, genetics, geography, body site, dietary pattern, lifestyle, and antibiotic treatment, resulting in a unique microbiome profile for each individual [1, 14]. Different kinds of bacteria in accordance with a certain proportion of combination distribution, interdependence and mutual constraints, forming a dynamic balance.Data on microbial composition can be collected in various ways, 16s rRNA gene sequences to gain knowledge on bacterial archaeal genotypes, 18s rRNA genes for microbial eukaryotes, and size fractionation for viruses. Metagenomics can reveal the functionalities of microbes, while metatranscriptomics can show the active gene expression within the microbiota [15]. Circadian

rhythm is regulated by a series of activator and repressor genes, further influencing several biological and physiological functionalities, including behavior, body temperature, immunity, and the sleep/wake architecture, in accordance with the circadian environment of Earth. Circadian rhythm is mainly controlled by gene regulation. To initiate the circadian cycle, it is required to activate the circadian locomotor output cycles protein kaput (Clock), brain and muscle ARNT-like 1 (Bmal 1), and neuronal PAS domain containing protein 2 (nPas2) genes, accompanied by the regulation of multiple proteins-period circadian protein homologue 1, 2, and 3 (Per1-3, cryptochrome 1 and 2 (Cry1 and Cry2), Rev-erbα (Nr1d1), Rev-erbβ (Nr1d2), and differentially expressed chondrocytes protein 1 and 2 (Dec1 and Dec2) [14].

The circadian rhythm shapes the diurnal oscillation of the gut microbiota via the gut-brain axis. Microbial rhythms are clock-dependent, as the Clock network mutation is associated with the altered microbial rhythmicity. About 20% of commensal species of the gut microbiota in rodents show diurnal oscillation, modulating the central clock in SCN [8]. Deletion of Bmal genes abolishes the rhythmicity of fecal microbiota, as well as altering the bacterial abundance in feces samples [16]. The peripheral rhythms displayed in other body parts, such as the small intestine and liver, needed for fine-tuning the central clock, can be clock-independent, especially when responding to environmental cues like food ingestion [4]. Intriguingly, in the absence of the central clock genes, the behavioral rhythms of the host remain normal [17]. In a 2016 study conducted by M. Cui and H. Xiao, which aims to explore how the circadian rhythm shapes the gut microbiota, which in turn affects radiosensitivity. They measured the relative level of Clock, Bmal1, and Per1 protein to show the rhythmic expressions of the genes regulating the circadian rhythm (Figure 4), indicating the disturbance of the circadian cycle under altered light/dark cycles [18]. Light is the main inducer of circadian rhythm via activation of the suprachiasmatic nucleus (SCN) in the hypothalamus [14]. Furthermore, androgen and gut microbiota, in both mice and humans, are suggested to have two-way crosstalk, indicating the sex-dependent differences in microbiota of different sexes [14]. As the GBA contains various pathways connecting the gut and the brain, the link responsible for the changes in the gut microbiota can be multiple.

The vagus nerve, as one of the multiple direct and indirect pathways within the GBA (Figure 4), is known to take part in the effects of gut microbiota on circadian rhythm [9]. Short-chain fatty acids, particularly butyric acid, may regulate the production of brain-derived neurotrophic factors via the vagus nerve and induce biosynthesis of neurotransmitters in the central nervous system [19]. Some scholars have pointed out that There is a kind of KLE1738 bacteria in the human gut that lives exclusively on the human brain chemical GABA, and GABA is the only nutrient that supports the growth of KLE1738, and the content of GABA in the brain is very likely to be affected by the consumption of GABA KLE1738 and the production of GABA The proportion of gut bacteria affected. Gut microbiota dysbiosis is often accompanied by decreasing vagal function [19]. The gut microbiota and their metabolites can impact the afferent pathways of the vagal nerve, influencing sleep/wake regulation [2].Muramyl peptides, the building blocks of peptidoglycan, and lipopolysaccharide (LPS), from Gram-negative bacterial cell wall, induce fatigue in the host, partly mediated by the TNF-α and LPS activation of vagal afferents 20]. Vagotomised mice, however, do not display inflammation associated with sleep deprivation [8]. The vagal afferent, compared to the vagal efferent, is much more well-studied. Thus, as part of the gut-brain axis, the efferent pathways of the vagus nerve may potentially take part in the diurnal oscillation and alterations induced by light in the gut microbiota.

