Effects of Nanobubble Water on Cultivation of *Lactobacillus acidophilus* 1028 and Community of Gut Microbiota in Mice

January 2020

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A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Environmental Studies
(Doctoral Program in Sustainable Environmental Studies)

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Abstract

All higher animals are associated with a diverse microbial community that is composed of not only bacteria but also archaea, viruses, fungi, and protozoa. The microbes colonize virtually every surface of the human body. By far the most often colonized organ is the gastrointestinal tract (GIT). The gut microbes have a closed relationship with host health and disease. Many internal and external factors can affect the composition of gut microbiota of the host.

Water is the most important factor for life, and its changes in properties can affect the composition of gut microbiota. Nanobubble water (NBW) is a kind of water that has many nanobubbles (NBs) in it. NBW has been applied in many fields due to its unique properties. The most promising field is biology. Recently, many works reported the promotion effects of NBW on plants, animals and cells. According to our literature review, still, little information is available on the effects of NBW on microorganisms, especially on the gut microbes.

Therefore, it is meaningful to investigate the potential effects of NBW on host health and disease through the modulation of gut microbes. In this study, the properties and stability of NBW were firstly researched. Then, with *Lactobacillus acidophilus* (LA)1028 being used as the model strain, the *in vitro* effects of four kinds of NBW prepared with different gases on the strain growth performance were explored. At last, an *in vivo* assessment was conducted on the impacts of NBW on the mice's physical signs through modulation of gut microbes.

Firstly, the properties of NBs and NBW were investigated, then the effects of NBW on the growth and metabolism of strain LA 1028 were explored. Results indicate that nitrogen NBW (N_2-NBW) has the highest absolute value of zeta potential, NB density and water mobility ((-25.3 ± 5.43) mV, (5.73 ± 1.0) $\times 10^7$ particles/mL and (3200 ± 139.6) ms, respectively), while the lowest was detected in carbon dioxide NBW (CO_2-NBW) ((-6.96 ± 2.36) mV, (3.39 ± 1.73) $\times 10^7$ particles/mL and (2764.6 ± 40.1) ms, respectively). Moreover, the NBs could stably exist in the N_2-NBW and hydrogen NBW (H_2-NBW) during 30-days' storage. Except CO_2-NBW, all the other NBW showed a promotion effect on the growth of the strain at lag and logarithmic phases. Among them, the N_2-NBW has the best performance, achieving the highest increase ratio of 51.1% after 6 h cultivation. The kinetic models (Logistic and Gompertz) indicate that the culture with N_2-NBW has the shortest lag phase

and the maximum specific growth rate when compared with H_2-NBW and DW groups under the same cultivation conditions. Preliminary analysis of the mechanism suggest that these effects were related to the properties (zeta potential and density) of NB, which might affect the transport of substances. These results suggest that NBW has the potential for promoting the production efficiency of probiotics via fermentation.

Secondly, the effects of NBW on the gut microbiota of the host were investigated. The mice were fed with the standard diet (SD) and separately supplemented with N_2-NBW (SD-N_2 group), H_2-NBW (SD-H_2 group) and deionized water (SD-C group) for five weeks. At the end of the experiment, the composition of fecal microbiota was analyzed using the 16S rRNA gene sequencing. Results indicate that the species diversity in the SD-N_2 group was significantly increased when compared with that in the SD-C group, while the SD-H_2 group had no difference with the SD-C group. Compared with the SD-C group, the ratio of *Firmicutes* to *Bacteroidetes* in the SD-N_2 group was significantly increased. That's mainly due to the relative abundance of S24-7 was significantly reduced at the family level and the relative abundance of *Clostridium* and *Coprococcus* was significantly increased at the genus level. In the SD-H_2 group, the relative abundance of *Mucispirillum* and *Helicobacter* was significantly lower than that in the SD-C group. Overall, these results indicate that supplementation with NBW to mice can alter the composition of gut microbiota in mice.

Thirdly, the effects of NBW on the process of obesity in mice under high-fat diet (HFD) were researched. In the experimental groups, HFD fed mice were supplemented with N_2-NBW (HFD-N_2 group) or H_2-NBW (HFD-H_2 group), while the mice fed with SD (SD-C group) or HFD (HFD-C group) were supplemented with deionized water as control groups. After ten weeks, the concentrations of total cholesterol, alanine aminotransferase and lipopolysaccharide in the mice serum of the HFD-N_2 group were significantly lower than those in the HFD-C group. 16S rRNA gene sequencing revealed that supplementation with N_2-NBW to the HFD fed mice significantly inhibited the increase of *Firmicutes/Bacteroidetes* ratio, which was mainly due to the increased abundance of S24-7 and the decreased abundance of *Allobaculum* and *Staphylococcus*. The Spearman's correlation analysis indicates that the alteration in gut microbiota has a closed relationship with the changes of obesity-associated markers. These results demonstrate that

supplementation with N_2-NBW potentially alleviates the development of obesity in HFD fed mice through modulation of gut microbiota.

This study demonstrated the promotion effects of NBW on the growth of strain LA 1028 and the modulation effects on the composition of gut microbiota in the host for the first time. Providing a new perspective of NBW on the production of probiotic and the prevention of obesity, which can be supplied with a factual basis for the application of NBW in the food and medicine fields.

Keywords: Nanobubble; Nanobubble water; Zeta potential; Probiotic; Gut microbiota; Obesity.

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Abbreviations

Air-NBW	Air nanobubble water
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ALP	Alkaline phosphatase
ALB	Albumin
CO_2-NBW	Carbon dioxide nanobubble water
COG	Clusters of orthologous groups of proteins
DW	Deionized water
GIT	Gastrointestinal tract
HFD	High-fat diet
H_2-NBW	Hydrogen nanobubble water
HDL-C	High density lipoprotein cholesterol
LA 1028	*Lactobacillus acidophilus* 1028
LDL-C	Low density lipoprotein cholesterol
LPS	Lipopolysaccharide
NBs	Nanobubbles
NBW	Nanobubble water
N_2-NBW	Nitrogen nanobubble water
OTU	Operation taxonomic units
ROS	Reactive oxygen species
SD	Standard diet
SCFAs	Short chain fatty acids
TG	Triglyceride
TC	Total cholesterol
TP	Total protein

Chapter 1 Introduction

1.1 Gut microbes

1.1.1 General information of gut microbes

All higher animals are associated with a diverse microbial community that is composed mainly of bacteria but also includes archaea, viruses, fungi and protozoa. The total number of bacteria that colonize in the adult intestinal is about 10^{14}, which is outnumber the human cells in the body by 10-fold [1-3]. There is a large amount of microbial colonize in the surface of the body where is exposed to the external environment [4], especially on skin and in the genitourinary, gastrointestinal, and respiratory tracts [5-8]. Up to now, the gastrointestinal is supposed to be the most heavily colonized organ. This is mainly because the special structure of the intestine can provide ample room for the growth of microorganisms, and the gut as the main digestive organ of the host can provide adequate nutrition for the growth and reproduction of microorganisms [4].

The majority of these bacteria are anaerobic bacteria, which are about100-1000 times the total number of aerobic and facultative anaerobic bacteria [9-11]. The gut microbiota of human and rodents is dominated by the *Bacteroidetes* and the *Firmicutes*, the sum relative abundance of the two phyla is appropriately above 90%, followed by *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, and *Cyanobacteria* [12]. The genomes of these bacteria store a large amount of genetic information, containing more than 5 million genes, encoding about 100 times the number of genes by human genomes [13, 14]. The microbial density and diversity increase from the proximal to the distal gut and along the tissue-lumen axis [4]. Estimates of the number of bacterial species in the human gut are 500 to 1,000 species [15]. Nevertheless, a recent analysis suggested that the human gut microbiota is composed of more than 35,000 bacterial species [16].

1.1.2 The roles of gut microbes in host health and diseases

The importance of gut to human health has long been recognized, Hippocrates has said ‘death sits in the bowels’ and ‘bad digestion is the root of all evil’ in 400 B.C [17]. The development of gene sequencing science and technology promote the awareness of gut microbes play an important role in human health and disease.

1) **Health**

The neonatal gut does not mature in structure and function, and the composition of gut micro-organisms play a key role in its subsequent development [18, 19]. Recent research has examined several aspects of the host-microbiota interactions that promote functional and structural maturation of the gastrointestinal tract (GIT), including peristalsis and surface maturation [20], barrier fortifications and regenerative capacity [21-23].

Gut microbes can inhibit the pathogenic bacteria in the intestine by competing for colonization sites, consuming energy substances and producing antibacterial substances, playing a protective role on the host.[4] Besides, the study of metabonomic showed that compared with the contribution of gut microbiota to metabolic processes, our own contribution is remarkably small. Gut microbes exert their effects mainly through two processes [4]. One process is nutrient acquisition. Many bacterial species have implicated in uptake and deposition of dietary fiber to SCFAs and dietary lipids. These processes modulate the energy balance and prevent the accumulation of potentially toxic[24, 25]. Moreover, the nutrient metabolism by resident microbes is not only for the host's benefit but also for the microbes itself, to maintain its numbers and fitness [26]. Another process is xenobiotic processing. Due to the symbiotic relationship between human and gut microbes, to investigate the drugs, dietary and environmentally harmful substances on the human body must also evaluate these compounds' effects on the gut microbes to fully understand nutrition and metabolism [4].

The importance of gut microbes to host is not only reflected in the gut, but also in the regulation of extraintestinal processes and organ systems. Through the study of germ-free (GF) animal experiments, highlighting the contributions of the indigenous gut microbes to their development and maintenance. The studies have shown the important role of gut microbiota in the central and peripheral neural processes [27], the host response to stress [28] and regulation of mood and behavior [27, 29-33].

2) Diseases

The presence of intestinal microbes is critical to the health of the host, however, sometimes microbes present in the gut can also cause disease in the host. The most typical example is *Helicobacter pylori* (*H. pylori*) induced gastric cancer [34]. As we all know, the gastric acid can inhibit almost all of the bacteria. However, *H. pylori* can colonize in the stomach and induce gastritis which is the strongest singular risk factor for gastric carcinoma [35]. Besides, gut

microbes have a direct relationship with the development of disorders of GIT. IBD (inflammatory bowel disease), which includes Crohn's disease (CD) and ulcerative colitis (UC), has long been suspected that the abnormal interaction between intestinal microorganisms and hosts causes inflammation of intestinal mucosa [4].

Despite the impact on the intestinal disease, gut microbes also have effects on the GIT accessory organ disease [36-39]. Indole could be synthesized by some intestinal bacteria such as *Escherichia coli*. The produced indoles would be generated to indoxyl sulfate in the liver. It has been reported that indoxyl sulfate could induce the senescence and dysfunction of proximal tubular cells [40, 41]. Currently, diseases related to intestinal microorganisms focus on multifactorial diseases and diseases of remote organ systems, such as obesity [42], allergy [43, 44], diabetes [45, 46], familial Mediterranean fever [47, 48] and autism [49, 50], etc.

1.1.3 Factors affecting the composition of gut microbes

The gut microbiota is dynamically balanced during the whole life of the host. The structure of gut microbiota can be altered by many factors, which are mainly divided into two major categories: internal and external.

1) Internal factors

Antibacterial substance. The host could produce some molecules such as mucus, antimicrobial peptides (AMPs) and immunoglobulin A (IgA) to encourage the growth of microbes that can colonize in their intestinal and inhibit or remove pathogens from the body. These molecules play an important role in different parts of the intestinal. In the small intestinal, the most important molecules are antimicrobial peptides (AMPs). The AMPs were mainly produced by intestinal epithelial cells and paneth cells [51]. These proteins including defensins, cathelicidins, C-type lectins, ribonucleases, and S100 proteins, in which defensins and cathelicidins are the two major groups of human AMPs [52]. These proteins could rapidly kill or inactivate the pathogens. Besides, the dominant species in the gut microbiota have been demonstrated could resist the high concentration of AMPs to grow well in the intestinal. For example, the *Bacteroides* resists the AMPs through producing the dephosphorylate lipid A [53]. In the large intestinal, the mucous plays a vital role in the modulation of gut microbiota. The structure of mucus is two layers, in which the outer layer contact with microbes. It is decorated by O-glycans which act as the nutrient

source and binding site for the gut microbiota. The gut microbes are selected and shaped by the mucus and O-glycans [54]. In addition, the antibacterial lectins, IgA and miRNAs also have been certificated could involve in the immune system to modulate the gut microbiota [55, 56].

Aging. Before birth, the bacteria that exist in the amniotic fluid and placenta could be transferred to the forthcoming baby [57]. At birth, the mode of birth plays an important role in the composition of gut microbiota in newborns. The differences between normal vaginal delivery and cesarean on the gut microbiota of newborns have been investigated [58]. Results indicated the newborns via vaginally had bacterial communities the same as their mother's vaginal microbiota, while by cesarean the gut microbiota were similar to the microbes of the skin surface. In a study about the gut microbiota changes with age, 367 fecal samples of healthy Japanese subjects from the age of 0 to 104 years were analyzed by 16S rRNA gene sequencing method. Results indicated the composition of gut microbiota changes with age and there are some patterns and transition points [59].

Lifestyle. Normally, the composition of the gut microbiota of individuals is stable. However, the homeostasis of gut microbiota can be altered by the temporary or long-term change of the host lifestyle. During a 50-day trip from the American metropolitan to Southeast Asia, the subject had a two-fold increase in the *Bacteroidetes* to *Firmicutes* ratio, which changed to original status after returning [60]. The long-term proper physical exercise can bring positive effects on the gut microbiota of the host. The proper exercise could reduce the stool stay time to decrease the contact time between the pathogen and the mucus layer [61]. Besides, exercise could improve the diversity of gut microbiota, and increase the abundance of beneficial bacteria such as *Akkermansia muciniphila* [62]. These beneficial changes will keep the host health.

2) External factors

Diet. The foremost factor can influence the composition of gut microbiota is diet. Carbohydrates, protein and fat are the three main components of the diet. Approximately 12-18 g of protein and 40 g of carbohydrate reach the colon every day while most of the fats are absorbed in the small intestinal [63]. The carbohydrate reaches the colon mainly be classified into three categories: the resistant starches (RS), non-starch polysaccharides (NSP) and oligosaccharides. It has been demonstrated that the change of amount and/or type of these carbohydrates not only affected the composition of gut microbiota but also influenced the metabolic products of the gut

microbial. These alterations to host health were rapidly and profoundly [64, 65]. The main end products of protein fermentation are short-chain fatty acids (SCFAs), branched-chain fatty acids (BCFAs), ammonia, phenolic compounds, amines and hydrogen sulfide (H_2S). These products can influence the composition of gut microbiota and host health. For example, ammonia has been certificated related to tumor growth and the alteration of the morphology of intestinal tissues [63]. The amount of fat in the diet is crucial for the effects of fat on the composition of gut microbiota. A small amount of fat will be digested and adsorbed in the small intestinal, but the consumption of a high-fat diet will significantly change the composition of gut microbiota compared with the normal diet. For example, the ratio of *Firmicutes* to *Bacteroidetes* was significantly increased in the gut microbiota of mice under high-fat diet feeding has been demonstrated in many reports about obesity prevention [66, 67].

Environment pollutants. Environment pollutants not only cause serious damage to our living environment but also directly or indirectly affect human being's health. The gut microbiota is sensitive to the common environmental pollutants such as heavy metal, organic pollutants and pesticides [68-71]. In addition, some air pollutants that have been demonstrated could also alter the composition of gut microbiota to influence the host health. Normally, the air pollution was recognized as an important factor induce the disease of cardiovascular and respiratory. However, epidemiological studies have indicated that air pollution has a closed relationship with gastrointestinal diseases such as inflammatory bowel disease (IBD) and irritable bowel syndrome [72, 73]. The particulate matter is the main pollutant of air pollution. It has been reported that particulate matter could alter the composition and function of gut microbiota and increase gut permeability [74].

1.2 Nanobubbles (NBs)

1.2.1 Definition and classification

NBs have been applied in many fields due to the unique properties. With the continuous innovation of theoretical research and detection technology, the list of possible applications is tremendous. Since the proposal of the NBs contribute to the attraction between hydrophobic surfaces in water [75]. many researchers have carried on studies on the NBs. Until now, the researchers did not arrive at a consensus regarding the definition of NBs. Based on Agarwal et al [76]. definition, the diameters of NBs below 200 nm, the range between 200 nm and 10 μm

represent microbubbles. In addition, Wu et al. [77] defined the NBs or sub-micron bubbles as the sizes of bubbles are less than a micron and categorized the bubbles they generated with a size <500 nm as NBs. Moreover, recently, Parmar et al. [78] defined the NBs as bubbles having a diameter of less than a few hundreds of nanometer. Through summarizing literature, the maximum of bubbles sizes below a micron suggested reasonable to represent NBs. Although the size distribution has an important influence on the properties of the bubble, it is obviously insufficient to define the NBs only from the size distribution. A detailed description of the definition and classification of bubbles is given [79]. According to their generalization, NBs could be defined as being invisible compared with microbubbles and macro-bubbles, Brownian motion and very stable in liquids with residence time in the range of hours to months, diameter less than one micron.

According to the different forms, NBs are generally divided into two categories: surface NBs and bulk NBs.

Surface NBs are nanoscopic gaseous domains found at the solid/liquid interface [80]. They are spherical cap-shaped bubbles, typically a few tens of nanometers in height and a few hundred nanometers in width. It was first hypothesized in 1994 to explain the long-range hydrophobic attraction until 7 years later it was found experimentally through using atomic force microscopy (AFM) [81]. After that, the knowledge on surface NBs growing rapidly, but there was always a doubt about the surface NBs are nanoscopic gaseous bubbles until Zhang et al. showed in their work as the presence of rotational fine structure from carbon dioxide saturated water [82] rather than being simply bulk containment [83]. Besides, a number of detecting techniques, include quartz crystal microbalance (QCM), surface plasmon resonance (SPR) and rapid cryo-fixation [82, 84-86], have been confirmed the surface NBs occurred naturally.

Bulk NBs are nanoscopic spherical bubbles in dense suspension in bulk liquids [80]. Reports of bulk NBs preceded reports of surface NBs. The literature does contain a report from the 1960s that strongly inferred the role of bulk NBs. The existence of surface NBs is now firmly established following many different investigations from a number of groups. Far less common are reports of the existence of bulk NBs. The reasons may be less appropriate techniques. The earliest evidence of bulk NBs was reported by Johnson et al. that the bubbles produced by shear in seawater were observed to be stable for more than 22 h [87]. The most startling evidence was reported that small nitrogen, methane and argon bulk NBs of 50 nm could be stable for 2 weeks [88]. After that, through rapid cryogenic freezing technology to prepare the sample, imaged by transmission

electron microscopy (TEM) and scanning electron microscope (SEM) were reported to reveal the bulk NBs [89, 90]. However, sample freezing leads to unavoidable perturbation of the sample. Some researchers argued that the observed features were only defects induced by the freezing. Perhaps Kobayashi et al. [91] presented the most direct evidence that bulk NBs consist of gas through using an instrument called Archimedes to detect the mass density relative to the solvent of individual nanoparticles.

1.2.2 Stability and negative surface charge of NBs

The NBs exhibits several unique physical and mechanical characteristics, such as smaller or virtual disappearance of buoyancy, extremely high surface area/volume ratio, negative zeta potential, enhanced solubility of oxygen in water, generation of free radicals, and slow rising velocity [89, 92-96]. The most important are stability and negative surface charge.

1) Stability

The most fascinating feature of NBs is stability. The existence of NBs as a stable entity in solutions under atmospheric conditions has been subjected to controversies for a long time due to classical thermodynamics [76, 77]. In accordance with the classical thermodynamic theory, NBs cannot exist or be thermodynamically stable [97, 98].

But according to the studies, once NBs formed are highly persistent [96] and stable for hours [99], days [88, 94, 98] and even months [100] under the right conditions [95, 101]. The fundamental question always raised up by researchers “why NBs could exist for a long time?” Due to the difference between mobility and existence in the liquid, the researchers have different explains to clarify their stable mechanism. For surface NBs, there are mainly two possible explanations that have been put forward. One, by Ducker, is that a trapped layer of contaminant coats each bubble, which decreases the surface tension and provides a barrier to diffusion [102]. The other, by Brenner and Lohse, surface NBs exist in a dynamic equilibrium (in fact the influx is at the three-phase line and the outflux is through the spherical cap in the model of Brenner and Lohse) [103]. For details to review the article [104]. There are mainly three combined factors to keep the bulk NBs could be kinetically stable. Firstly, since NBs are small, the buoyancy force on bulk NBs is also small. They have no propensity to rise in solution. Secondly, repulsive hydrodynamic and electrostatic double-layer forces will act to stabilize NBs. Finally, a degree of metastability can be achieved when bubbles form from a supersaturated solution [80]. Different

researchers have come up with different mechanisms for different experimental conditions. Each view has its pros and cons. At present, there is still no definite mechanism convincing everyone to explain the reasons for the stable existence of NBs.