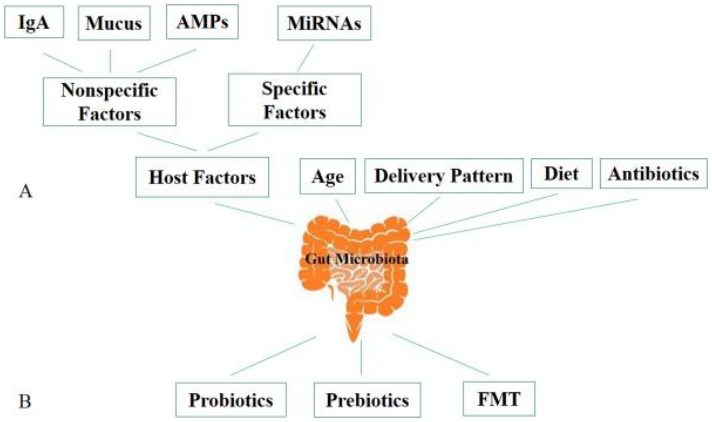

Fig. 3 Factors affecting gut microbiota [21].

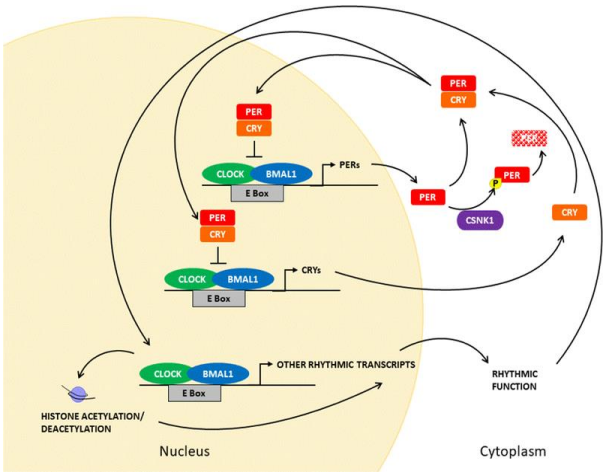

Fig. 4 Genetic regulation of circadian rhythm [22].

3. Research Proiect

3.1. Research Purpose

Though some studies are dedicated to the effect of disturbed circadian rhythm through shifts in the light/dark cycle on the gut microbiota [18, 23-25], the specific role of the vagus nerve in mediating these effects is undetermined. Therefore, the purpose of the research is to investigate the role of the vagus nerve in the impact of circadian rhythm on gut microbiota.

(1)To test the hypothesis that the diurnal rhythmicity of gut microbiota is shaped by the host circadian rhythm via the vagus nerve. The vagotomized mice and non-vagotomized mice will be put under a certain light/dark cycle. The composition and diversity of the gut microbiota from fecal samples will be measured at fixed time intervals. The proteins responsible for the circadian cycle will also be tested in peripheral blood at the same time.

(2)To test the hypothesis that, when performing fecal transplant from vagotomized mice to normal mice, diurnal rhythmicity of the gut microbiota can reoccur under the induction of host circadian rhythm via the vagus nerve. The fecal microbiota transplant (FMT) will be done from vagotomized mice to normal germ-free (GF) mice, expecting the recurrence of the rhythmicity of gut microbiota. For another group, the FMT will be operated from vagotomized mice to vagotomized GF mice as a control group. The composition and diversity of the gut microbiota from fecal samples will then be tested at fixed time intervals. The proteins responsible for the circadian cycle will also be tested in peripheral blood at the same time.

(3)To test the hypothesis that the composition of gut microbiota is shaped by the host circadian rhythm induced by the shifts in the light/dark cycle via the vagus nerve. The mice will be put under different light/dark cycle patterns, with both vagotomized mice and normal mice in each group. After

the testing of proteins responsible for the circadian cycle in peripheral blood at fixed time intervals in a significant amount of time to induce changes in host circadian rhythm, the composition and diversity of gut microbiota will be measured from fecal samples.

3.2. Materials and Methods

3.2.1. Animals

196 Specific pathogen-free (SPF) C57BL/6 mice and 28 GF C57BL/6 mice aged 10 weeks, both containing equal numbers of male and female mice, will be used for all the experiments. The mice will be maintained under standard laboratory conditions (12h light/12h dark, lights on at 7 AM, temperature 22℃, air humidity 40%-70%, free access to food and water).

3.2.2. Subdiaphragmatic vagotomy

Mice will be anesthetized with ketamine/xylazine. After an abdominal incision of the skin and abdominal wall, the liver and intestine will be carefully retracted, allowing access to the lower esophagus and stomach. The dorsal and ventral branches of the vagus nerve will be exposed under a surgical microscope. These will be dissected and all neural and connective tissue surrounding the esophagus below the diaphragm will be removed, transecting all small vagal branches. In sham-operated mice, vagal trunks will be similarly exposed but not cut. All mice will be monitored daily for 14 days after surgery for recovery.

3.2.3. DNA extraction and 16s rRNA sequencing

Total microbial DNA will be extracted from the fecal sample using a DNA extraction kit. The integrity of the DNA will be tested by agarose gel electrophoresis and the quantity of the DNA will be detected by UV spectrophotometer. The DNA will then be amplified by PCR with the primers specific to the V3 region of the 16s rRNA gene. To optimize the DNA outcome, magnetic microbeads will be applied to bind the DNA impurities. Fluorescent nucleotides will be added by DNA polymerase. Image array will be used to determine the nucleotides added.

3.2.4. Western blots

Western blots will be used to detect the level of translation of Clock, Bmal1, and Per1 proteins in peripheral blood samples. Gel electrophoresis will be applied to separate the different types of proteins in the samples by size. The separated proteins will be transferred to the surface of a membrane. After the primary antibody binding and second antibody binding, the levels of translation of the proteins will be visualized.

3.2.5. Stool sample collection

The fresh fecal samples of mice will be collected after defecation. The fecal samples will then be placed into sterilized screwcap microtubes immediately and stored at −80 °C until use.

3.2.6. Fecal transplant

Fecal samples from normal SPF C57BL/6 mice will be transplanted into the GF C57BL/6 mice, allowing the gut microbiota from normal SPF C57BL/6 mice to colonize the GI tract of the GF mice. After the dilution and homogenization of the stool samples, the samples will be filtrated and centrifuged. The fecal sample will then be suspended in cryoprotectant for later use. Once the donor stool is well prepared, the donor feces will be administrated to the GF mice via colonoscopy.

3.3. Research Design

3.3.1. Experiment 1

Control group: 28 SPF C57BL/6 mice, containing equal numbers of male and female mice, will be put under 12 hours light/12 hours dark (L12D12) condition for 3 days, starting from 7 AM.

Experimental group: 28 SPF C57BL/6 mice, containing equal numbers of male and female mice, will be vagotomized as previously described. The recovery period will be 14 days. 28 SPF C57BL/6 vagotomized mice will be put under 12 hours light/12 hours dark condition for 3 days, starting from 7 AM This will be the experimental group (as described in Figure 5).

Relative abundance of the Clock, Bmal1, and Per1 proteins will be measured from the peripheral blood every 6 hours. Fecal samples will be obtained every 6 hours. 16s rRNA sequencing will be applied to test the genotypes of bacteria in the stool samples. The diversity of the microbial community will be measured via evenness, species richness, dominance. Shannon Diversity Index, UniFrac and Jaccard Index will be used to compare the microbial communities.