2) Negative surface charge

Zeta potential is a physical property exhibited by any particle in suspension and used for the measurement of the magnitude of the electrostatic repulsion or attraction between particles and bubbles [78, 93]. It can be used for the optimization of suspensions and emulsions and offers insights into the interaction mechanism between particles and bubbles [78]. The zeta potential of a bubble is an important factor in many engineering applications, as it determines the interaction of the bubble with other materials such as oil droplets and solid particles [105, 106]. Generally, both the NBs and MBs are negatively charged in the pH range of 2–12. At a neutral pH, the negativity of the zeta potential was shown in some researches, range from -50 mV to -20 mV [107-114]. Basically, two explanations are proposed on the mechanism of the negative zeta-potential of bubbles in relation to OH^- ions. These mechanisms that induce selective adsorption of OH^- ions are based on the hydration energies of H^+ and OH^- and the water molecular dipole orientation at the interface. The tendency of the H^+ ions to stay in the bulk water phase is high exposing the OH^- ions to the gas phase. The other justification is that an electric double layer is formed because of the structural orientation of water dipole at the interface with hydrogen pointing to the bulk water phase and oxygen atoms pointing towards the gas phase [79].

1.2.3 The application of NBs

1) Non-biological fields

Cleaning. The traditional cleaning method requires a large amount of cleaning agent, which not only increases the cost of production but also affects the environment. It has been demonstrated the NBs could be used to prevent the surface fouling and clean the already fouled surface [115]. The NBs could improve the cleaning efficiency on the surface of the ceramic membrane and stainless steel [116, 117]. Besides, the combination of NBs and the common surfactant sodium dodecyl sulfate (SDS) could effectively clean the hydrophilic protein-coated surface [118]. These results indicated the NBs could be used as an efficient, environment-friendly and low-cost cleaning agent.

Wastewater treatment. The unique interface properties such as stability, negative surface

charge, oxidative potential, large specific surface area and high density of NBs can improve some wastewater treatment process. The production of reactive oxygen species (ROS) at the collapse of NBs has been confirmed [119-121]. ROS can degrade organic pollutants and inactivate pathogens in the wastewater. These effects could improve the oxidation, disinfection and surface foulant mitigation during wastewater treatment. Besides, the gas in the bubble could determinate the ability of oxidation in the wastewater treatment. Ozone NBs have been certificated more effective in the oxidation of organic pollutants (e.g. trichloroethene) than air NBs [122]. In addition, NBs have a larger specific surface area than normal bubbles, which will lead to increased efficiency of gas distribution into the water, thus improving the efficiency of water treatment processes that rely on efficient gas transfer [123]. The ability of NBs to burst and dissolve in water rather than at the surface may also have the secondary benefit of transferring more hydrophobic pollutants from wastewater into the air as aerosols [124]. This will be beneficial to reduce the molecular weight of organic pollutants in water [125]. Furthermore, because the surface of the NBs is negatively charged, it can absorb positively charged particles in water. This will reduce the electrostatic repulsion of the NBs adsorbing positive particles and the NBs on the surface of the adsorbent, then promote the formation of "gas bridge" [126]. This effect has been proved to promote the rapidly coagulation and flocculation of particles in chemical mechanical planarization (CMP) wastewater [127, 128]. In summary, the unique properties of NBs make them a kind of chemically-free technology with broad application prospects in wastewater treatment.

2) Biological fields

Plants. NBs have been shown to promote some plant seeds germinate rate. Liu et al. dipped the seeds of barley and spinach in the nitrogen and air mixture gas nanobubble water (NBW) to demonstrate the biological activity of NBW through detecting the germinate rates [94, 119]. Results indicated the germinate rate in the NBW was improved 15-25% than that in the normal water. Ahmed et al. also proved the NBW exhibited a 6-25% higher germinate rate on the seeds of lettuce, carrot, fava bean, and tomato than that in the tap water, and the nitrogen NBW has the best performance [129]. At the same time, NBW significantly promoted the height, leaves and fresh weight of vegetables than normal water [130, 131].

Animals. Ebina et al. [130] cultured the sweetfish and rainbow trout in the air NBW for 3 weeks and 6 weeks, respectively. At the end of the experiment, the total weight of sweetfish

increased from 3.0 kg to 10.2 kg in the air NBW while that cultured in the normal water only increased from 3.0 kg to 6.4 kg. Besides, the weight of rainbow trout culture in the normal water was increased by 14.2% compared with that cultured in the normal water. In addition, they also detected the effects of free oral intake of oxygen NBW on the growth of male DBA1/J mice. Results suggested that the oxygen NBW was significantly increased the body weight (23.5 vs. 21.8 g; $p < 0.01$) and length (17.0 vs. 16.1 cm; $p < 0.001$) of mice than that intake of normal water [130].

Anticancer. Hypoxia is a key factor in the treatment of solid tumors. Some researchers have demonstrated that oxygen NBW can effectively improve hypoxia during treatment. Owen et al. [132] studied the treatment of oxygen NBW in a mouse xenograft tumor model for human pancreatic cancer via gavage. They found the mice have the oxygen NBW have a reduction of 75% and 25% in the transcriptional and translational expression of hypoxia-inducible-factor-1α (HIF1α) respectively. Besides, in the oxygen NBW group, the expression of vascular endothelial growth factor (VEGF) was decreased and the expression of arrest-defective protein 1 homolog A (ARD1A) was increased. The same effects were confirmed in the experiment of Mahjour et al. [133] The tumor size in the drinking oxygen NBW group was significantly decreased along with the treatment period when compared with the drinking normal water group.

Bacteria. Ozone is used for disinfection because of its strong oxidation and this effect can be improved by ozone NBW. The ozone NBW could act as a new antibacterial agent to periodontal therapy.[134] It has been demonstrated that after exposure the representative periodontopathogenic bacteria, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* to ozone NBW only 0.5 min, the numbers of colony dropped to the limitation of detection. Meanwhile, the viability of the oral tissue cell was not influenced by 24 h exposure. However, some studies in Japan have revealed that the disinfection effects could be decreased by the existed digestive enzymes. The same effects were also demonstrated in the detection of ozone NBW against *Helicobacter pylori* [135]. It has been reported that the pepsin in the stomach could suppress the disinfection of ozone NBW to *H. pylori* but the ozone NBW could sustain the disinfection activity at a wide pH range (2.0-7.4). In addition, the ozone NBW has no cytotoxicity on mammalian cells and tissue.

1.3 The objectives and contents of this study

NBW has been applied in many fields, in which the most promising is biology. Microbes are the most important part of the composition of biological. However, there are fewer reports about the effects of NBW on the growth and metabolism of microbes, especially on gut microbes. Taking into consideration the biological activities of NBW and the important role of gut microbes in the health of the host, it is meaningful to investigate the effects of NBW on gut microbes. In this study, the effects of NBW on gut microbes were investigated through the *in vitro* and *in vivo* test, the objectives and contents of this study are as below.

1.3.1 The objectives

The aims of this study are:

1) To study the effects of NBW on the growth and metabolism of model strain in the gastrointestinal by in vitro tests.
2) To verify NBW can affect the composition of gut microbiota in mice through *in vivo* test.
3) To evaluate the alleviation effects of NBW on obesity through altering the composition of gut microbiota.

1.3.2 The contents of this study

The structure of this study is shown in Fig. 1-1.

At first, the properties of four gas types (air, N_2, H_2 and CO_2) NBW were investigated including the NB density, size distribution, zeta potential, stability and water mobility. Thereafter, the medium with and without NBW addition was applied to explore its effects on the growth and metabolism of *Lactobacillus acidophilus* 1028 (LA1028) through evaluating the strain growth, lactic acid production and glucose consumption.

Then, supplementation with two kinds of NBW from different gases (nitrogen (N_2) and hydrogen (H_2)) in addition to deionized water to mice under standard diet for five weeks. The faecal microbiota was analysed using 16S rRNA gene sequencing at the end of the experiment.

At last, the mice were supplemented with NBW under high-fat diet (HFD) for ten weeks. At the end of the experiment, the obesity-associated markers including lipids and lipopolysaccharides in the serum, and short chain fatty acids (SCFAs) in the feces of mice were detected. Besides, the composition of fecal microbiota was analyzed using 16S rRNA gene sequencing.

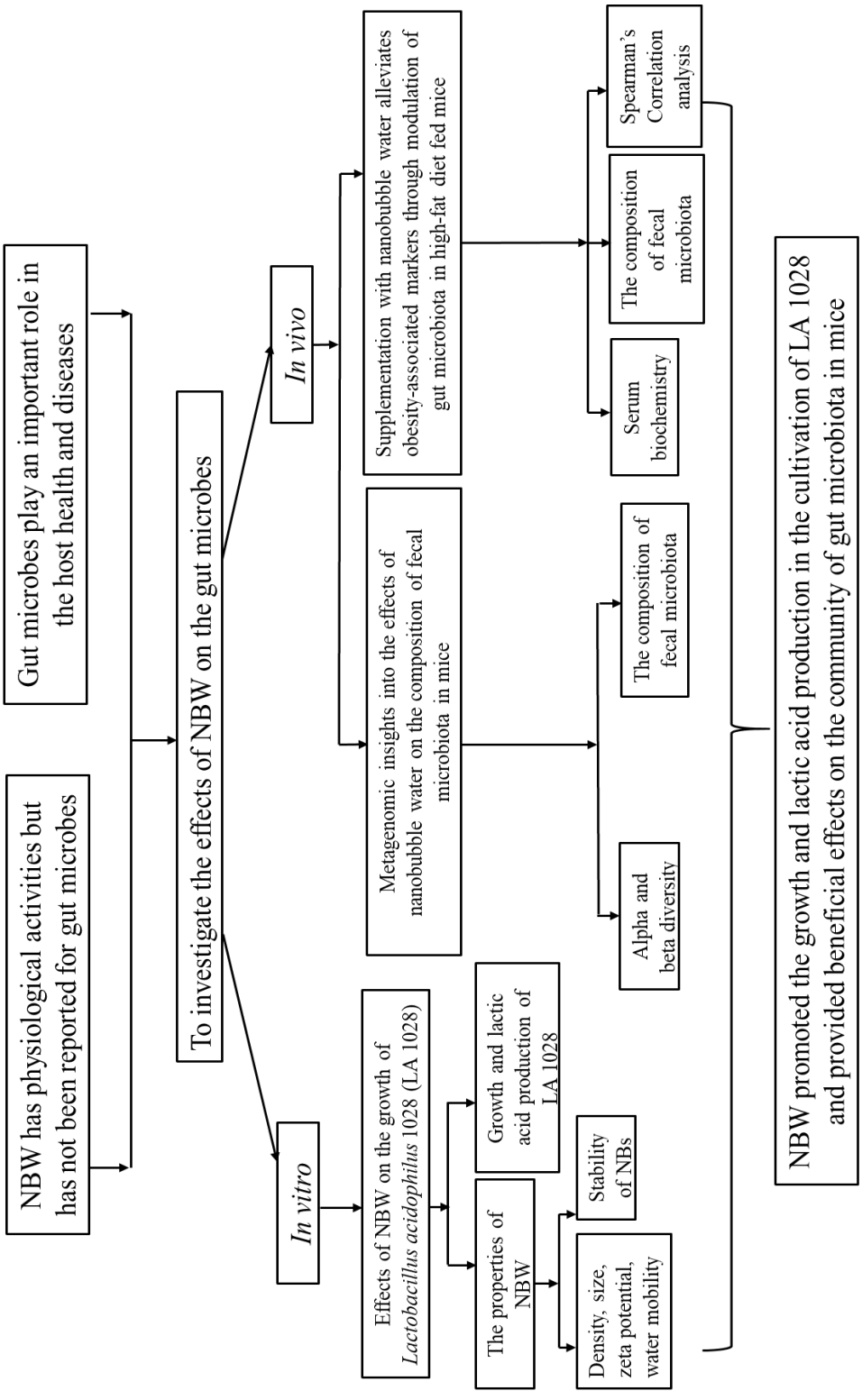

Figure 1-1 The structure of this study

Chapter 2 Effects of nanobubble water on the growth of *Lactobacillus acidophilus* 1028 and its lactic acid production

2.1 Introduction

Probiotics that exist in a variety of environments from the human gastrointestinal tract to the dairy products can confer a health benefit to the host when administered in adequate amounts [136-138]. With the increasing disposable income and lifestyle changes, the customer's growing awareness of the probiotic role in health maintenance contributes a lot to the amplification of the probiotics market. It has been valued at approximately USD 40.09 billion in 2017 and is expected to generate revenue of around USD 65.87 billion by the end of 2024 [139]. Thus, it is urgently to improve the yield of probiotic to satisfy the increasing demand of the market. From the ancient fermentation foods to the current industrial production of probiotic products, they are almost exclusively produced through fermentation technologies [140]. Microbial fermentation is a process in which the strain, culture medium, reactor and culture condition combine and interact with each other. The optimization strategies of probiotics cultivation usually focus on these four aspects to obtain higher biomass or metabolites. Many trials have been attempted in the selection and cultivation of acid/bile-tolerant strains to enhance their survival rate under the simulated gastrointestinal environment [141-143], increase the level of inoculum or co-culture to eliminate antagonistic effects of substances [144, 145]. Besides, there are numerous researches focusing on amelioration of the components of the medium to promote the growth of probiotics [146, 147] and reduce the cost [148, 149]. Lactic acid is a major end product of the metabolism of many probiotics, which would inhibit the strain growth with its concentration increase in the batch fermentation [150]. Fed-batch and continuous culture systems individually or combined with extractive fermentation approaches can be applied to partially overcome this kind of product inhibition problems to obtain high-cell-density cultivation [150, 151]. Researchers always paid more attention to the medium pH [152], temperature, dissolved oxygen (DO) and fermentation time during the optimization of operation condition parameters, in order to increase the survival rate of lactic acid bacteria and achieve the best economic benefits [153, 154].

It is worth noting that water, the major solvent for liquid fermentation is an important growth factor for microorganisms. Water is not only an integral part of biomolecules structured organization but also an essential factor for their functioning. It is inappropriate to discuss biological processes without assessing the role of water [155]. However, the effects of water on the growth of probiotics have rarely been addressed. Up to the present, previous reports mainly tried to alter water activity and examined the influence on the growth and metabolism of probiotics instead of improving the properties of water itself [156, 157].

Nanobubble water (NBW) is produced by mixing the gas with water through different kinds of nanobubble (NB) generators, resulting in the introduced gas existing in the form of suspended nanoscale bubbles in the water. The most promising discovery of NBW is its physiological activity and potential application in biological fields. Recently, many works reported that the application of some kinds of NBW increased the seed germination rate [119-121], promoted the growth of plants [129, 130], accelerated the mouse and shellfish growth [130], and inhibited tumour cell development individually or cooperated with other substances [132, 134, 158]. According to the literature review, the effect of NBW on microorganisms is the major aspect of its biological application, which still has rarely been reported. The available one is relating to the treatment with pathogenic bacteria [134, 135].

In summary, taking the physiological activities of NBW and the mounting demand of the probiotics market into consideration, it is meaningful to examine the effects of NBW on the growth of probiotics. In this study, the properties of four gas types (air, N_2, H_2 and CO_2) NBW were investigated including the NB density, size distribution, zeta potential, pH and DO. Thereafter, the medium with and without NBW addition was applied to explore its effects on the growth of *Lactobacillus acidophilus* 1028 (LA1028) through evaluating the strain growth, lactic acid production and glucose consumption. To the best of our knowledge, this is the first trial of NBW effects on the growth of probiotics, aiming to amplify the application of NBW in the field of food and medicine.

2.2 Materials and methods

2.2.1 Generation of NBW

1.5 L deionized water (DW) was first added into a transparent 2.0 L plastic beaker, which could be recycled through the micro- and nano-bubble generator (HACK FB11, JAPAN) with the introduction of air (in the laboratory room), CO_2, N_2, and H_2 (with a purity of 99.999%, Taiyo Nippon Sanso Co. Ltd., Japan) individually. And the produced NBW was labelled as Air-NBW, CO_2-NBW, N_2-NBW, and H_2-NBW, respectively. The generator blended the water and gas with high speed to produce NB as previously described [159]. To avoid possible impurities from the equipment, the generator was washed with water exchanged as described before the production of test NBW [160]. The outlet pressure was kept at 0.25±0.2 MPa to maintain the milky state of the water during a 20 min production duration. After being produced, the beaker with the NBW was statically placed on the table to let the milky water become transparent with visible most microbubbles disappeared while the NB still in the water (as shown in the data below).

2.2.2 NBW properties

The produced NBW was fully filled into a 6 mL glass screw jar and then the bubble size and density were measured with the nanoparticle tracking analysis method using the equipment named NanoSight (NanoSight-LM10, MALVERN, UK). Zeta potential was evaluated by the zeta potential analyser (NanoZS, MALVERN, UK). pH and dissolved oxygen (DO) of NBW were detected by the corresponding meters (METTLER TOLEDO FE20 and HACK HQ40d) respectively. The water mobility was determined as previously reported with some modification [94]. Briefly, the spin–spin relaxation times (T_2) were measured using a pulsed spectrometer (JNM-MU25A, JEOL, Japan) operated at 25 MHz and at a constant temperature of 20℃. The pulse sequence used for T_2 was the Carr–Purcell–Meiboom–Gill sequence, the volume of water was 0.9 mL. Each test was performed in triplicate.

2.2.3 Strain and medium

Lactobacillus acidophilus 1028 (LA1028) was obtained from the Japan Collection of Microorganisms (JCM). The composition of culture medium was MRS medium (Table 2-1).

2.2.4 Strain culture and experimental design

The stock culture of strain LA1028 kept frozen at -80℃ was activated. Then the bacteria were incubated on the agar plate at 37℃ for 48 h to get the single colony. One colony was chosen to inoculate in the MRS broth and static cultivation at 37℃ for 24 h. After that, 1 mL was inoculated into 100 mL fermentation MRS medium and cultivated at 37 ℃ for 24 h. The samples were collected in a certain time interval. Biomass was indicated by the optical density at 600 nm (OD_{600}) using the spectrometer (UV1800, Shimadzu, Japan).

Previous report shows that the NB density decreased in 0.02 MPa vacuum pump and water bath at 30℃ [94]. To avoid the high temperature and pressure effects on the density of NB in water during the autoclave process, the components of fermentation medium were sterilized separately. The NBW was filtered through the 0.45 μm mixed cellulose ester syringe filter, which was termed as the NB density of filtered NBW as 100%. The sterilized DW was added to the filtered NBW at different volume ratios (30%, 60% and 90%) to make the test water with different NB densities. The concentrated solutions of casein peptone, glucose, beef extract and yeast extract were separately prepared, then sterilized and added into the final medium to get the proper concentration, respectively. The inorganic salts in the MRS medium were mixed together to prepare the concentrated solution and filtered through 0.45 μm syringe filter before being added to the final medium. The growth of LA1028 in the MRS medium with addition of DW and different volume ratios of the four kinds of NBW were recorded.

Table 2-1 The composition of MRS medium.

Components	mass	unit
Casein peptone, tryptic digest	10.0	g
Beef extract	10.0	g
Yeast extract	5.0	g
Glucose	20.0	g
Tween 80	1.0	g
K_2HPO_4	1.0	g
Sodium acetate	5.0	g
Diammonium citrate	2.0	g
$MgSO_4 \cdot 7H_2O$	0.2	g
$MnSO_4 \cdot 5H_2O$	50.0	mg
Distilled water	1.0	L
Adjust the pH to 6.5. Sterilize by autoclaving at 121℃ for 15 min.		

The fermentation medium was the same as MRS medium except the glucose concentration was 10 g/L. Add 15 g agar in 1 L medium to get the MRS agar medium. Casein peptone, beef extract, and yeast extract were purchased from BD Biosciences, and all the other reagents were obtained from Wako Pure Chemical Industries, Ltd.

2.2.5 Lactic acid and glucose concentration

Lactic acid was quantified using the spectrometer method described elsewhere with some modifications.[161] A solution of 0.2% iron (III) chloride（$FeCl_3$•$6H_2O$）was firstly prepared. The calibration curve was obtained using lactic acid as standard reagent. 10 g/L of lactic acid solution was diluted using DW to 5 g/L, 2.5 g/L, 1.25 g/L, and 0.625 g/L, respectively, with DW as control. 100 μL solution was vortexed with 4 mL of 0.2% $FeCl_3$•$6H_2O$ for 10 s, and then statically stood for reaction for 15 min. The absorbance of the solution was measured at 390 nm by using UV-1800 (Shimadzu, Japan). The samples were measured as the calibration curve method. Glucose concentration was quantified with the dinitro salicylic acid (DNS) method.

2.2.6 Kinetic models

Predictive modelling is promising for the dramatically changing quantitative food microbiology. Models are used to describe the microorganism behaviour under different physical or chemical conditions such as temperature, pH and water activity [162, 163]. Among the various models applied to the fitting of growth curve, the Logistic and Gompertz model are widely used as the three-parameter model is simpler and easier to use. Their estimates have more degrees of freedom, and it is very important that all the three parameters can be given a biological meaning [163]. In this study, the following modified Logistic equation (Eq. (2-1)) and Gompertz equation (Eq. (2-2)) were employed:

$$y = \frac{A}{\left\{1 + exp\left[\frac{4\mu_m}{A}(\lambda - t) + 2\right]\right\}} \qquad (2-1)$$

$$\mathrm{y} = Aexp\left\{-exp\left[\frac{\mu_m \cdot e}{A}(\lambda - t) + 1\right]\right\} \qquad (2-2)$$

where y is the OD_{600} value at time t, A is the maximum OD_{600} value during the fermentation time, μ_m(h^{-1}) is the maximum specific growth rate, t (h) is the duration of the test, and λ (h) is the length of lag phase that is defined as the delayed period of the strain adapts to the new environment and starts to reproduce. The calculated correlation

coefficients (R^2) were compared to indicate which kinetic model is the best fitting to the experimental results.