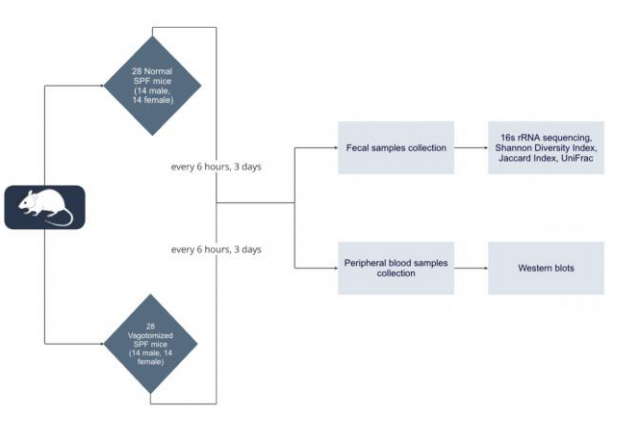

Fig. 5 Procedures of the experiment 1.

3.3.2. Experiment 2

After 3 days of data collection in the vagotomized mice, the fecal samples from 14 of the same SPF C57BL/6 vagotomized mice will be transplanted to 14 normal GF C57BL/6 mice respectively at the beginning of the new light/dark cycle as shown in Figure 6. At the same time, fecal samples from another 14 SPF C57BL/6 vagotomized mice same as in experiment 1 will be transplanted into 14 vagotomized GF C57BL/6 mice. The fecal samples will be transplanted between mice of the same sex. All the GF C57BL/6 mice will be put under 12 hours light/12 hours dark (L12/D12) condition for 7 days, starting from 7 AM.

Relative abundance of the Clock, Bmal1, and Per1 genes will be measured from the peripheral blood every 6 hours. Fecal samples will be obtained every 6 hours. 16s rRNA sequencing will be applied to test the genotypes of bacteria in the stool samples. The diversity of the microbial community will be measured via evenness, species richness, dominance. Shannon Diversity Index, UniFrac and Jaccard Index will be used to compare the microbial communities.

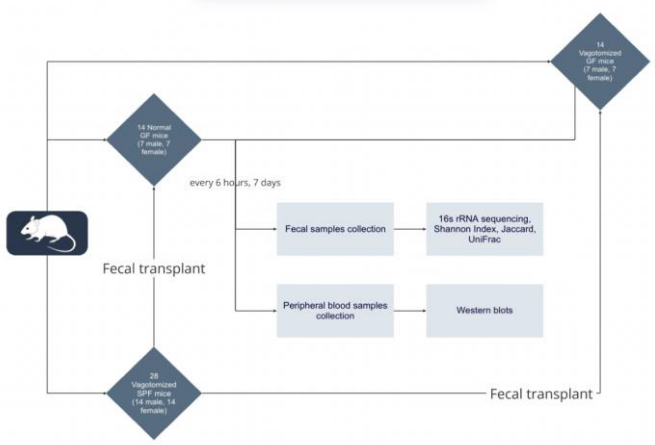

Fig. 6 Procedures of the experiment 2.

3.3.3. Experiment 3

As mentioned in Figure 7, 140 SPF C57BL/6 mice will be placed under 5 distinct light/dark cycles: L24/D0, L18/D6, L12/D12, L6/D18, L0/D24. 28 mice will be clustered into a group, consisting equal numbers of male and female. In each group, 7 male mice and 7 female mice will be vagotomized, 7 of each will be normal.

Relative abundance of the Clock, Bmal1, and Per1 genes will be measured from the peripheral blood every 6 hours for 14 days to validate the alteration of circadian rhythm induced by shifts in the light/dark cycle until the diurnal oscillation of the Clock, Bmal1, and Per1 genes become stable. Fecal samples will then be obtained. 16s rRNA sequencing will be applied to test the genotypes of bacteria in the stool samples. The diversity of the microbial community will be measured via evenness, species richness, dominance. Shannon Diversity Index, UniFrac and Jaccard Index will be used to compare the microbial communities.

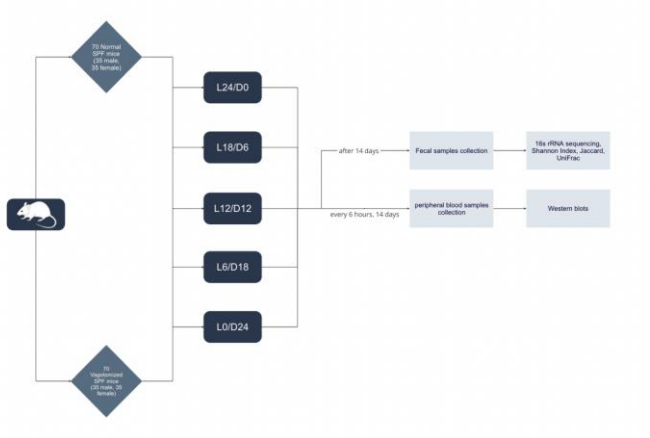

Fig. 7 Procedures of the experiment 3.

3.4. Statistical Analysis

All results were expressed by mean ± standard error of the mean (SEM). Statistical differences among the groups were analyzed using a one-way ANOVA using SPSS 25.0 software and GraphPad Prism 8 software. Results with P values of < 0.05 were considered statistically significant when testing for differences among groups.

3.5. Expected Result and Limitation

3.5.1. Experiment 1

It is expected that gut microbiota in the normal mice will show diurnal oscillation in accordance with the circadian rhythm, whereas the gut microbiota in vagotomized mice would be less obvious. It is important to notice that the rhythmicity of gut microbiota would not disappear completely, since the vagus nerve is not the only pathway between the gut and brain. The positive result in experiment 1 will address the role of the vagus nerve in maintaining the impact of circadian rhythm on the gut microbiota. The role of the vagus nerve in initiating this impact, however, would not be determined. And if the negative result is obtained, it would only disprove the vagus nerve's role yet prove other pathways.

3.5.2. Experiment 2

The positive result would be that the diurnal rhythmicity of the gut microbiota recurs when the FMT is performed from the vagotomized mice to normal germ-free mice. Nevertheless, when the FMT is done from vagotomized mice to vagotomized mice, the diurnal rhythmicity of the gut microbiota would continue to be less obvious compared to the normal SPF mice. This is caused by the nature of the gut microbiota as an ecosystem and the subtle differences between the hosts. If the positive result is presented, the role of the vagus nerve in initiating the impact of the circadian rhythm on the gut

microbiota will be proved, filling the gaps within experiment 1. The limitation of the research would be that the negative result would not be able to demonstrate any relationship between circadian rhythm and gut microbiota, neither proving nor disproving the hypothesis, supported by the fact that the gut microbiota is a dynamic ecosystem undergoing natural selection on its own level, indicating its proclivity to maintain the "status quo", regardless being part of the host physiology [14].

3.5.3. Experiment 3

The diversity of the gut microbiota in normal mice would be expected to be the highest in the L12/D12 cycle. Except for that, the normal mice in the group with a longer exposure to light would have less diverse microbiota based on the previous studies [8, 21]. If the hypothesis is correct, only the normal mice would display changes in the composition and diversity of the gut microbiota, whereas the gut microbiota in vagotomized mice would undergo significant but less drastic changes, since the vagus nerve is only one of the pathways in the GBA. If the negative result is gained, in which the gut microbiota of the vagotomized mice show hardly any difference, then the role of the vagus nerve in maintaining the impact of circadian rhythm on the gut microbiota will be disproved. One limitation would be that the time for the circadian rhythms to adjust to the altered light/dark cycle may not be long enough, causing the changes in gut microbiota to be induced only partially.

4. Conclusion

The limitation of the study project includes the inaccuracy caused by the stool sample collection. In this case, some microbes may attach to the epithelial of the intestine, making it difficult to include these microbiomes in the fecal samples. In addition, it is impossible to distinguish the microbial species from different areas of the GI tract, given the fact that the composition and diversity of the gut microbiota vary among all parts of the GI tract. Furthermore, the technology applied in this research to test the genotypes of the prokaryotes is the 16s rRNA sequencing, which limited the resulting microbial profile to only include bacteria and archaea. Last but not least, the microbial profile from the rodents would not be able to apply to humans, causing the result of similar research in humans to be different.

The relationship between gut microbiota, vagus nerve and circadian rhythm deserves further research and exploration. High-quality basic and randomized, double-blind and individualized clinical studies on the above issues will provide more new ideas for future research

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