2.2.7 Statistical analysis

All the fermentation experiments were performed in triplicate. The data were presented as mean ± SD and used for results and discussion. All statistical analyses were performed through one-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) test by using SPSS 19.0. Significant difference was assumed at $p < 0.05$, while $p < 0.01$ denoted highly significant difference.

2.3 Results and discussion

2.3.1 Nanobubble (NB) and nanobubble water (NBW) properties

The NB density, size and zeta potential, pH and DO of NBW were detected as shown in Table 2-2. No bubble was detected in DW. The NB density ranged between (1.45-6.73) $\times 10^7$ particles/mL, with their size distribution at 150-400 nm. The N_2-NBW has the highest NB density, which is significantly different from the CO_2-NBW and Air-NBW ($p < 0.05$), while no significant difference in NB density was found between N_2-NBW and H_2-NBW. These observations were probably brought about by the different solubility of the test gases in the water. As it is known, among the four kinds of gases, CO_2 has the highest solubility in water under the same condition, which is followed by air, H_2 and N_2. Therefore, the higher solubility the gas has, the more volume of gas would dissolve into the water under the same constant gas flowrate during the generation. In addition, bubble breakage and coalescence were also observed during the NB production by the generator, into which the low-pressure gas was injected with water to form NBW by the mechanical vibration and pressure reduction [164, 165]. The gas from the broken bubbles would continue to dissolve into the water if unsaturated or to mix with water through the generator to produce NB. In this context, less CO_2-NB was formed during the operation cycle compared with other three kinds of gases.

Except for the long-term stability of NB in the liquid, one of the most attractive properties of NB is its negatively charged surface. From Table 2-2, the DW zeta potential is nearly zero while all the four kinds of NBW have negative zeta potential that is in agreement with many reports [90, 129, 160, 164, 166]. The N_2-NBW has the highest

absolute value of zeta potential ((-25.3±5.43) mV), indicating its highly significant difference from other three types of NBW ($p < 0.01$). Air-NBW and H_2-NBW had similar zeta potentials, (-13.2±1.85) mV and (-16.2±6.35) mV, respectively, while the lowest absolute value of zeta potential, i.e. (-6.96±2.36) mV was detected in the CO_2-NBW. Several pervious works claimed that the pH, ion concentration or surfactants could effectively affect the zeta potential value of NBW [90, 129, 166]. As only different gas was used in the production of the four kinds of NBW and the equipment parameters kept constant during the production, their different zeta potential could be attributed to the different gas type applied. Soluble CO_2 in the water would form carbonic acid, which can partially dissociate to give H^+ resulting in significantly decreased pH in the water. Previous works on the effects of pH on zeta potential of NB indicate that the absolute value of NB zeta potential would reduce with the decrease of pH value [129, 159, 166-170]. This might be the main reason for the lowest zeta potential in the CO_2-NBW. Overall, the different properties of the four kinds of NBW are associated with the gas type, which is especially affected by the gas solubility.

Fig. 2-1 shows the water mobility of four kinds of NBW after generation. Compared with the water mobility of DW, the water mobility of NBW was significantly increased. My results are consistent with the previous reports [94]. The weak molecular interactions such as the hydrogen bonding, molecule mobility and steric effects can be detected by the proton-nuclear magnetic resonance [171]. The increase in T_2 represented the high mobility of water molecular, it would take more time to return the equilibrium state under the effects of magnetic field. These results suggested that the NBs in the water altered the properties of water.

Table 2-2 The NB density, size, zeta potential of NBW prepared with different gases

	DW	Air-NBW	N_2-NBW	H_2-NBW	CO_2-NBW
NB density (x 10^7 particle/mL)	n.d.	3.59±1.14 [b]	5.73±1.0 [a]	4.73±0.35 [ab]	3.39±1.73 [b]
NB size (nm)	n.d.	199.7±22.1 [b]	313.0±52.1 [a]	206.0±26.5 [b]	230.7±81.2 [ab]
Zeta potential (mV)	-0.985±2.28 [d]	-13.2±1.85 [b]	-25.3±5.43 [a]	-16.2±6.35 [b]	-6.96±2.36 [c]

Data are expressed as mean ± SD, and data with dissimilar letters differ, $p < 0.05$. n.d., not detectable

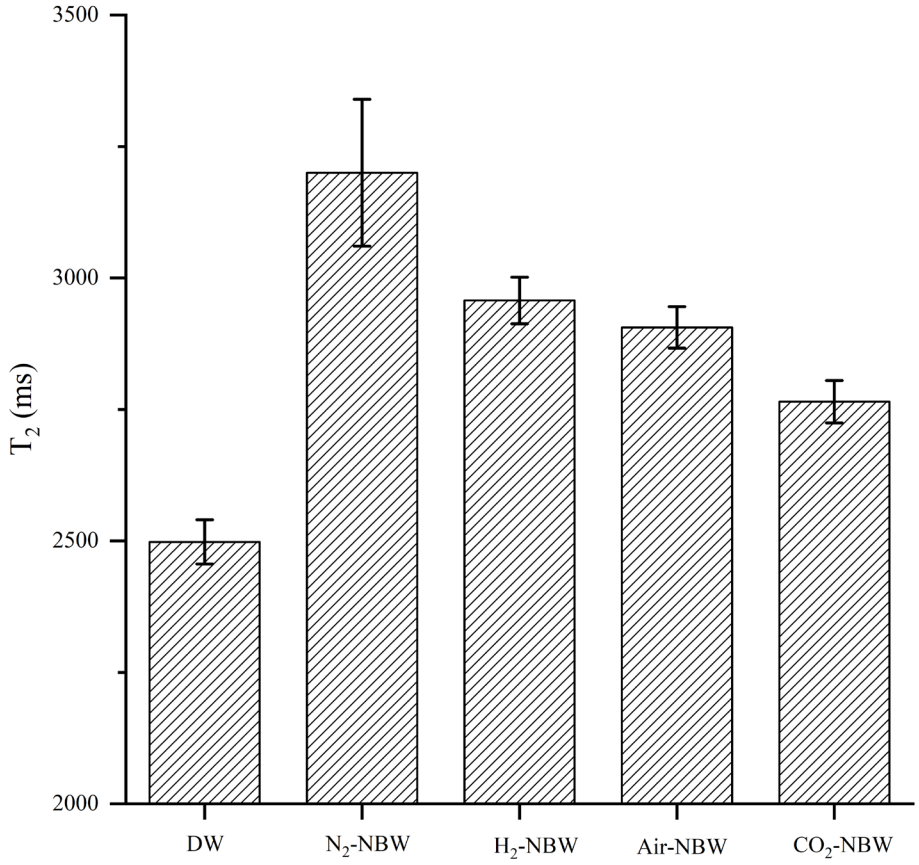

Figure 2-1 The water mobility of NBW.

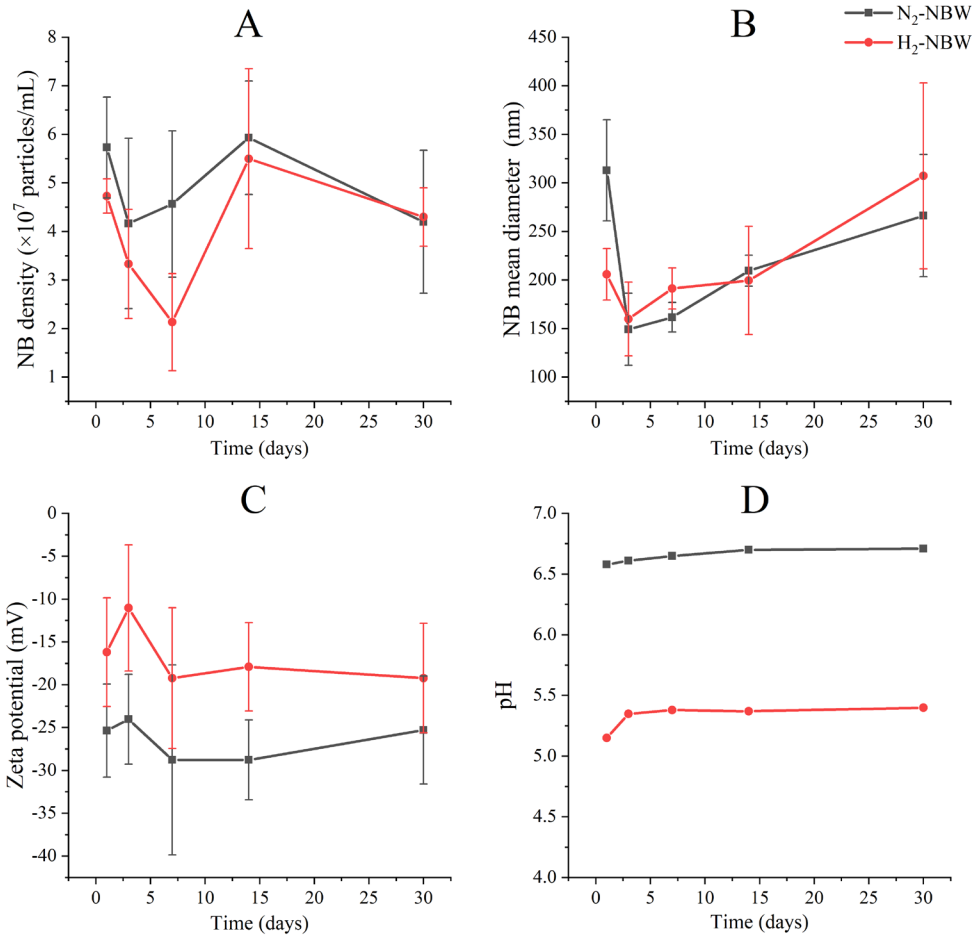

Figure 2-2 The stability of NBW during 30 days storage. The changes of density (A), diameter (B), zeta potential (C) of NB and pH of NBW (D).

Fig. 2-2 shows the change of NB density, size and zeta potential of N_2-NBW and H_2-NBW during 30 days storage at 25℃. There was no significant difference in the NB density of two kinds of NBW during 30 days. However, there was a slightly decrease in the concentration of two kinds of NBW in first week. Besides, the NB size of N_2-NBW was significantly decreased from day 1 to day 3 while the H_2-NBW has a slightly decrease. Since the generation of NBs were accompanied by the production of microbubbles, the simultaneous reduction of NB density and size in the first week might be related to the reduction of the number of microbubbles. The zeta potential of N_2-NBW and H_2-NBW was not altered during 30 days storage, around -25 mV and -15 mV, respectively. In addition, there was no change in the pH of two kinds of NBW during storage. These results indicated that the NBs could stable exist in the water for 30 days.

2.3.2 Effects of NBW type and volume percentage in medium on the growth of strain LA1028

Fig. 2-3 shows the growth of LA1028 by using different NBW at different volume percentages in the culture medium. The results show that addition of 30%, 60%, and 90% of Air-NBW could improve the growth of strain LA1028 during cultivation from 6 h to 18 h (Fig. 2-3A). According to the statistical analysis, significant difference ($p < 0.05$) in LA1028 growth was observed between the 90% Air-NBW group and the DW group after cultivation for 6 h, increasing by 21.5% in terms of OD_{600} value; while the addition of 30% and 60% Air-NBW showed little improvement effect at the same time. However, during the cultivation from 9 h to 15 h, the addition of Air-NBW had obvious improvement effect on LA1028 growth, increasing by 21.6%, 23.8% and 29.7% at 15 h under 30%, 60%, and 90% Air-NBW addition, respectively. Probably the growth of bacteria entered into their stationary phase after 21 h cultivation, little difference was noticed among the test groups.

Fig. 2-3B shows the effects of CO_2-NBW addition on the stain growth during 24 h cultivation. As it can be seen, almost the same growth trend was observed between the CO_2-NBW and Air-NBW tests. In addition, no significant difference was detected among the CO_2-NBW test groups during the whole growth phase.

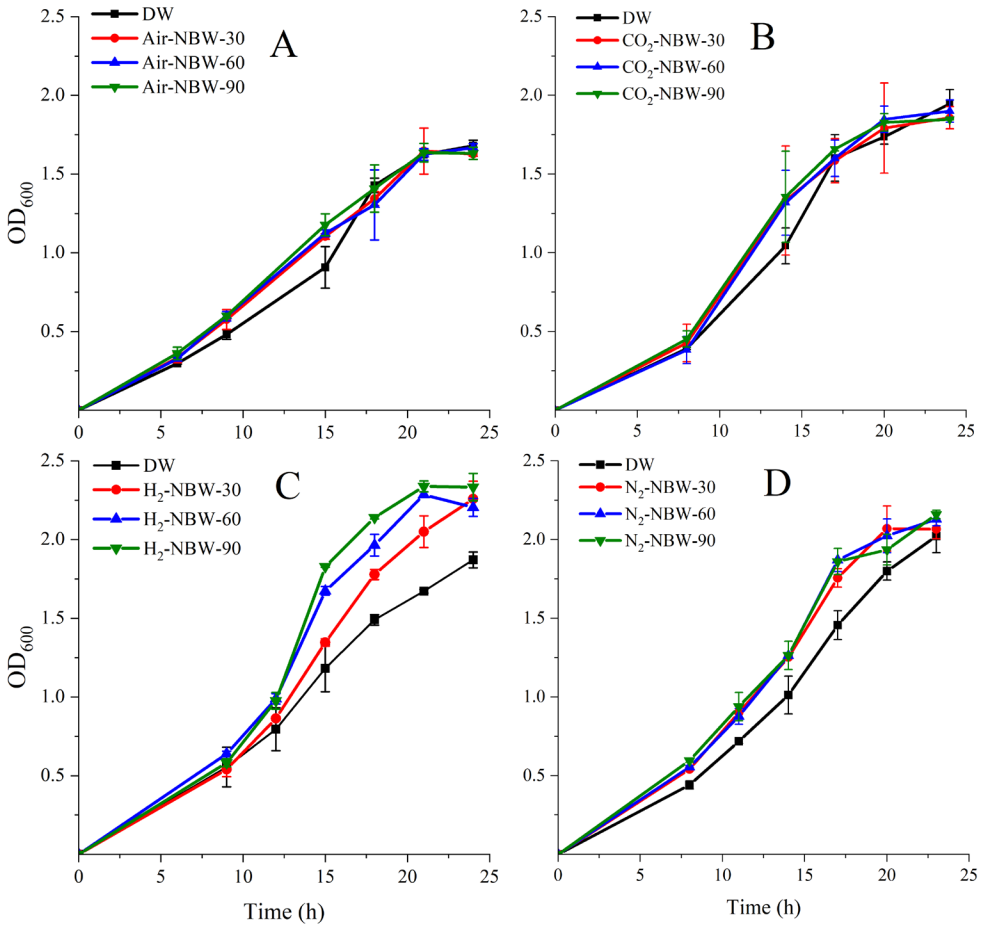

Figure 2-3 Growth of strain LA1028 under the addition of different gas NBW at different volume percentages. (A) Air-NBW, (B) CO_2-NBW, (C) H_2-NBW, and (D) N_2-NBW. Y in NBW-Y denotes the volume percentage of NBW in the test was Y%.

The effect of H_2-NBW addition on the strain growth is shown in Fig. 2-3C. During the initial 9 h cultivation, no significant difference was observed among the test groups; while the strain growth started to accelerate from 12 h, which continued to increase till 21 h. After cultivation for 21 h, no difference was noticed among the H_2-NBW groups, while their strain growth was significantly higher than the DW group. Obviously, the H_2-NBW showed better improvement effect on the strain growth, which followed a volume percentage dependent manner. The highest increase ratios under 30%, 60% and 90% of H_2-NBW addition were 22.6%, 41.6% and 54.7% in comparison to the DW group, respectively.

N_2-NBW also showed promotion effect on the strain growth as shown in Fig. 2-3D. The N_2-NBW group's growth showed highly significant difference ($p < 0.01$) from the DW group at the beginning of strain growth, which is different from those in Air-NBW and H_2-NBW addition tests. However, there was no significant difference between the N_2-NBW and DW group tests at stationary phase (after 20 h). Addition of 90% N_2-NBW exhibited a highly significant increase ($p < 0.01$) compared with the 30% N_2-NBW addition, which also obviously improved in comparison to the 60% N_2-NBW addition after cultivation for 8 h. After that, the N_2-NBW addition tests reflected almost similar stain growth till the end of cultivation. Compared with the DW group, the N_2-NBW test groups were averagely increased by 23.2%, 25.2% and 29.5% during the period from 8 h to 20 h cultivation when 30%, 60% and 90% of N_2-NBW were added into the culture medium, respectively.

The above results show that except CO_2-NBW, other three kinds of NBW promoted the growth of strain LA1028 in a volume percentage dependent manner. More specifically, this promotion effect occurred at the lag and logarithmic phases.

2.3.3 Comparison of effects on the growth and metabolism of strain LA1028 between N_2-NBW and H_2-NBW

Taking the effects of the four kinds of NBW on the growth of strain LA1028 into consideration, H_2-NBW and N_2-NBW were chosen to conduct this specific study, in which the effects of 90% NBW addition on growth, glucose consumption and lactic acid production of strain LA1028 were examined.

Fig.2-4 show the effects of H_2-NBW and N_2-NBW addition at 90% volume percentage on the growth of strain LA1028. The NBW group tests showed growth promotion compared with the DW group, in which N_2-NBW demonstrated a better performance than H_2-NBW.

A highly significant difference ($p < 0.01$) in the growth was discerned between the tests of N_2-NBW and DW addition from 6 h to 15 h. After that, although the OD_{600} value of N_2-NBW group tests were still higher than the DW group, their gap became smaller along with the fermentation progressed and finally to almost no difference at the stationary phase.

The N_2-NBW group tests achieved the highest increase ratio of 51.1% at 6 h compared with the DW group. Addition of H_2-NBW also promoted the growth of strain LA1028 at 6 h and 15 h ($p < 0.05$). After that, however, almost no difference was observed between the H_2-NBW and DW groups. The H_2-NBW group tests obtained the highest increase ratio of 34.2% at 6 h compared with the DW group.

As for the two NBW addition tests, the highest increase ratios were acquired at 6 h, with significantly increased growth of the strain in comparison to the DW group before 15 h, proving again that the effect of NBW addition on the growth of strain LA1028 mainly occurs at the lag and logarithmic phases. Besides, the bacteria at lag phase mainly adapt to the new environment and prepare enzymes or energy for logarithmic phase, which involves metal ion accumulation as previously described [172]. The existence of NB in the water would influence this preparation process as it possesses negative zeta potential. As Table 2-2 shows, there is no significant difference in NB density between N_2-NBW and H_2-NBW. Therefore, the significantly higher absolute value of zeta potential of N_2-NBW than H_2-NBW might be the major reason for their different promotion effects under the same test condition.

Fig. 2-5A demonstrates the changes of lactic acid concentration during the cultivation. It is observed that NBW addition groups have higher lactic acid production than the DW group during the cultivation, and a significant improvement ($p < 0.05$) in strain growth was observed between N_2-NBW and DW groups from 6 h to 21 h (except at 15 h); while H_2-NBW group showed the promotion effect on strain growth only at 6 h. No significant difference in the lactic acid concentration was noticed among the test groups after fermentation for 21 h. As seen, the promotion effects of NBW on the lactic acid production also mainly occurred at the lag and logarithmic phases. From the observation and the strain growth curve, the lag phase period was less than 6 h. The lactic acid concentrations in the DW group at 6 h were significantly lower ($p < 0.05$) than those in the N_2-NBW and H_2-NBW groups (0.62 ± 0.12 g/L, 1.58 ± 0.54 g/L and 1.66 ± 0.56 g/L, respectively).

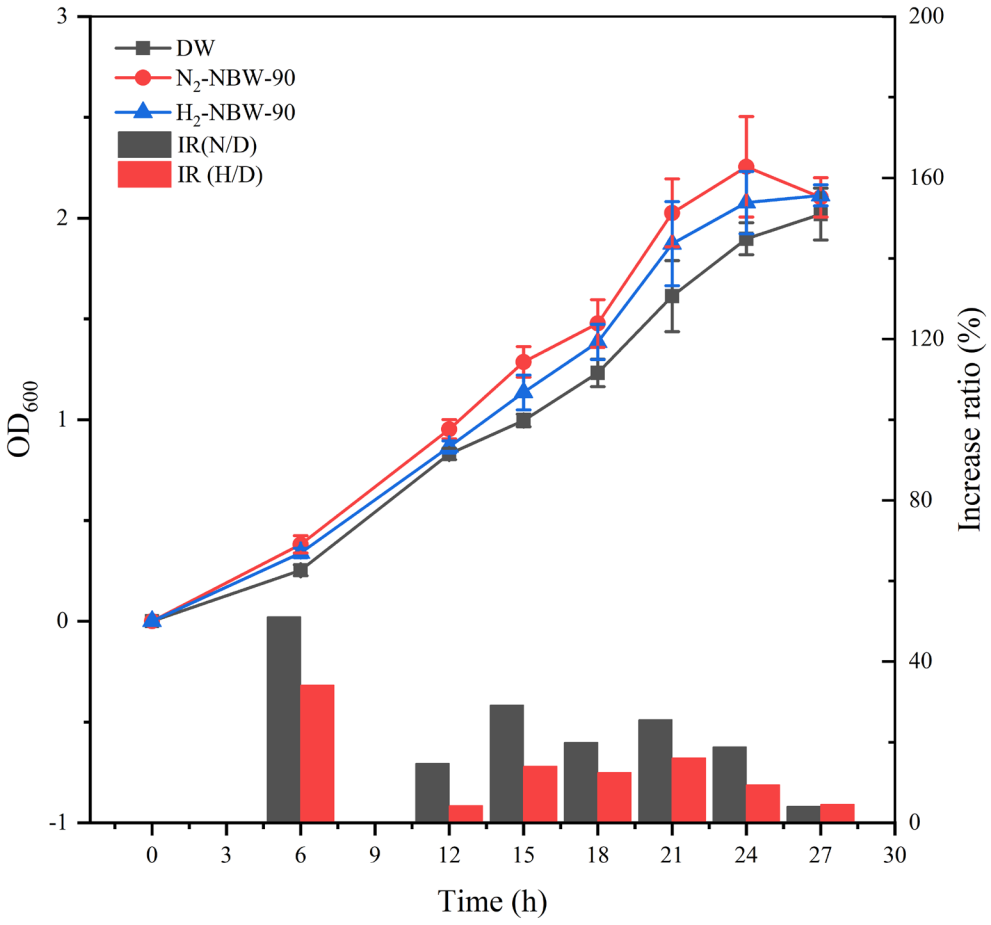

Figure 2-4 The growth curve and increase ration of strain LA1028 under the N_2-NBW and H_2-NBW (90% volume percentage). IR: increase ratio; H/D, H_2-NBW/DW test; N/D, N_2-NBW/DW test.

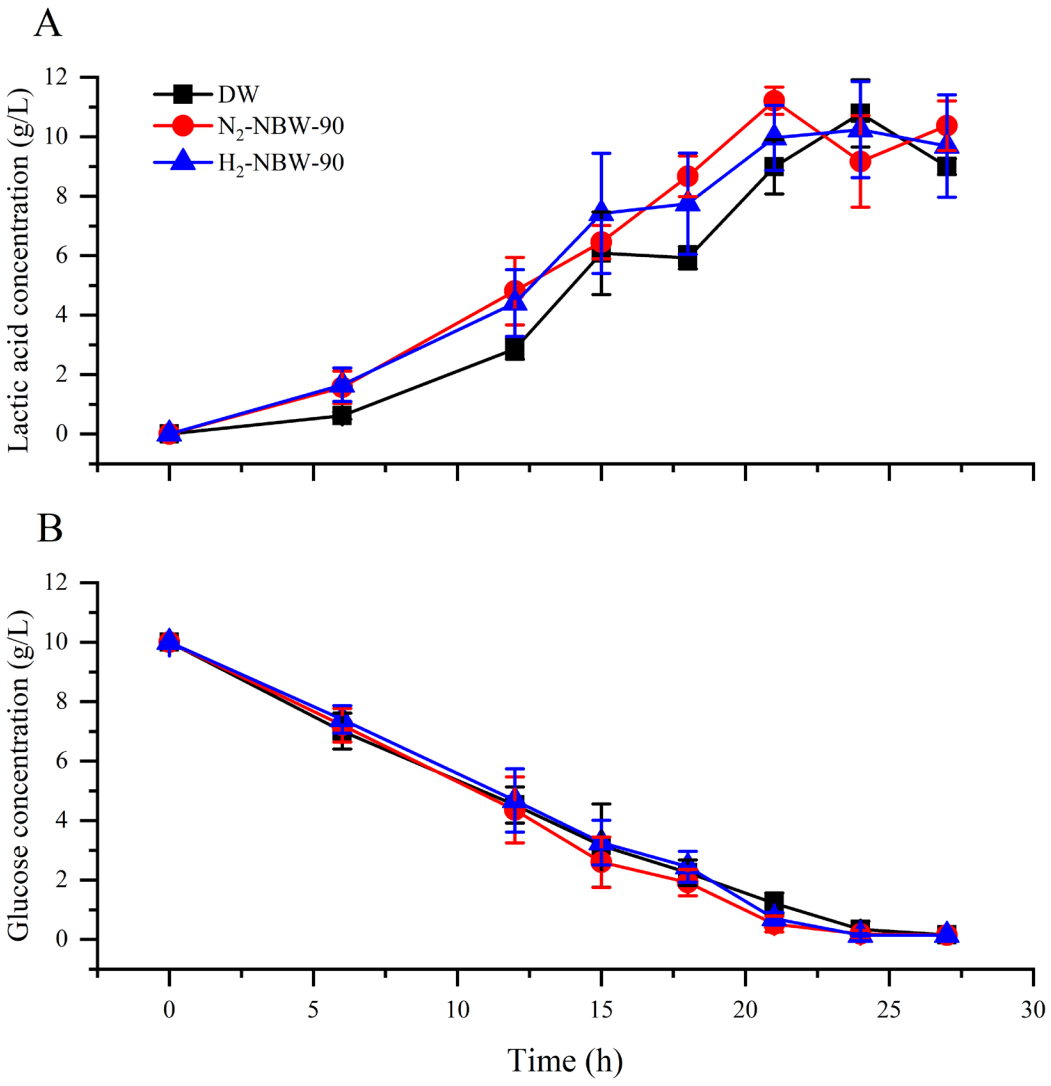

Figure 2-5 The concentration of lactic acid (A) and glucose (B) during cultivation.

Theoretically, one mole of glucose can be converted into two moles of lactic acid in homolactic fermentation, and the energy produced during the process can be used for bacterial growth and metabolism. Since the molar mass of glucose is twice that of lactic acid, the concentration of the glucose consumed should be equal to the concentration of lactic acid produced. However, no significant difference was detected in glucose concentration among the test groups during the lag phase and logarithmic phase as shown in Fig.2-5B. The carbon source materials used by the strain during the lag phase are mainly converted to the necessary enzymes and energy to supply for the physiological and regulatory processes responsible for adaptation to the new environment [172]. These results indicate that with the accumulation of lactic acid at the lag phase, the NBW groups probably produce more energy than the DW group so that the strain growth and reproduction are accelerated. That is, the NBW group could adapt to the new environment faster than the DW group.

2.3.4 Kinetic analysis of the growth of strain LA1028 in N_2-NBW and H_2-NBW

The relevant parameters obtained by fitting the modified Logistic and Gompertz models are shown in Table 2-3. The values of R^2 calculated from the Logistic and Gompertz models are both higher than 0.96 and almost equal to each other, indicating that the experimental data well fitted to these two models under the test conditions. From the results of the models, no significant difference has been found in the maximum OD_{600} value (A); while the duration of lag phase (λ) of N_2-NBW group is highly significant shorter than other groups ($p < 0.01$). In addition, the maximum specific growth rate (μ_m) of N_2-NBW group is significantly higher than the DW group ($p < 0.05$), while no significant difference is found between N_2-NBW and H_2-NBW groups. The duration of lag phase (λ) and the maximum specific growth rate (μ_m) can respectively represent the status of strain growth during the lag phase and logarithmic phase. Therefore, the predicted values from the modified Logistic and Gompertz models also demonstrate that NBW addition could mainly affect the lag phase and logarithmic phase growth of LA1028, and N_2-NBW may have the best promotion effect on its growth.

Table 2-3 Parameters estimated from the modified Logistic and Gompertz models based on OD_{600} values from the LA1028 fermentation with and without NBW addition.

Condition	DW	N_2-NBW	H_2-NBW
Logistics model			
A	2.33±0.16 [a]	2.38±0.82 [a]	2.40±0.12 [a]
λ(h)	5.72±0.16 [a]	5.10±0.16 [b]	5.60±0.11 [a]
μ_m(h^{-1})	0.11±0.01 [b]	0.13±0.01 [a]	0.12±0.14 [ab]
R^2	0.9807	0.9654	0.9810
Gompertz model			
A	2.90±0.30 [a]	2.72±0.19 [a]	2.90±0.38 [a]
λ(h)	4.55±0.10 [a]	3.90±0.14 [b]	4.35±0.14 [a]
μ_m(h^{-1})	0.10±0.06 [b]	0.12±0.01 [a]	0.11±0.01 [ab]
R^2	0.9859	0.9638	0.9809

Data in the table are expressed as mean ± SD, and different letters indicate significant difference at 5% level ($p < 0.05$).

2.3.5 Analysis on the mechanisms

The promotion effects of NBW were mainly observed at lag and logarithmic phases of the strain growth, which was demonstrated by the strain growth, lactic acid production and kinetic analysis. However, what's the mechanism involved in this phenomenon?

The process of strain cultivation is very complex. Currently there is no effective and stable detection methods to determine the change of properties and quantities of NB during strain cultivation because the high conductivity of medium would interfere with the measurements [173]. Besides, many particles smaller than NB are also co-existing in the medium. In this study, a preliminary analysis was conducted based on the observed phenomena and theories.

At first, can NB be stably present in the fermentation system? Although the composition of the MRS medium is complex, it can be mainly classified into inorganic salts/acids and organic substances. The colloidal stability of NB in the presence of pH, inorganic salts and normal organic matters has been thoroughly studied and reported. It has been clarified that the NBs have high stability under high ionic strength and surfactant concentration condition [129]. Compared with the ionic strength in the experiment (300 mM NaCl), MRS medium has a lower ionic strength. The ionic strength equation (Eq. (2-3)) as described elsewhere [174].

$$I = \frac{1}{2}\sum_{i=1}^{n} c_i\, z_i^2 \qquad (2-3)$$

where I (mM) is the solution ionic strength, C_i (mM) is the molar concentration of ion, Z_i the charge of the ion. The MRS medium ionic strength value was calculated equal to 177.6 mM, between the ionic strength of the 50 mM $CaCl_2$ and 300 mM NaCl in the experiment [129]. Therefore, theoretically the salts in the MRS medium have less effects on the NB stability. Besides, the organic matters adsorption on the surface of NB could act as a "skin" that enhances its stability as reported elsewhere [175]. Moreover, most of the organic components that have charge in the MRS medium mainly belong to proteins, polypeptides and amino acids which normally possess negative charge because of their isoelectric point [176] lower than the initial pH 6.5 in the MRS medium. Thus, theoretically the NB could stably present in the MRS medium at the beginning of the cultivation.

Then, Why the promotion effects of NB on the growth of strain LA 1028 were observed at the lag and logarithmic phases? The possible mechanisms are illustrated in Fig. 2-6.

NB could act as carriers to accelerate the metal ions adsorption onto activated carbon as described elsewhere [177]. In their study the tested NB had a negative zeta potential of (-8.6±1) mV. In my study, all the 4 kinds of NBW exhibited negative zeta potential and had the higher absolute value than the reported value (except CO_2-NBW) as shown in Table 2-2. From this viewpoint, the NB in water can attract the positively charged substances such as metal ions in the medium. I propose that the existing NB in the water could offer a new way for the substances to transport into bacteria in addition to the traditional diffusion. It has been described that most Gram-negative and Gram-positive bacterial cell surface possesses net negative electrostatic charge [178]. This means that there are repulsive forces between the NB and LA 1028 cell surface. However, once positively charged particles or metal ions are adsorbed on the surface of the NB, the absolute value zeta potential of the NB will decrease, possibly breaking the repulsion equilibrium with the strain LA 1028 to transfer the adsorbed substances onto the bacterial surface for utilization. After these positively charged substances are detached from the surface of the NB, the repulsive force between the surface of the strain LA 1028 and NB will repel NB into the solution. The NB works just as a "bus" as described elsewhere [177]. Therefore, when the pH is not decreasing to affect the NB to stably present in the solution, the processes of adsorption and release will recirculate and accelerate the strain growth and reproduction. However, the NB stability will be reduced with the accumulation of lactic acid, because the H^+ ions could significantly decrease the absolute value of zeta potential that will induce the NB coaggregation and breakage. Thus, the decrease in NB density has limited effects on the stationary phase.

2.4 Summary

In this study, the effects of four kinds of NBW on the growth of strain LA1028 were investigated. Results indicate that the gas itself determinates the properties of NBW, which is especially affected by the gas solubility, the NBs could stable exist in the water for 30 days and increase the water mobility of NBW. Besides, the promotion effects of NBW were mainly observed at lag and logarithmic phases of the strain growth, which were manifested by the strain growth, lactic acid production and kinetic analysis. Then, the mechanisms

were preliminarily analysed, suggesting that the NBW properties including NB zeta potential, density are probably responsible for the promotion effects on the growth of strain LA1028. The actual mechanisms should be further investigated. Results from this study indicate that NBW has the potential for promoting the productivity of probiotics in the cultivation, which provides a new prospective for the application of NBW.

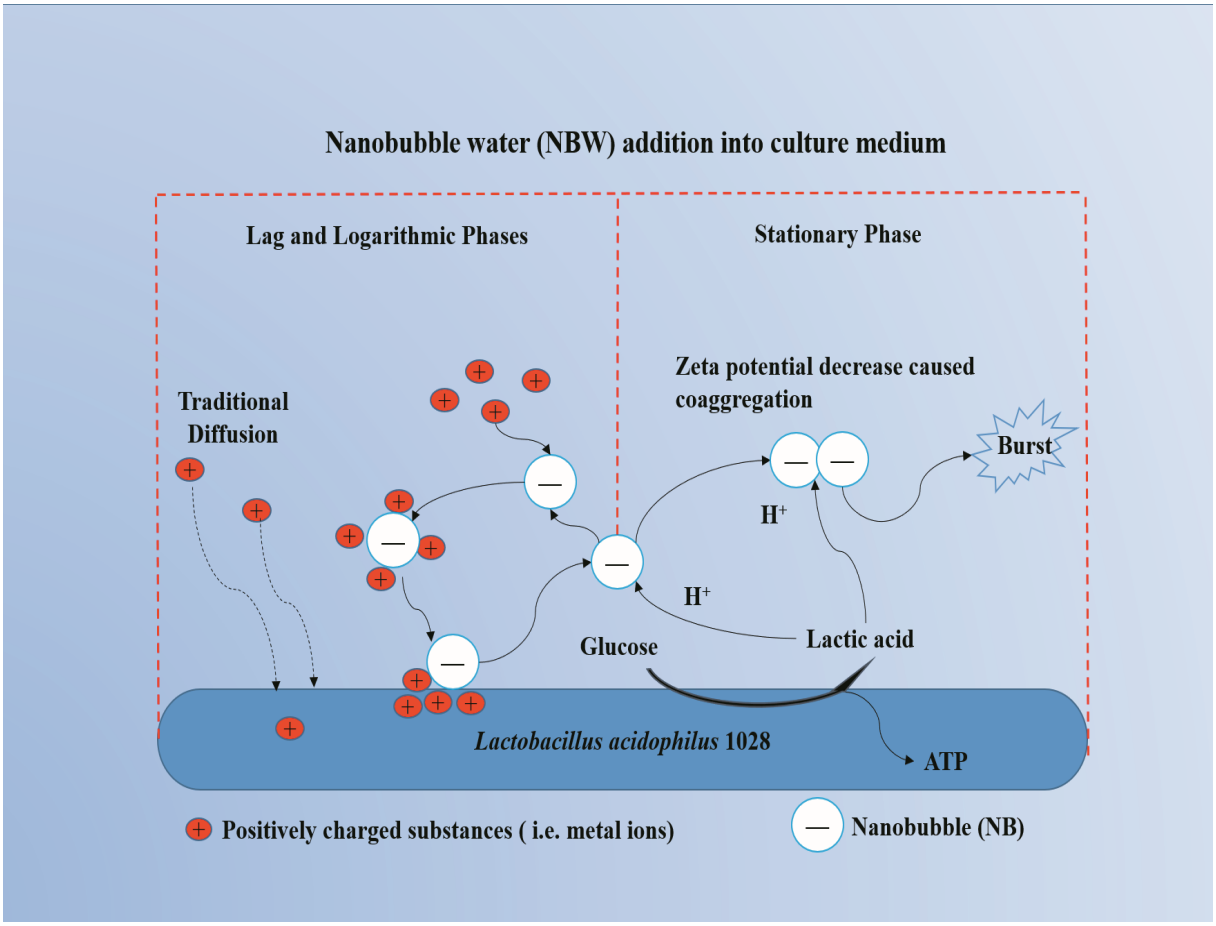

Figure 2-6 The possible mechanisms for the promotion effects of NBW on the growth of strain LA 1028.

Chapter 3 Metagenomic insights into the effects of nanobubble water on the composition of fecal microbiota in mice

3.1 Introduction

The surface area of human gastrointestinal around 250-400 m^2, which is the largest interface in the human body that interacts with the external environment [179]. Approximately 10^{14} microorganisms are colonized in the adult gastrointestinal tract, the number of which is 10 times the total number of human cells. Normally, the adult gut microbiota weighs more than 1 kg and encodes the number of genes more than 100 times that are encoded by the human genome [180, 181]. As early as ancient times, human beings had begun to realize the important role of the gut for health. In the theories of traditional Chinese medicines (TCM) and Chinese native religion, the site of intestinal is an important part of the human body and named "*Dantian*", which is considered to be closely related to human life activities and is referred as "the progenitor of life". Many traditional prescriptions of TCM recently have been proved to treat diseases through the modulation of gut microbiota in human body.[182-184] Hippocrates once said "death sits in the bowels" and "bad digestion is the root of all evil" in 400 B.C [17]. With the rapid development of DNA sequence technology and bioinformatics technology, the role of gut microbiota in maintenance the host health and in the development of diseases is gradually clarity.

Many factors can alter the composition of gut microbiota to influence the well-being of the host. Water is implicated in all aspects of life activities can also affect the gut microbiota of the host. There has been reported that the pH change of drinking water affected the incidence of diabetes and the composition of gut microbiota in diabetes-prone non-obese diabetic mice. The acidic water drinking decreased the relative abundance of Firmicutes and increased the relative abundance of Actinobacteria and Proteobacteria [185]. Besides, changed the acidic water to neutral water decreased the abundance of genus *Bacteroides* and some species of *Prevotella*, and increased the abundance of *Parabacteroides* [186]. Moreover, the temperature of the water was also reported to alter the composition of gut microbiota in the host. Supplementation warm water with the early postweaning rabbits during winter could significantly increase the body weight and feed conversion rate than that in the rabbits drinking the cold water. The relative abundance of *Copprococcus* in warm water drinking group was markedly improved at day 70 and had a

significantly low risk of diarrhea during 71-82 days [187]. The results of 16S rRNA gene sequencing showed that gut microbiota in rainbow trout was influenced by temperature. The fishes were fed in cold water (11℃) had higher bacterial diversity and abundance of lactic acid bacteria than that in warm water (18℃) [188]. These results indicated that the gut microbiota of the host could be affected by the alteration in the properties of water.

Nanobubbles (NBs) are the gaseous bubbles with the diameter below 1 μm. In recent years, NBs have attracted the attention of many researchers due to their unique properties (e.g. long-term stability in aqueous and negative surface charges) and showed great potential application in many fields such as environmental protection, industrial products cleaning and agricultural [115, 130, 131, 189]. Compared with the ultrapure water, the stable NBs that exist in water will endow the water with the properties as colloid [129, 190]. Besides, the water mobility which was measured by the relaxation time of water molecules in the low-field nuclear magnetic resonance (NMR) had been confirmed to increase in the nanobubble water (NBW) [94]. These results indicated that NBs exist in water changed the properties of water. Thus, I hypothesis that supplementation with NBW to the host can affect the composition of gut microbiota in the host.

In this study, supplementation with nitrogen (N_2) NBW, hydrogen (H_2) NBW and deionized water (DW) to mice with a standard diet for five weeks. At the end of the experiment, the fecal microbiota was analyzed using 16S rRNA gene sequencing. This is the first study to detect the effects of NBW on the gut microbiota of mice. This study expects to further explore the biological activities of NBW and supply the factual basis for developing the application of NBW in the food and medicine field.

3.2 Materials and methods

3.2.1 Water and diet

The production of NBW was referred to the subsection 2.2.1. The NBW was sterilized by the 0.45 μm syringe filter and autoclave (121℃, 15 min) was used to sterilize the deionized water. The sterilized water was placed under aseptic condition to room temperature before supply to mice. The standard diet (10% fat, 20% protein, and 70% carbohydrate, D 12450B) was purchased from Research Diets (New Brunswick, NJ). Water and diet were given *ad libitum*. The water and diet intake, body weight was weighted every three days.

3.2.2 Animals and experiment design

The six-week-old male BALB/c mice (n=30) were purchased from Jiangsu Laboratory Animal Center, Jiangsu, China. The mice were housed in stainless steel cage (n=5) with controlled environmental conditions (temperature 25℃, relative humidity 60%, and 12h light/dark cycle). All experiments involving mice were performed using protocols approved by the Ethics Committee of Jiangnan University, China (Protocol number: JN. No20180615c0600810) and the procedures were carried out following European Community guidelines (Directive 2010/63/EU) for the care and use of experimental animals.

After one-week acclimatization, the mice were randomly divided into three groups: SD-C group (standard diet and DW), SD-H_2 group (standard diet and H_2-NBW) and SD-N_2 group (standard diet and N_2-NBW). Experiment period was five weeks. The feces of each mice were collected into the sterilized 1.5mL centrifuge tube at the end of the trial and stored at -80℃ until further analysis. Five samples in each group were randomly selected to send to BGI Co., Ltd, China (Wuhan, China) for DNA extraction and 16S rRNA gene sequencing.

3.2.3 DNA extraction and 16S rRNA gene sequencing

The procedure was performed as described previously with some modification [191]. Briefly, the bacterial DNA of the mice fecal samples were extracted using E.Z.N.A.® Bacterial DNA Kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's instruction. The V4 region of 16S rRNA genes from the single fecal sample was amplified by polymerase chain reaction (PCR). The amplicons were purified by using Agencourt AMPure XP beads and dissolved in Elution Buffer. After proper labeling, the construction of libraries was finished and then quantified by the Agilent Technologies 2100 bioanalyzer before being sequenced pair-end on the Illumina HiSeq 2500 platform, with the sequencing strategy PE250 (HiSeq Reagent Kit).

3.2.4 Bioinformatics analysis

The sequences were filtered, merged and clustered into operational taxonomic units (OTU) at 97% similarity as previously described [192, 193]. Taxonomic informations were assigned to OTUs representative sequence using Greengene Database [165]. The alpha diversity was represented by the index of observed species, chao-1, Shannon and Simpson. The beta diversity was determined by visual assessment using principle coordinate analysis (PCoA) plots. The

composition of fecal microbiota at phylum, family, genus and species level were separately analyzed based on the relative abundance of the fecal microbial.

3.2.5 Statistical analysis

The statistical analysis was performed using the statistical software SPSS 25.0 (SPSS Inc., Chicago, IL, USA). At first, checked the normal distribution of data with the Shapiro-Wilk test. The distribution assumed data using the one-way ANOVA test, multiple comparison with the LSD test (Equal variances assumed) or Games-Howell test (Equal variances not assumed). Otherwise, using the nonparametric tests (the Kruskal-Wallis test). Unless otherwise indicated, the data are presented by mean ± SD, the significant difference was accepted at $p < 0.05$ and $p < 0.01$ was supposed to be highly significant difference.

3.3 Results

3.3.1 Microbial composition and diversity

The alpha diversity of each group is shown in Fig.3-1. There was no difference at the species richness (Fig. 3-1, A and B) between groups. However, compared with the SD-C group, the SD-N_2 group have a significant increase ($p < 0.05$) at the species diversity (Fig. 3-1, C and D). The alpha diversity in the SD-H_2 group was higher at the species diversity than that in the SD-C group but has no significant increase ($p \geq 0.05$). Fig. 3-2 shows the beta diversity in each group. According to the X-axis results, the distance between the SD-N_2 group and the SD-C/SD-H_2 groups is significantly different. From the Y-axis results, it can be seen that there is a significant difference in the distance between SD-H_2 and SD-N_2/ SD-C groups. Therefore, compared with the control group, drinking NBW could significantly change the diversity of fecal microbiota.

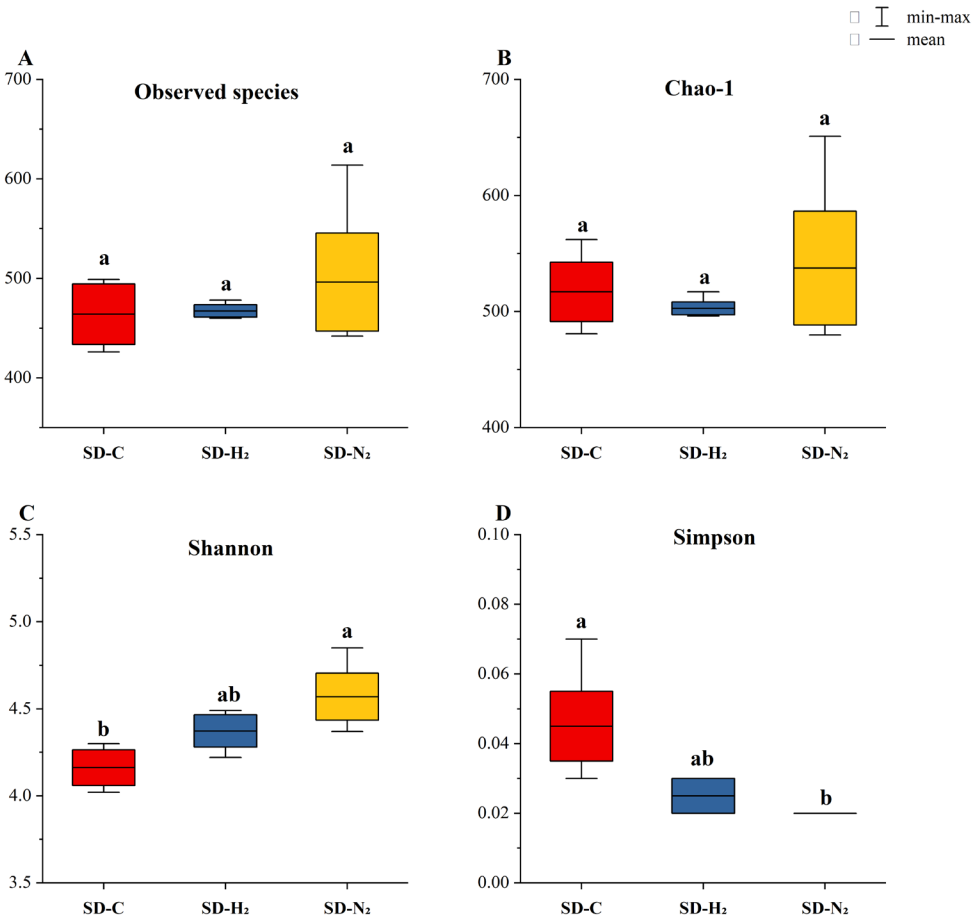

Figure 3-1 The indexes of alpha diversity in each group after five weeks. (A) Observed species; (B) Chao-1; (C) Shannon; (D) Simpson. Groups with dissimilar letters differ, $p < 0.05$.

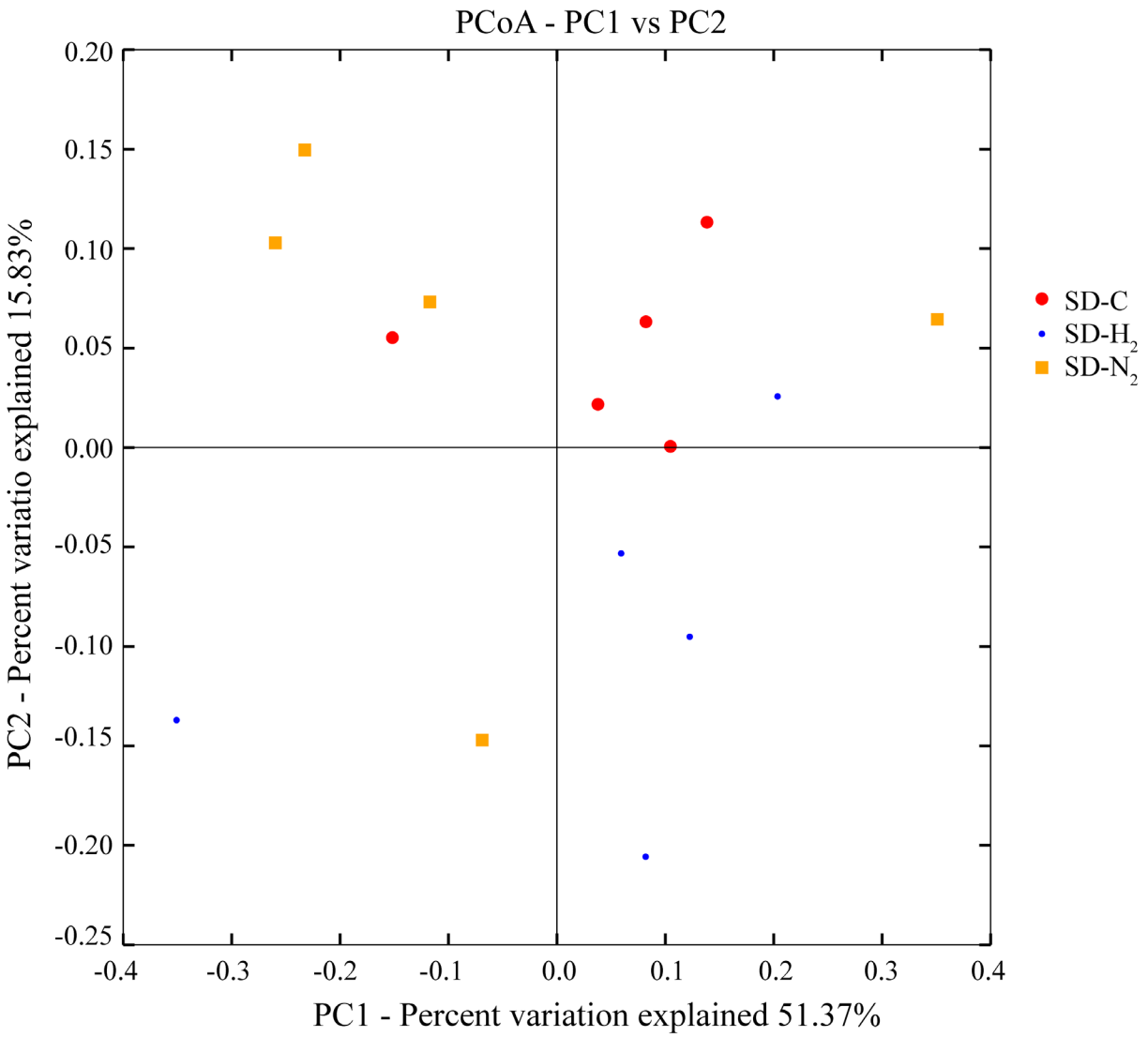

Figure 3-2 The Principle coordinate analysis (PCoA) plots based on weighted UniFrac metrics. Each colored symbol represents the composition of fecal microbiota of one mouse.

3.3.2 Relative abundance of fecal microbiota

At the phylum level, the most abundant phylum was *Bacteroidetes* (range, 52-74%; mean, 65.7 ± 7.1%), followed by *Firmicutes* (range, 16-32%; mean, 23.5 ± 5.7%), *Proteobacteria* (range, 4-10%; mean, 7.6 ± 2.8%), TM7 (range, 0.2-3.2%; mean, 1.8 ± 1.2%) and some phyla that the average abundance below 1% including *Deferribacteres*, *Tenericutes*, *Actinobacteria*, *Cyanobacteria*, *Fusobacteria* and others (Fig. 3-3A). The ratio of *Firmicutes* to *Bacteroidetes* (F/B) in three groups followed the descending order: SD-N_2 > SD-H_2 > SD-C, in which the SD-N_2 group was highly significantly higher than that in the other two groups ($p < 0.01$, Fig. 3-3B). There was a significant difference in the relative abundance of *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Deferribacteres* among three groups (Fig. 3-4). The relative abundance of *Bacteroidetes* in the SD-N_2 group was significantly lower ($p < 0.05$) than that in the SD-H_2 group (Fig. 3-4A). Compared with the SD-C group, the SD-N_2 group was significantly increased the abundance of *Firmicutes* (Fig. 3-4B) while the SD-H_2 group was significantly decreased the abundance of *Deferribacteres* and *Proteobacteria* (Fig. 3-4C D).

Bacteroidetes. This phylum including seven families: S24-7; *Rikenellaceae*; *Prevotellaceae*; *Porphyromonadaceae*; *Paraprevotellaceae*; *Odoribacteraceae and Bacteroidaceae*, and seven genera: *AF12*; *Bacteroides*; *Odoribacter*; *Parabacteroides*; *Prevotella*; *Rikenella* and "Others" (Fig. 3-5 and Fig. 3-7). At the family level, the relative abundance of S24-7 was highly significantly lower ($p < 0.01$) in the SD-N_2 group (44.1 ± 2.66 %) than that in the SD-H_2 and SD-C groups (53.8 ± 5.11 % and 55.4 ± 2.53 %, respectively. Fig. 3-6A).

Firmicutes. This phylum including thirteen families, and six genera: *Ruminococcus*; *Oscillospira; Lactobacillus*; *Dorea*; *Coprococcus* and *Clostridium* (Fig. 3-5 and Fig. 3-7). At the family level, the SD-N_2 group (10.8 ± 3.55 %) was significantly increased ($p < 0.05$) at the abundance of *Lachnospiraceae* than that in the SD-C group (4.42 ± 0.99 %) (Fig. 3-6B). At the genus level, the relative abundance of *Clostridium* and *Coprococcus* was significantly increased ($p < 0.05$) in the SD-N_2 group (Fig. 3-8, A and B). The SD-H_2 group has a higher abundance of these two genera than that in the SD-C group. However, there was no significant difference between them. Besides, a significantly lower relative abundance of *Oscillospira* was observed in the SD-H_2 group (2.72 ± 1.04 %) than that in the SD-N_2 group (4.63 ± 1.34 %) (Fig. 3-8C).

Proteobacteria. This phylum including five families: *Helicobacteraceae*; *Enterobacteriaceae*; *Desulfovibrionaceae*; *Alealigenaceae* and *Aeromonadaceae*, four genera: *Sutterella*; *Helicobacter*; *Flexispira* and *Desulfovibrio* (Fig. 3-5 and Fig. 3-7). At the genus level, the relative abundance of *Helicobacter* in SD-H_2 group was significantly lower ($p < 0.05$) than that in the SD-C and SD-N_2 groups (Fig. 3-8E).

Deferribacteres. This phylum only has one family (*Deferribacteraceae*) and one genus (*Mucispirillum*) (Fig. 3-5 and Fig. 3-7). The SD-H_2 group (0.154 ± 0.157 %) was significantly decreased ($p < 0.05$) at the relative abundance of *Mucispirillum* than that in the SD-C group (1.11 ± 0.55 %) (Fig. 3-8D).

The SD-H_2 group was significantly decreased ($p < 0.05$) at the relative abundance of *Mucispirillum_schaedleri* than that in SD-C group (Fig. 3-9). There was no difference at the abundance of the other identified species between three groups.

3.4 Discussion

During the experiment, there was no significant difference between groups in the body weight of mice (Table 3-1 and Fig. 3-10), which was inconsistent with the previously report that NBW could accelerate the growth of mice [130]. However, the results of 16S rRNA gene sequencing suggested that the NBW definitely could alter the gut microbiota in mice.

The results of current study showed that supplementation with N_2-NBW to mice significantly decreased ($p < 0.05$) the relative abundance of S24_7 and increased the relative abundance of *Clostridium* and *Coprococcus*. The S24_7 is a prominent example of the uncultured constituents in gut microbiota and mainly colonized in the intestinal of homeothermic animals. Up to now, the exact interaction between this family and the host is remain unclear [194]. Several reports have shown that the abundance of S24_7 has a positively correlation with short chain fatty acids (SCFAs) producing [195-197]. However, the members of the family were recognized as the lipopolysaccharide (LPS)-producing bacteria has been reported [198]. Since there were no significant differences in physical signs between the groups, the effect of alteration in the abundance of S24_7 caused by N_2-NBW supplementation on mice should be further investigated. The *Coprococcus* is a genus of anaerobic cocci which belong to the family *Lachnospiraceae*. It has been reported that the genus *Coprococcus* was correlated with the production of SCFAs,

especially with propionate and hexanoate [199, 200]. It was also suggested as health-promoting microbes and was found with high abundance in the gut of healthy lean adults [201, 202]. Most of species in genus *Clostridium* are belong to the *Clostridium cluster XIVa* (also known as the *Clostridium Coccoides* group) and *Clostridium cluster IV* (also known as the *Clostridium leptum* group), which are the chief components of total bacteria in the gut microbiota and has been recognized as butyrate-producing bacteria [203-205]. The genus *Clostridium* was found mainly to inhabit the areas between the mucosal folds [206]. This special region allows *Clostridium* performs a critical role in the modulation of physiologic, metabolic and immune process in the gut [207]. In this study, supplementation with N_2-NBW to mice significantly increased the relative abundance of *Coprococcus* and *Clostridium* in fecal microbiota of mice. Thus, we suggest that the N_2-NBW could exhibit some beneficial effects on the gut microbiota of host.

Compared with the SD-C group, the relative abundance of *Deferribacteres* and *Proteobacteria* were significantly decreased ($p < 0.05$) in the SD-H_2 group at the end of experiment. The typical genus and species in the phylum *Deferribacteres* are *Mucispirillum* and *Mucispirillum schaedleri* (*M. schaedleri*), respectively. Therefore, the significantly decreased abundance of *Deferribacteres* in the SD-H_2 group was resulted from the decreased abundance of *M. schaedleri*. *M. schaedleri* is a non-spore-forming, flagellated anaerobe. It is the core member of the murine gut microbiota, and commonly was found in mice not humans. It is normally considered as the opportunistic pathogen, associated with inflammation in many mouse models [196, 208]. Besides, the results of the composition of fecal microbiota suggested that the decreased relative abundance of *Helicobacter* resulted in the significantly lower ($p < 0.05$) abundance of phylum *Proteobacteria* in the SD-H_2. A total of 37 species in the genus *Helicobacter* have been validated until 2017 [209]. The most representative and widely been studied species is *Helicobacter pylori* (*H. pylori*). Early in 1994, *H. pylori* has been categorized as group I carcinogen by the World Health Organization (WHO) [210]. *H. pylori* infection not only induces the gastrointestinal diseases (e.g. chronic gastritis, gastric cancers and inflammatory bowel diseases) but also related to extra-gastric diseases (e.g. iron deficiency anemia and idiopathic thrombocytopenic purpura) [209, 211, 212]. In addition, some cases of non-*H. pylori* Helicobacter (NHPH) infections was reported in some animals and humans [213-215]. Thus, the *Mucispirillum* and *Helicobacter* have been implicated in the development with some diseases. In this study, supplementation with H_2-NBW to mice

significantly decreased the abundance of these two genera indicated that the H_2-NBW might also have beneficial effects on the gut microbiota of host.

According to the previous discussion, the gut microbiota of mice was definitely altered in the NBW drinking group. However, how the NBW could modulate the composition of gut microbiota in mice? The mechanism was preliminarily analyzed.

It has been reported that the NB in water could affect the physical properties of water, which is characterized by increasing water mobility.[94] My result was consistent with the report that the water mobility of N_2-NBW and H_2-NBW was higher than DW (Fig. 2-1). Water mobility has been usually used to assess the translocation of water molecules in the food [216]. The mobility of water possible to influence the components' structural change of food. Water can work as a plasticizer to influence the mobility of starch molecules, and in turn, this reaction also determines the water molecules' position in the gel network. The gel hardness has a closed relationship with the mobility and amount of water in the less mobile state [217]. Therefore, the alteration in water mobility of NBW could change the composition of gut microbiota by affecting the food properties. Besides, the increased mobility of water has been reported might accelerate the mass transfer rate of nutrients and indirectly stimulate the activity of microorganisms [159]. From this point of view, the presence of NB might influence gut microbial growth and metabolism. The different values of water mobility might influence the effects, this might bring about the different performance between N_2-NBW and H_2-NBW. To sum up, the impact of water mobility on the food properties and substances transport might be responsible for the alteration of gut microbiota under HFD feeding with NBW. The real mechanism of NBW on the modulation of gut microbiota should be further investigated.

3.5 Summary

In this study, supplementation with NBW to mice certainly altered the composition of fecal microbiota. Compared with the composition of fecal microbiota in SD-C group, the abundance of two beneficial genera in SD-N_2 group were increased while the abundance of two pathogenic genera abundance in the SD-H_2 group were reduced. Besides, preliminary analyzed the mechanism suggested that the increased water mobility of NBW might influence food component structure and substances transport, these alterations changed the composition of gut microbiota of mice.

Table 3-1 Food and water daily intake in different groups.

	SD-C	SD-H_2	SD-N_2
Food (g/day/cage)	19.48 ± 0.90	20.13 ± 0.52	19.17 ± 1.42
Water(g/day/cage)	15.20 ± 0.34	15.48 ± 1.68	15.1 ± 0.19

Data are expressed as mean ± SD.

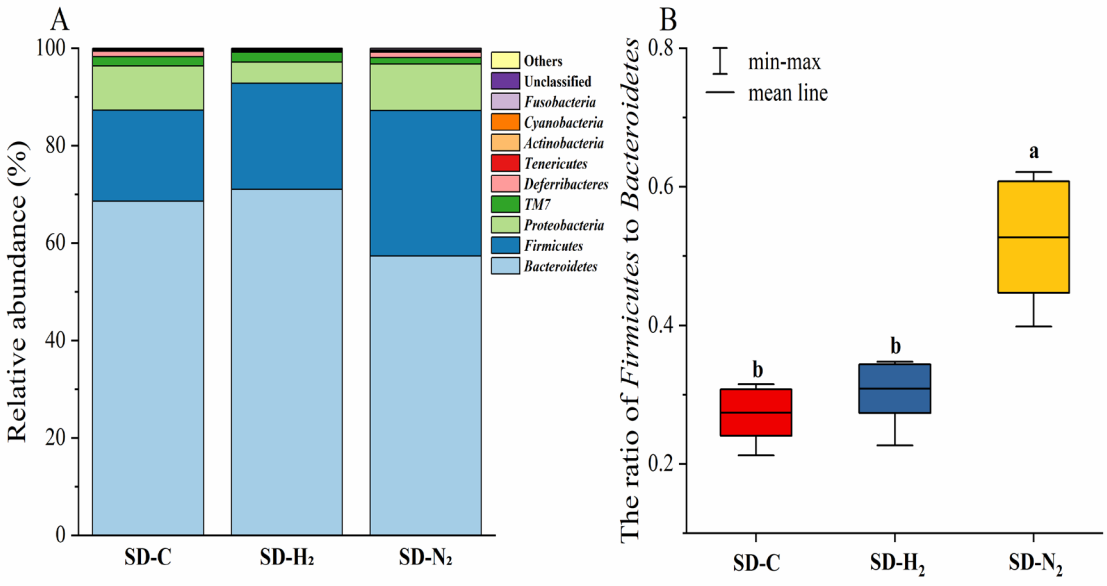

Figure 3-3 The composition of fecal microbiota in each group after supplementation with NBW and deionized water for five weeks. (A) The composition of fecal microbiota at phylum level; (B) The ratio of *Firmicutes* to *Bacteroidetes*. Groups with dissimilar letters differ, $p < 0.05$.

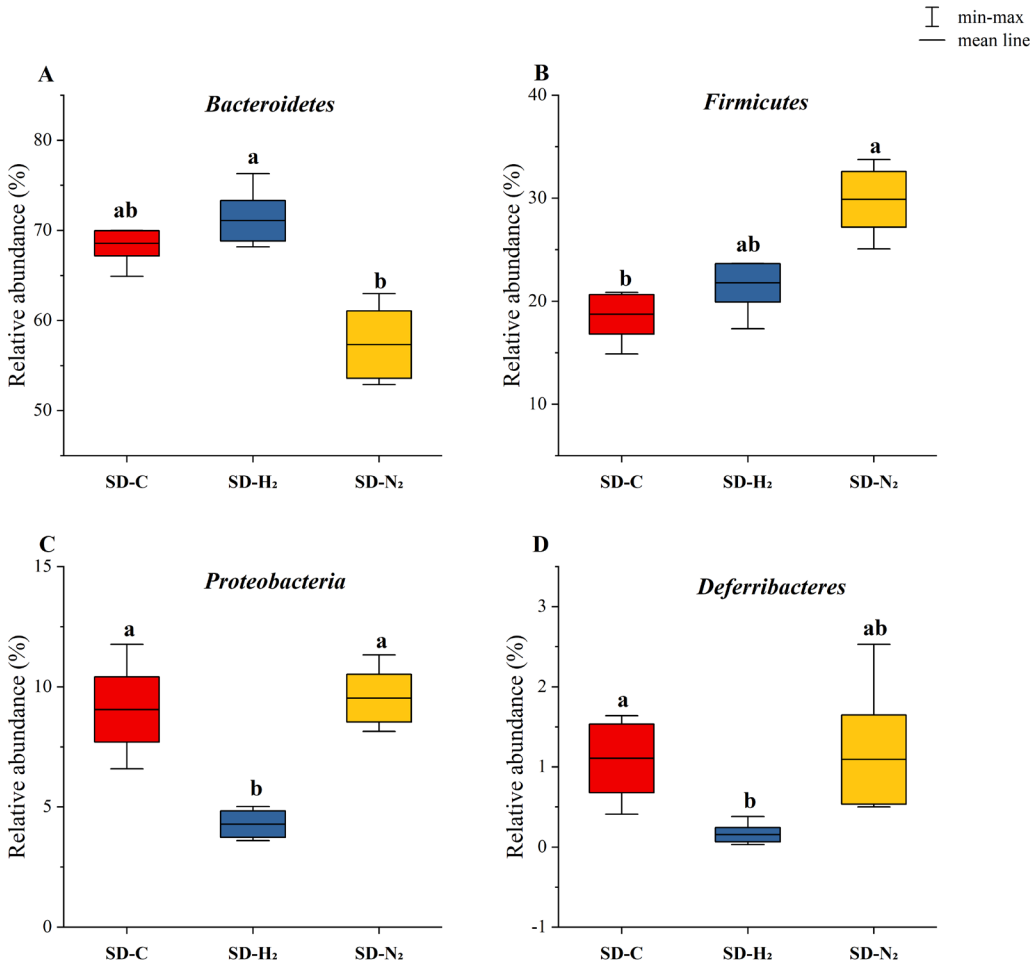

Figure 3-4 The relative abundance of *Bacteroidetes* (A), *Firmicutes* (B), *Proteobacteria* (C) and *Deferribacteres* (D) in different groups after supplementation with NBW and deionized water for five weeks. Groups with dissimilar letters differ, $p < 0.05$.

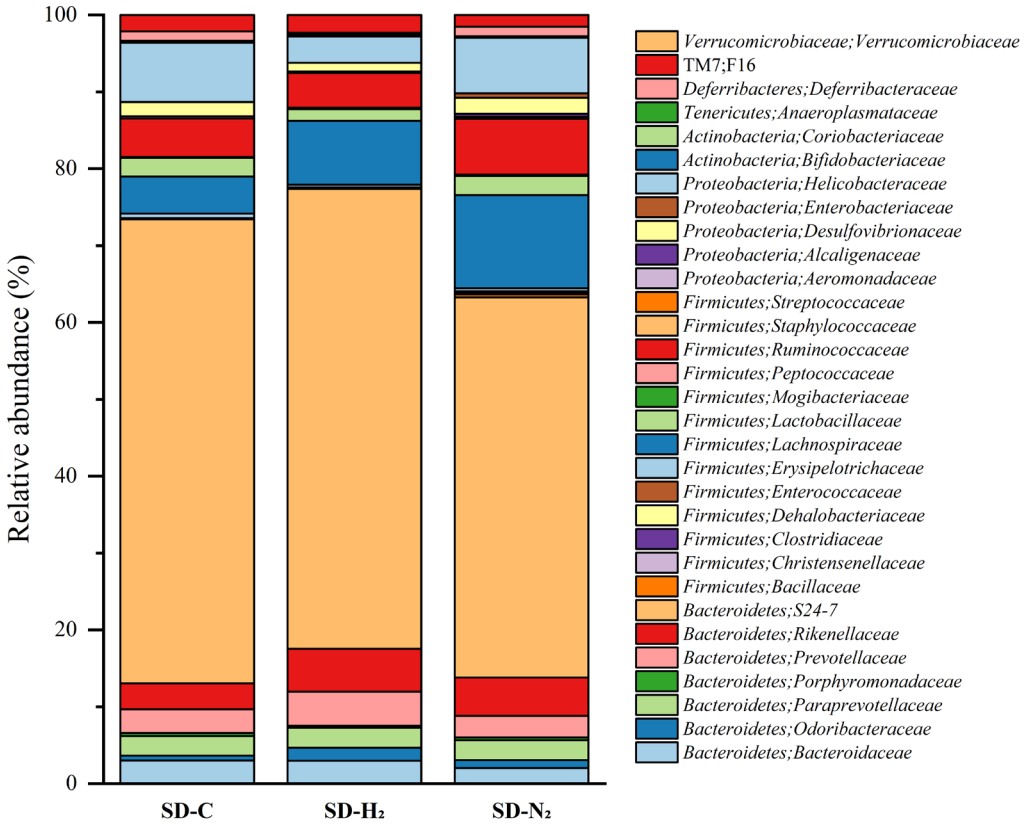

Figure 3-5 The composition of fecal microbiota at family level after supplementation with NBW and deionized water for five weeks.

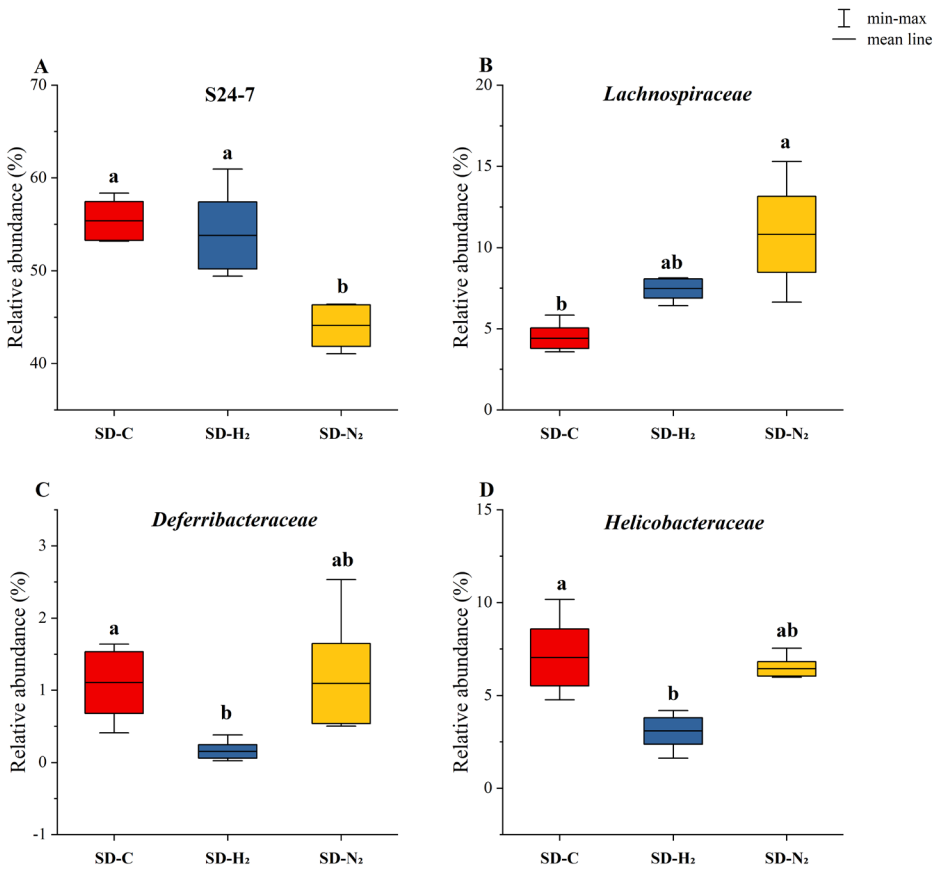

Figure 3-6 The relative abundance of S24-7 (A), *Lachnospiraceae* (B), *Deferribacteraceae* (C) and *Helicobacteraceae* (D) in different groups. Groups with dissimilar letters differ, $p < 0.05$.

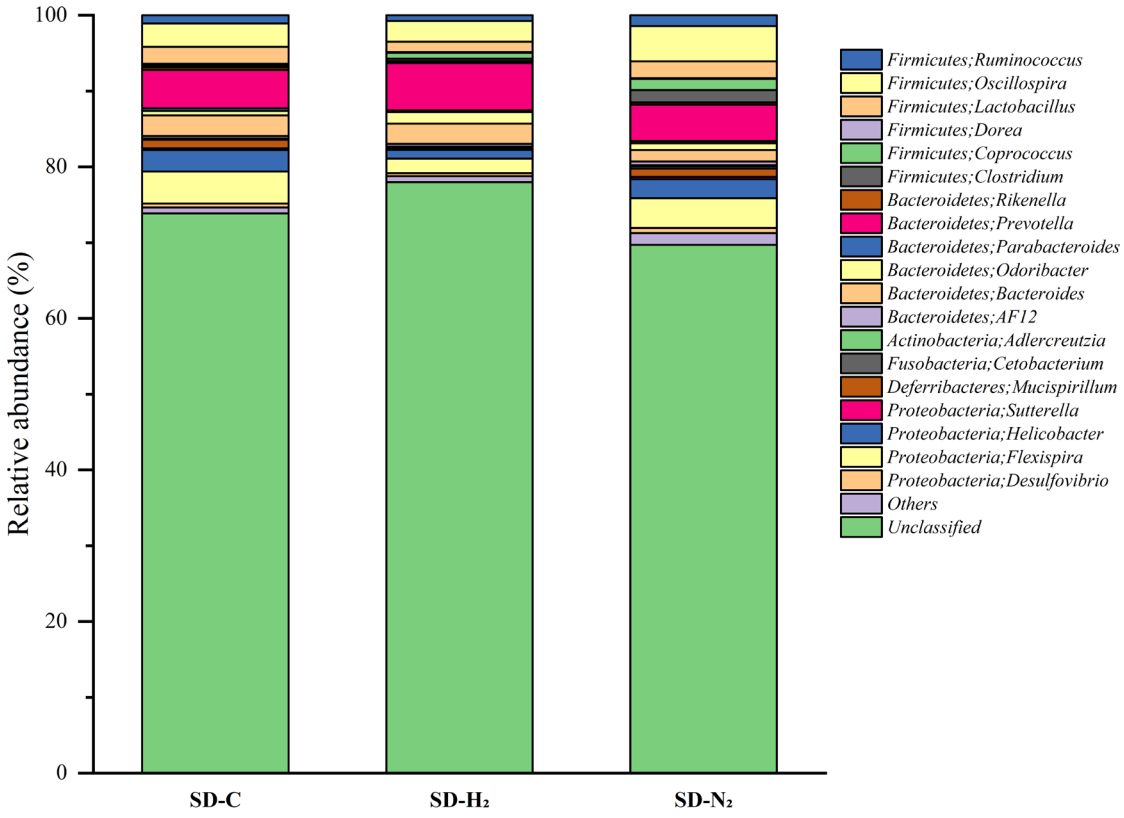

Figure 3-7 The composition of fecal microbiota at genus level after supplementation with NBW and deionized water for five weeks.

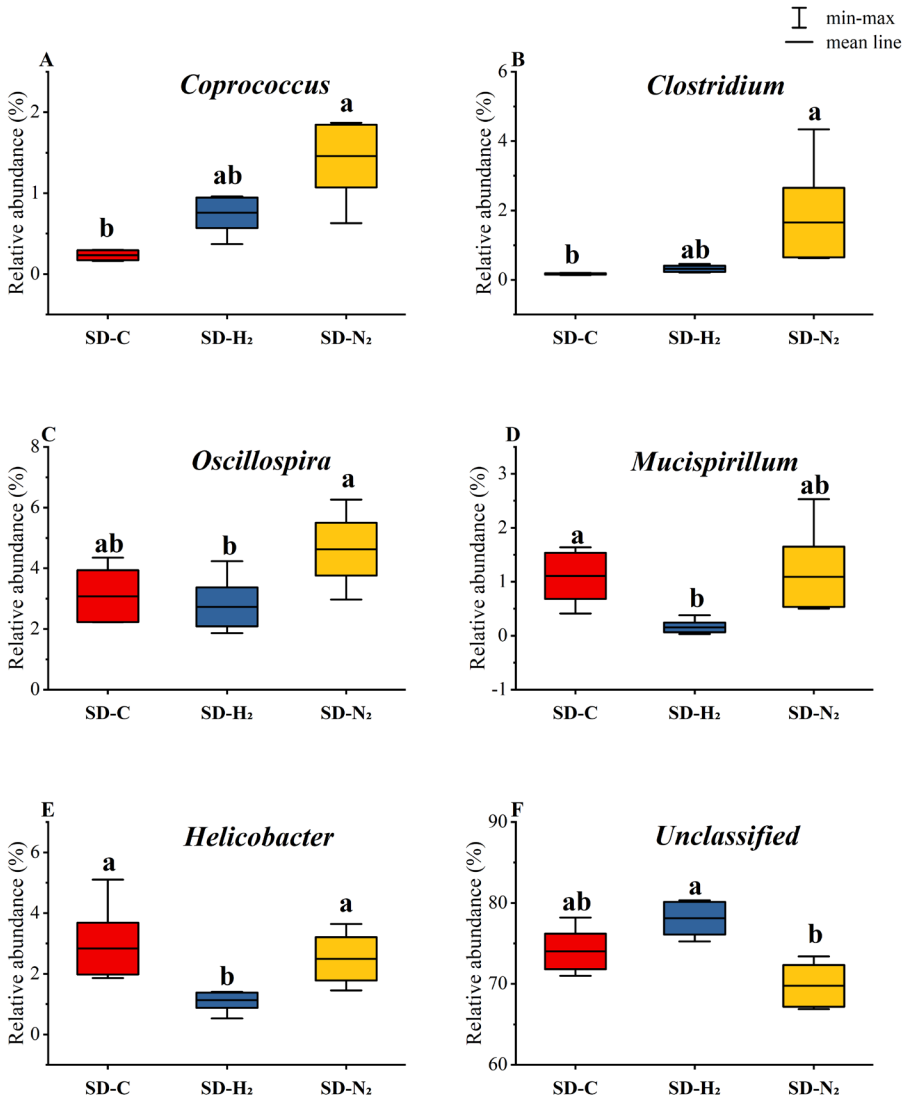

Figure 3-8 The relative abundance of *Coprococcus* (A), *Clostridium* (B), *Oscillospira* (C), *Mucispirillum* (D), *Helicobacter* (E) and Unclassified (F) in different groups. Groups with dissimilar letters differ, $p < 0.05$.

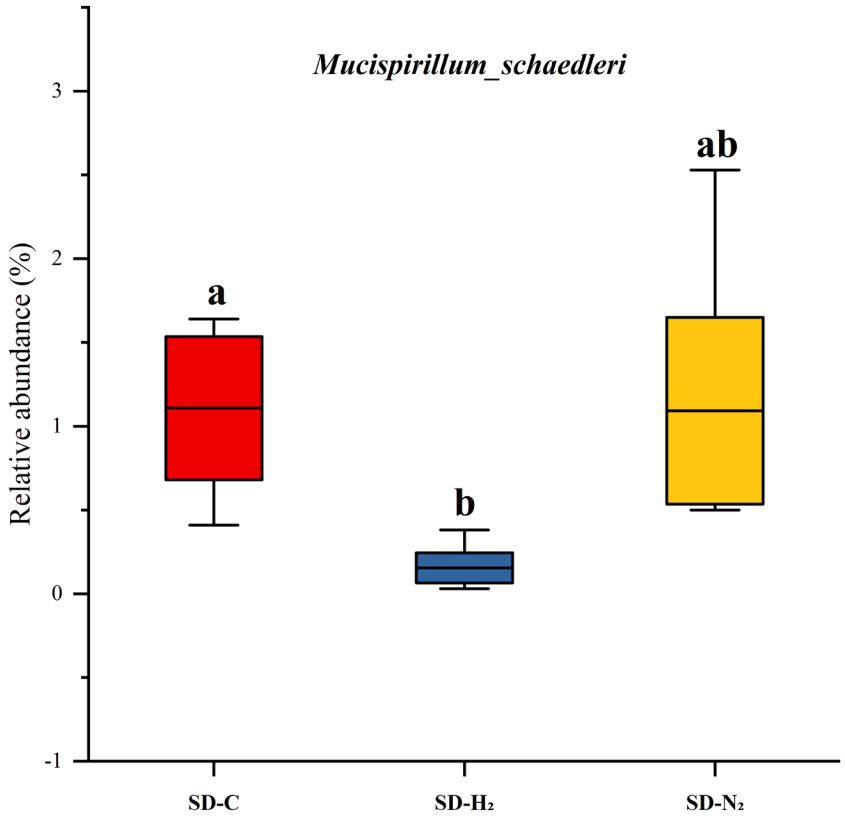

Figure 3-9 The relative abundance of *Mucispirillum_schaedleri* in different groups after supplementation with NBW and DW for five weeks. Groups with dissimilar letters differ, $p < 0.05$.

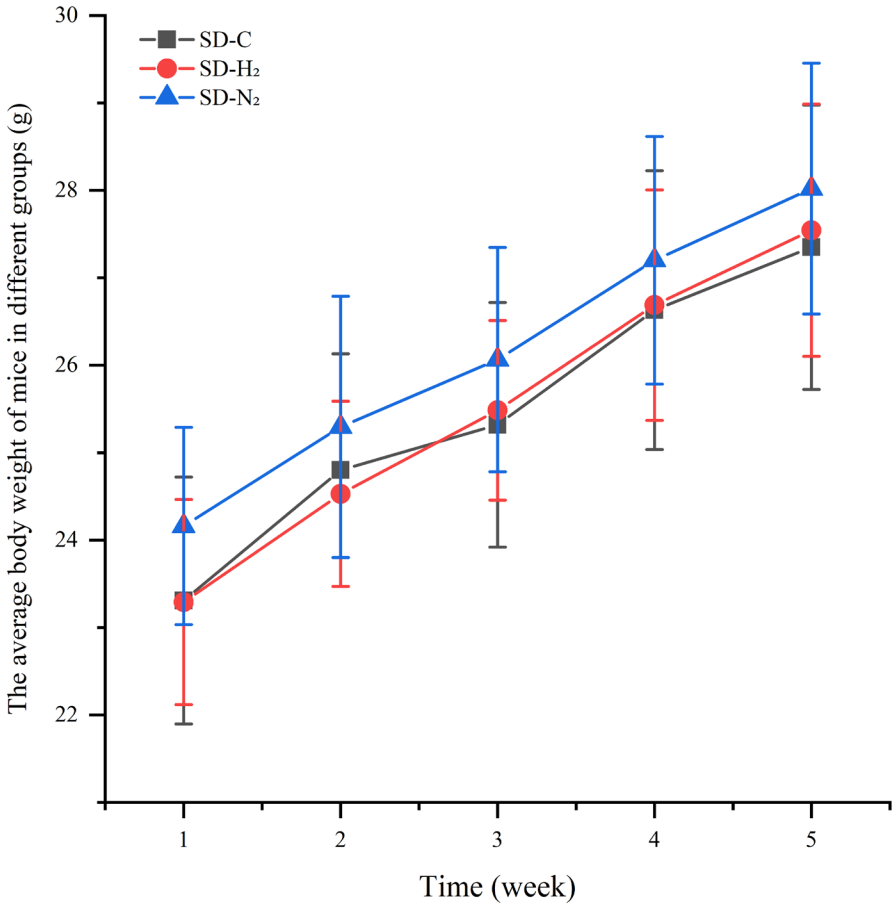

Figure 3-10 The average body weight of mice in different groups during the five weeks experiment period.

Chapter 4 Effects of NBW on the physical signs and gut microbiota of the mice in high-fat diet

4.1 Introduction

The prevalence of obesity continues to grow worldwide, and especially speeds up since 1995 [218]. Obesity can induce a variety of chronic diseases such as cardiovascular disease and diabetes, which will not only bring about great harm to the health of individuals but also increase the cost of medical care. It has been predicted that the costs associated with obesity in the U.S. would occupy 10% of the national healthcare costs in 2035 [219]. The gut microbiota has been demonstrated to play an important role in the development of obesity mainly through the regulation of energy intake, lipid metabolism and inflammatory response [220-224]. Therefore, current researches usually focus on preventing and ameliorating the obesity by modulating the gut microbiota of host.

Nanobubble water (NBW) has been widely studied in many fields such as organic pollutants degradation, heavy metal ions adsorption, no detergent cleaning and mineral floatation due to the unique properties [115, 177, 189, 225]. The most interesting one is its application in the field of biological. For plants, the seeds of barley, spinach, lettuce, carrot, fava bean, and tomato were reported have a higher germination rate in NBW than that in normal water [94, 119, 120, 131]; NBW significantly promoted the height, leaves and fresh weight of vegetables than normal water [130, 131]. For animals, the weight of sweetfish and rainbow trout cultured in air NBW were separately increased about 60% and 15% than cultured in normal water. Free oral take of oxygen NBW significantly elevated the body weight and length of mice than that of normal water [130]. In addition, the NBW was also investigated in the fields of medical. The oxygen NBW was used to overcome the tumor hypoxia to inhibit the breast or pancreatic cancer cell growth in mice via the gavage or free oral intake, and results revealed that the expression of promote tumor growth factors decreased while the expression of inhibit tumor growth factors increased [132, 133]. The tumor size of the mice in oxygen NBW drinking group was detected significantly decreased than that in normal water drinking group [133]. In addition, in Chapter 2 I have demonstrated the NBW could accelerate the growth of *Lactobacillus acidophilus* at the lag and logarithmic phases. In Chapter 3, I had found the NBW has the potential to alter the composition of gut microbiota in

mice under standard diet.

Taking into consideration the biological activities of NBW and the important role of gut microbiota in the development of obesity, I hypothesis that supplementation with NBW could alleviate the process of obesity induced by high-fat diet (HFD) through modulation of gut microbiota. In this study, the male BALB/c mice were supplemented with NBW under HFD feeding for ten weeks. At the end of the experiment, the obesity-associated markers including lipids and lipopolysaccharides in the serum, and short chain fatty acids (SCFAs) in the feces of mice were detected. Besides, the composition of fecal microbiota was analyzed using 16S rRNA gene sequencing. This study expects to supply a new view to ameliorate the obesity induced by HFD, further investigate the biological activities of NBW to supply a factual basis for the application of NBW in the medicine and food fields.

4.2 Materials and methods

4.2.1 Nanobubble water (NBW) generation

This part can be referred to the subsection 3.2.1.

4.2.2 Animals and diet

Six-week-old male BALB/c mice were bought from Jiangsu Laboratory Animal Center (Jiangsu, China) and housed in individual cages under controlled environmental conditions (temperature 25°C, relative humidity 60%, and 12 h light/dark cycle). The mice were maintained for one week to acclimate to the housing environment prior to the experiment. The deionized water (DW) was sterilized by autoclave (121°C,15 min) and the produced N_2-NBW and H_2-NBW were sterilized by 0.45 μm mixed cellulose ester (MCE) syringe filter. All experiments involving mice were performed using protocols approved by the Ethics Committee of Jiangnan University, China (Protocol number: JN. No20180615c0600810) and the procedures were carried out following European Community guidelines (Directive 2010/63/EU) for the care and use of experimental animals.

Twenty mice were randomly divided into four groups: SD-C (standard diet and DW, control), HFD-C (high-fat diet and DW, control), HFD-N_2 (high-fat diet and N_2-NBW) and HFD-H_2 (high-fat diet and H_2-NBW). The standard diet (10% fat, 20% protein, and 70% carbohydrate, D 12450B) and the high-fat diet (45% fat, 20% protein, and 35% carbohydrate, D12451) were purchased from

Research Diets (New Brunswick, NJ). Water and diet were received *ad libitum* during the whole experimental period.

4.2.3 Sample collection

Food, water intake, and body weight were measured every three days for 10 weeks using a weighing balance (METTLER ME4002). Fecal samples were collected separately from individual mice into sterilized 1.5 mL centrifuge tube and randomly selected three samples from each group for fecal microbiota composition analysis, the rest were stored at -80°C until further analysis. All the mice were fasted for 12 h (overnight) and sacrificed at the end of the trial. Blood collections were drawn from individual mice into sterilized tubes and immediately centrifuged at 3×10^3 rpm for 10 min at 4°C. The serum layer was transferred into a 1.5 mL tube and stored at -80°C until further analysis. The liver and epididymal fat were removed and weighed. The index of the liver and the epididymal fat were separately calculated as the ratio of the weight of liver or epididymal fat in per body weight.

4.2.4 Biochemistry analysis of serum

The serum lipids [triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C)], hepatic function biomarkers [alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (TP), and albumin (ALB)] and the glucose concentration were tested using the serum analyzer (BS-420, Shenzhen Mindray Biomedical Electronics Co., Ltd., China).

All the above serum biochemistry markers were analyzed using commercially available kits (Wuxi Shanhe Group Medical Equipment Co., Ltd., Wuxi, Jiangsu, China) according to the protocols recommended by the manufacturer. The lipopolysaccharide (LPS) of serum was detected by the ELISA test kit (Nanjing Senbeijia Biological Technology Co., Ltd., Nanjing, Jiangsu, China), followed by the manufacturer's instruction.

4.2.5 Determination of short chain fatty acids (SCFAs) in feces

SCFAs were measured as previously described with some modification [226]. Briefly, using a gas chromatography spectrometer (GC 2010plus, Shimadzu Corporation, Japan), which was equipped with a DB-PPAP column (30 m × 0.25 mm × 0.25 μm). The carrier gas was helium (flow

1 mL min^{-1}, split ratio 5:1, the volume of sample 1 μL). The injection temperature was 200°C. The ionization temperature was 250°C. The programming temperature was 80°C (1 min)-10°C/min-200°C (1 min). Fecal samples of about 50 mg (after freeze-drying) were soaked with a saturated NaCl solution and acidified with sulfuric acid (10%). The standard curve was made by the external standard method and the concentration of SCFAs were calculated according to the standard curve.

4.2.6 Fecal DNA extraction, amplification and sequencing

This part can be referred to the subsection 3.2.3.

4.2.7 Bioinformatics analysis

The raw data were filtered to obtain clean reads and merged into tags using Fast Length Adjustment of Short reads (FLASH, v1.2.11) [192]. The tags were clustered to operational taxonomic units (OTU) at 97% similarity with software USEARCH (v7.0.1090) [193]. The taxonomic information was annotated using the Ribosomal Database Project (RDP) Classifier against the Green Gene Database [165]. The significant difference in the composition of different groups was statistical analyzed based on the relative abundance of bacteria. COG (Clusters of orthologous groups of proteins) database was utilized to predict the function of the fecal microbiome by using the software PICRUSt.

4.2.8 Statistical analysis

This part can be referred to the subsection 3.2.5.

4.3 Results

4.3.1 Comparison of food intake, water intake, body weight and liver/fat index between experimental and control groups

Table 4-1 shows the average food and water intake per cage per day. The food intake ranged from 17 ~ 23 g while the water intake ranged from 11 ~ 15 g. There was no difference in food and water intake between groups, indicating that the mice have no preference for water and diet.

The body weight changes of mice in four groups for every two weeks are shown in Fig. 4-1, A. Mice in the different groups has the same initial body weight. The average body weight of mice in the HFD-C and the HFD-H_2 groups were significantly higher than that in the SD-C group during the experiment ($p < 0.05$). There was no difference in the mice body weight between the HFD-N_2

group and the SD-C group from during the experiment period. Besides, significantly ($p < 0.05$) lower body weight of the mice in the HFD-N_2 group than that in the HFD-C group was observed from the week 2 to week 6. At week 10, there was no significant difference in epididymal fat index among groups ($p > 0.05$). However, a significantly lower ($p < 0.05$) liver index was observed in the NBW groups than that in the HFD-C group (Fig. 4-1B).

4.3.2 Serum biochemistry analysis

The concentration of serum lipid is shown in Fig. 4-2A. The concentration of TC and HDL-C in the serum of mice in the HFD-N_2 group was highly significant lower ($p < 0.01$) than that in the HFD-C group. There was a decreasing trend in the concentration of TG and LDL-C in the mice serum after supplementation of NBW under HFD feeding.

To evaluate the effects of NBW on the hepatic function under HFD feeding, the serum level markers representing the hepatic status were detected. The activity of ALT in the serum of mice in the HFD-C group was significantly higher ($p < 0.05$) than that in the HFD-N_2 group, HFD-H_2 and SD-C groups (Fig. 4-2B). Although there was no significant difference in the activity of AST in the mice serum among the four groups, a decreasing trend of it was observed in NBW groups. Meanwhile, the concentration of TP, ALB and glucose in serum have no difference among groups (Fig. 4-3). Besides, the concentration of LPS in the mice serum was significant reduced ($p < 0.05$) when supplementation with NBW to the mice under HFD feeding (Fig. 4-2C).

4.3.3 Short chain fatty acids (SCFAs) analysis in the feces of mice

Mice feces in HFD-C and HFD-H_2 groups showed a significantly higher concentration of acetic acid (AA), propionic acid (PA) and butyric acid (BA) ($p < 0.05$) than that in HFD-N_2 group (Fig. 4-4). Compared with the HFD-C group, there was no significant difference in HFD-H_2 and HFD-N_2 groups at the concentration of I-BA, I-VA, and VA.

Table 4-1 Average food and water daily intake in different groups.

	SD-C	HFD-C	HFD-H_2	HFD-N_2
Food（g/day/cage)	19.89±0.92	19.55±3.21	19.67±2.09	18.90±2.02
Water (g/day/cage)	13.53±1.67	13.87±1.21	13.47±2.39	13.23±1.98

Data are expressed as mean ± SD.

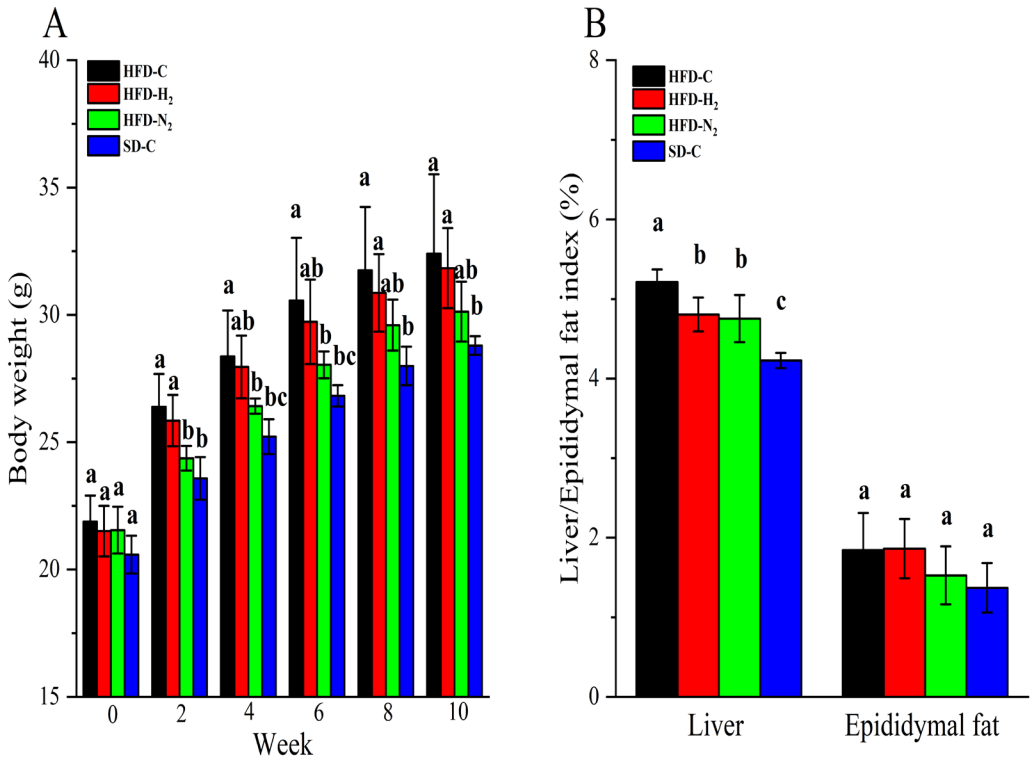

Figure 4-1 The body weight changes during 10 weeks (A) and the liver/epididymal fat indexes at week 10 (B) in each group. Data are expressed as mean ± SD, and groups with dissimilar letters differ, $p < 0.05$.

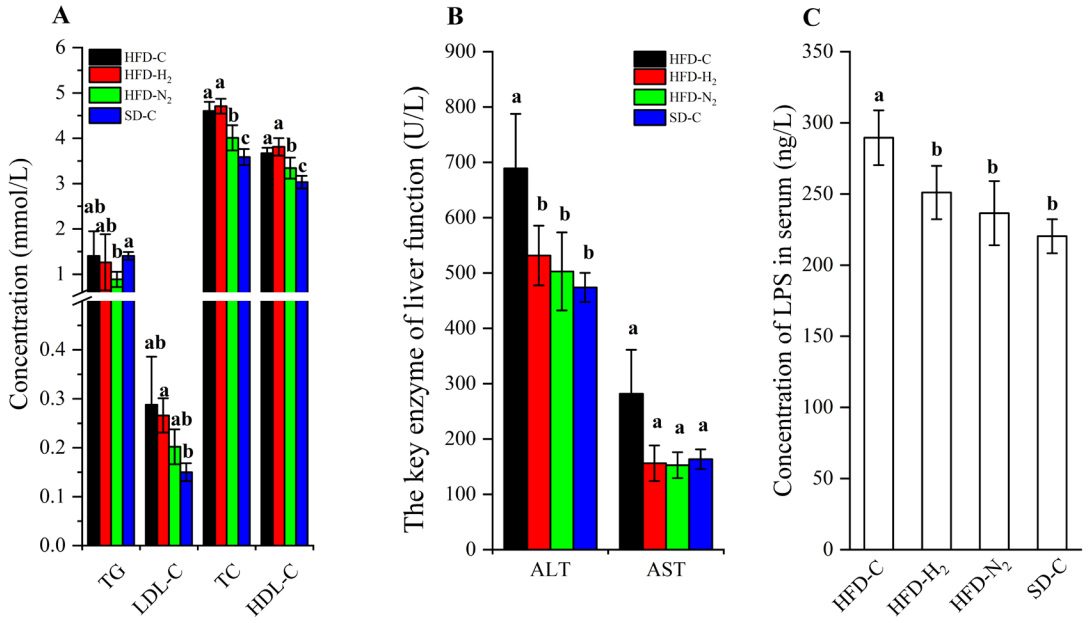

Figure 4-2 The serum biochemistry analysis at week 10 in each group. (A) serum lipids. (B) liver function biomarkers. (C) serum lipopolysaccharide (LPS). TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase. Data are expressed as mean ± SD, and groups with dissimilar letters differ, $p < 0.05$.

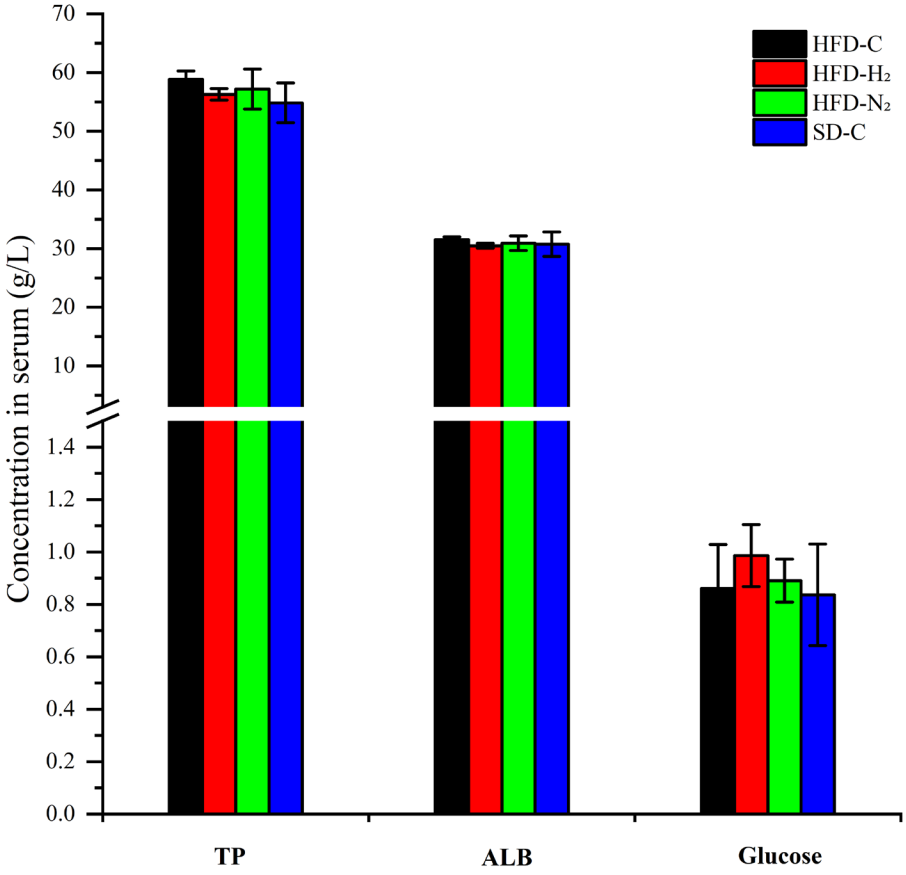

Figure 4-3 The concentration of TP (total protein), ALB (albumin)and glucose in serum of mice in each group at week 10.

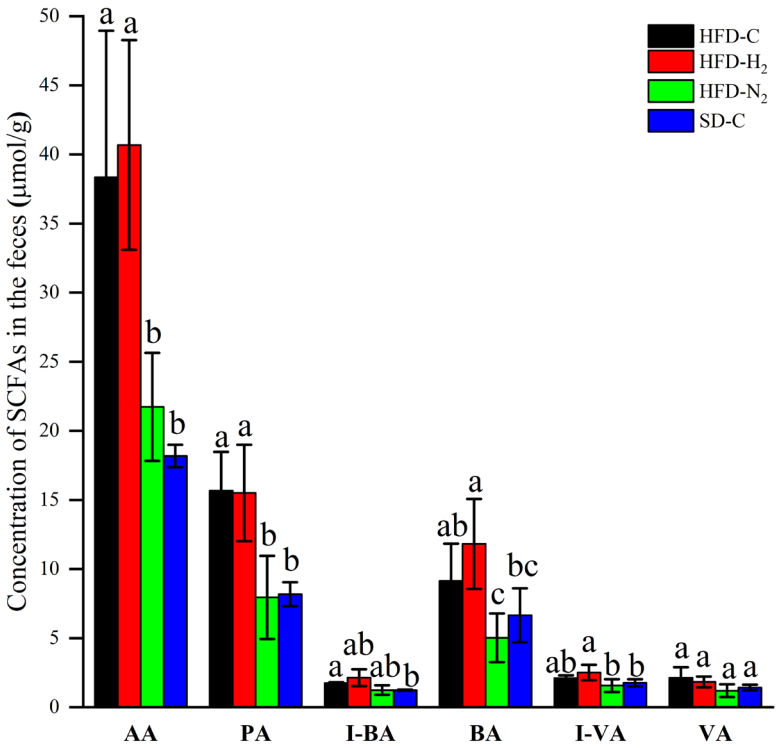

Figure 4-4 The concentration of SCFAs in the feces of mice at week 10. AA, acetic acid; PA, propionic acid; BA, butyric acid; I-BA, iso-butyric acid; VA, valeric acid; I-NA, iso-valeric acid. Data are expressed as mean ± SD, and groups with dissimilar letters differ, $p < 0.05$.

4.3.4 The composition of fecal microbiota

To evaluate the effects of HFD feeding with NBW on the gut microbiota of the mice, the fecal microbiota of each group at week 0 and week 10 was analyzed using 16S rRNA gene sequencing.

Results showed that there was no difference at the phylum level of fecal microbiota among groups at week 0 (Fig. 4-5A). After HFD feeding for 10 weeks, the relative abundance of phylum *Firmicutes* in HFD-C and HFD-H_2 groups was significantly increased ($p < 0.05$, Fig. 4-5A, B). Besides, the relative abundance of phylum *Bacteroidetes* in the HFD-C group was highly significantly lower than that in the HFD-N_2 group ($p < 0.01$, Fig. 4-5A, C). Although there was a significant difference in the relative abundance in the phylum *Cyanobacteria* among groups, the relative abundance of the phylum below 0.1% which was not discussed here. The relative abundance of most phyla increased at week 10 compared with that at week 0 but there was no significant difference between the four groups (Fig. 4-5A).

The ratio of *Firmicutes*/*Bacteroidetes* (F/B) increased greatly from week 0 to week 10 in the HFD-C group (0.34 ± 0.15 to 1.51 ± 0.99) and the HFD-H_2 group (0.28 ± 0.06 to 0.95 ± 0.35), but slightly improved in HFD-N_2 group (0.27 ± 0.09 to 0.343 ± 0.21) (Fig. 4-5D). The ratio of F/B in HFD-C group was significantly higher than that in SD-C and HFD-N_2 groups while it had no difference with HFD-H_2 groups at week 10 (Fig. 4-5D).

In the phylum *Firmicutes*, the HFD feeding significantly increased ($p < 0.05$) the relative abundance of *Allobaculum* (family *Erysipelotrichaceae*) in the HFD-C group, however, there was no significant difference between the NBW groups and the SD-C group (Fig. 4-6B, Fig. 4-8A). Besides, the relative abundance of genus *Staphylococcus* (family *Staphylococcaceae*) in the HFD-C group was significantly higher ($p < 0.05$) than that in the other three groups (Fig. 4-6C, Fig. 4-8B). In the phylum *Bacteroidetes*, the HFD feeding significantly decreased the relative abundance of *Prevotella* at the genus level, however, there was no significant difference between the HFD-N_2 group and the SD-C group (Fig. 4-8C). Furthermore, the relative abundance of genus *Adlercreutzia* (family *Coriobacteriaceae*, phylum *Actinobacteria*) in the HFD-C group was significantly lower than that in the SD-C and HFD-N_2 groups (Fig 4-6D, Fig 4-8D). There was no difference at the species level based on the statistical analysis of the relative abundance of identified species among groups.

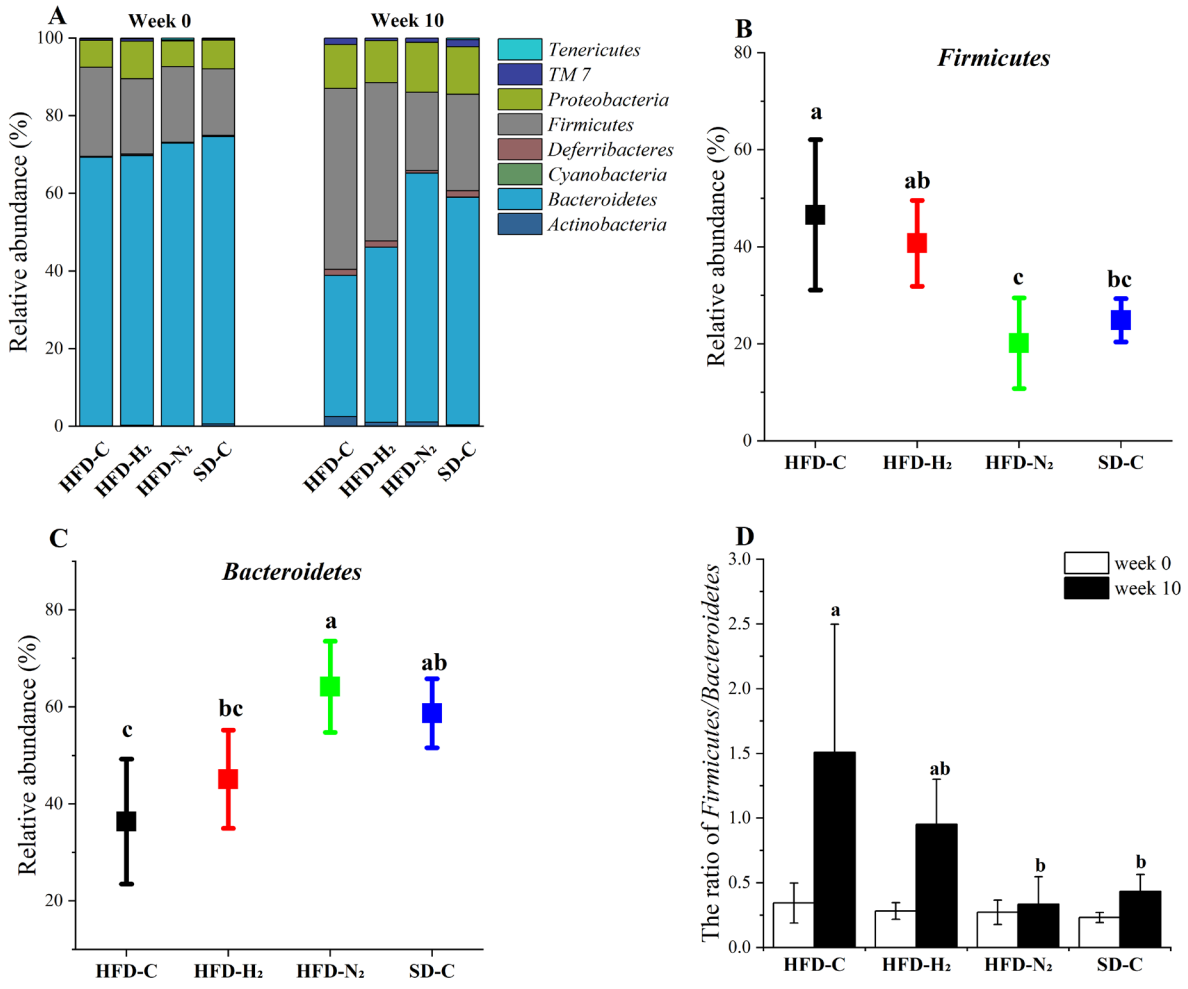

Figure 4-5 The fecal microbiota at the phylum level in different groups. (A) The composition of fecal microbiota at week 0 and week 10 in different groups. The relative abundance of phylum *Firmicutes* (B) and phylum *Bacteroidetes* (C) at week 10 in different groups. (D) The ratio of *Firmicutes*/*Bacteroidetes* at week 0 and week 10 in four groups. Data are expressed as mean ± SD, and groups with dissimilar letters differ, $p < 0.05$.

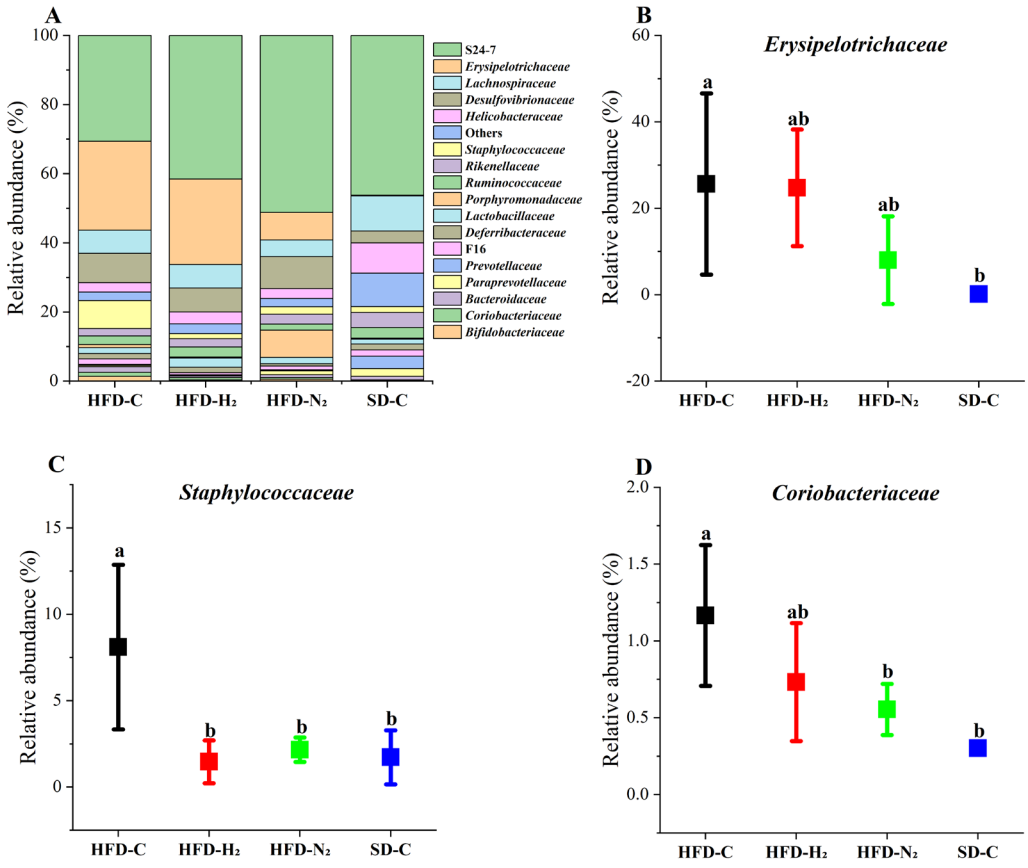

Figure 4-6 The fecal microbiota at the family level at week 10 (A) and the relative abundance of *Erysipelotrichaceae* (B), *Staphylococcaceae* (C), *Coriobacteriaceae* (D) among four groups. Data are expressed as mean ± SD, and groups with dissimilar letters differ, $p < 0.05$.

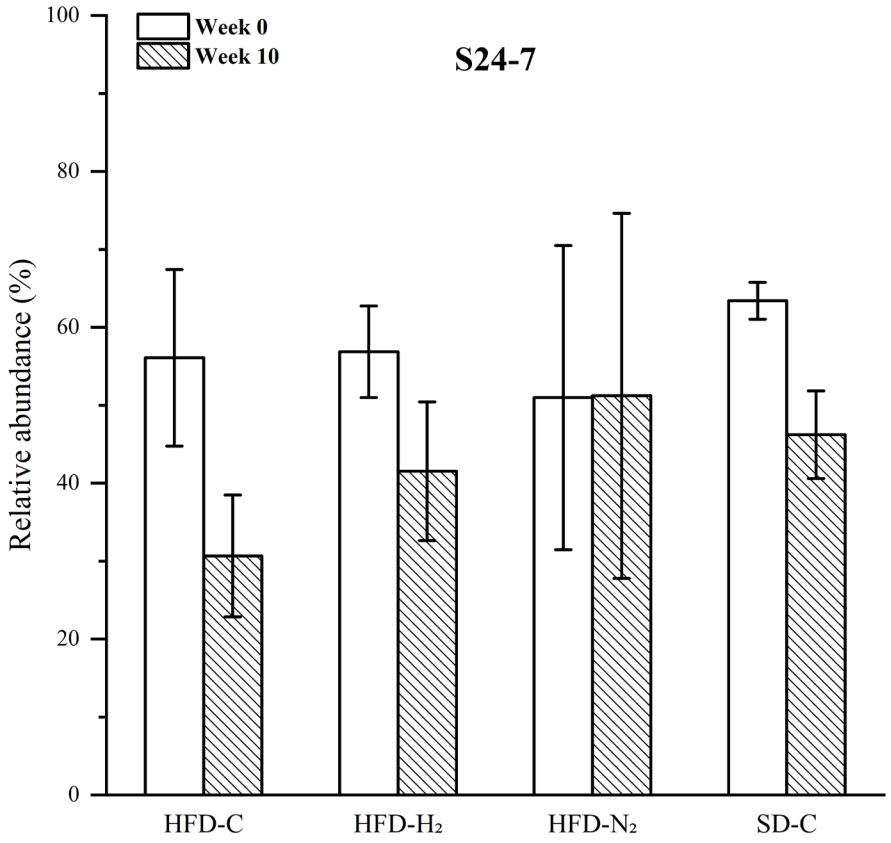

Figure 4-7 The relative abundance of family S24-7 at week 0 and week 10.

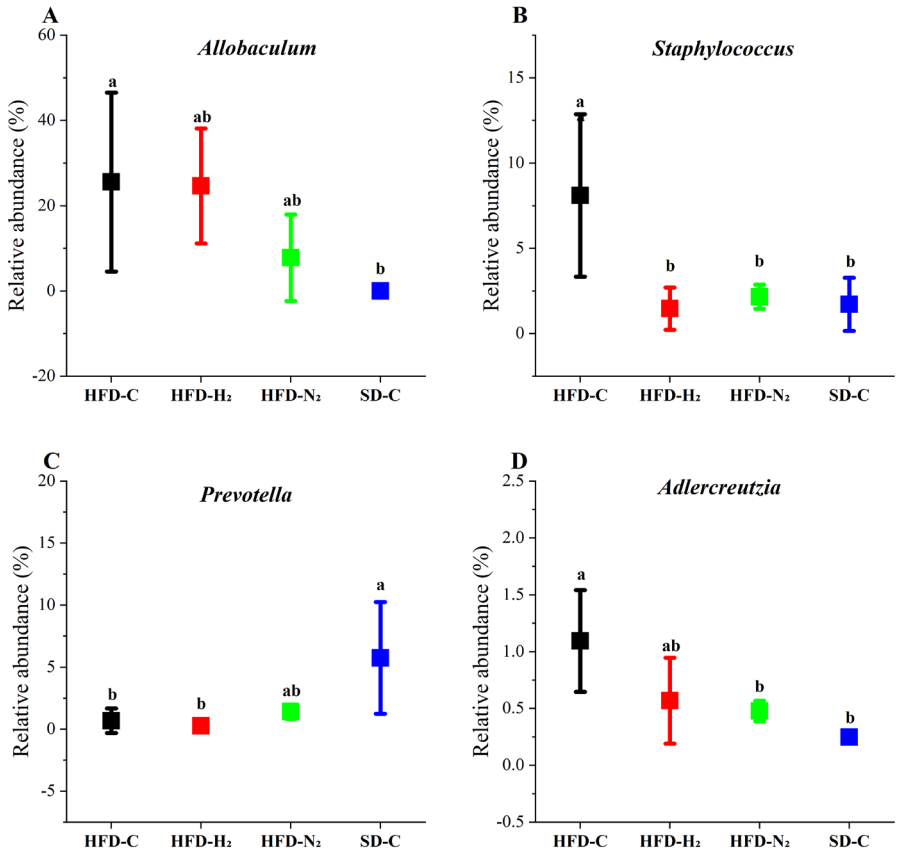

Figure 4-8 The relative abundance of genus *Allobaculum* (A), *Staphylococcus* (B), *Prevotella* (C) and *Adlercreutzia* (D) in different groups at week 10. Data are expressed as mean ± SD, and groups with dissimilar letters differ, $p < 0.05$.

4.3.5 The obesity-associated markers correlation with the gut microbiota at the genus level

Spearman's rho non-parametric correlation analysis was applied to investigate the relationship between the obesity-associated markers (including body weight, TC, LDL-C, ALT, AST, LPS and SCFAs) and the bacteria at the genus level (Fig. 4-9). Results found that the genus *Allobaculum* and genus *Adlercreutzia* had a positive relationship with these markers while the genus *Prevotella* had a negative relationship. Besides, the genus *Staphylococcus* only had a positive correlation with the concentration of HDL-C and TC.

4.3.6 Predicted function of fecal microbiota

The software PICRUSt firstly standardized the number of species of 16S rRNA gene sequencing raw data. Then, the information of species was analyzed based on the COG database to obtain a predicted function results at COG level 2. A total of 24 kinds of function were predicted. The relative abundance of six predicted functions that have a significant difference at COG level 2 among four groups show in Fig. 4-10.

The functions of "Intracellular trafficking, secretion, and vesicular transport", "Inorganic ion transport and metabolism" and "cell wall/membrane/envelope biogenesis" were significantly overrepresented in the HFD-N_2 group compared with that in the HFD-C group. The relative abundance of function "lipid transport and metabolism" in the SD-C group was significantly higher than that in the HFD-C group while having no difference with the HFD-N_2 and HFD-H_2 groups. Besides, there was a significantly decreased abundance of "function unknown" and "transcription" functions in the HFD-N_2 group than that in the HFD-C group.

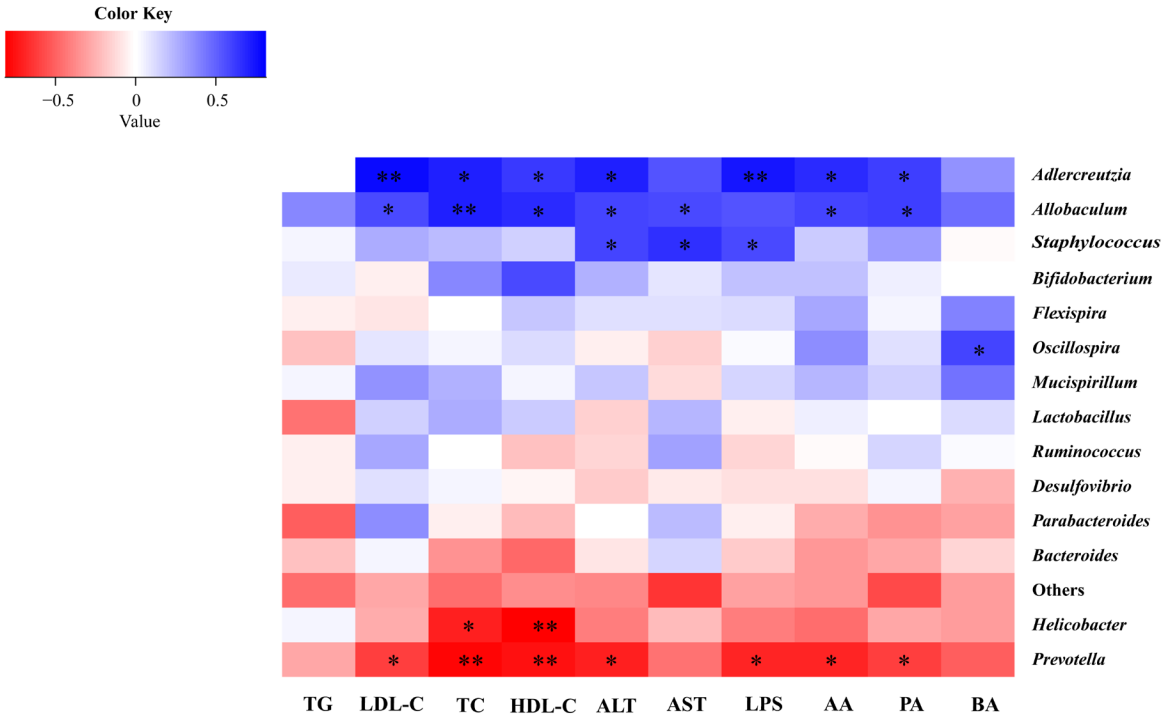

Figure 4-9 The Spearman's correlation analysis between obesity-associated markers and the fecal bacteria at the genus level. The color cell represents correlation R values from the negative to positive correlation (red-white-blue). Significant correlations are indicated by 0.01 < $p \leq 0.05^{*}$, $p \leq 0.01^{**}$. TG, triglyceride; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LPS, lipopolysaccharides; AA, acetic acid; PA, propionic acid; BA, butyric acid.

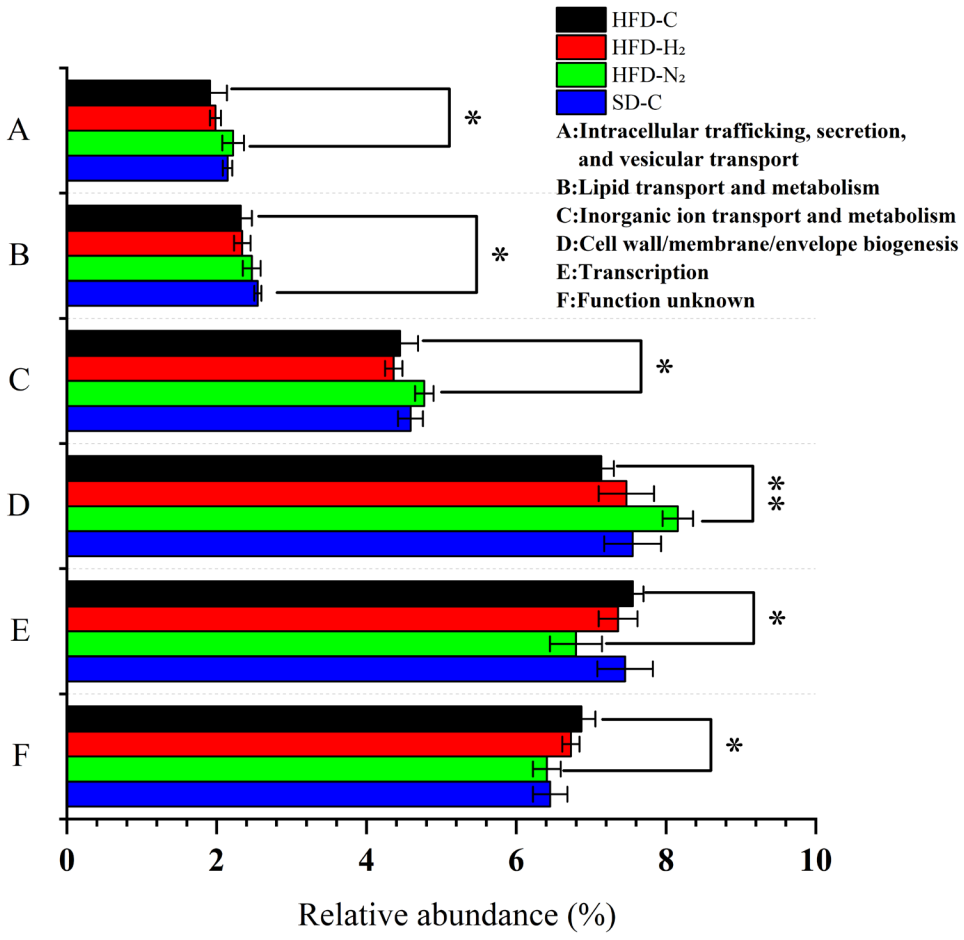

Figure 4-10 The significant difference in the relative abundance of predicted functions of fecal microbiota at COG level 2 among groups at week 10. The significant difference between HFD-C group and HFD-N_2 group are indicated by $0.01 < p \leq 0.05$*, $p \leq 0.01$.**

4.4 Discussion

HFD feeding can bring about a large alteration of gut microbiota that the relative abundance of phylum *Firmicutes* increase and the relative abundance of phylum *Bacteroidetes* decrease [66], which is consistent with our results. In this study, the sum of the average relative abundance of the family *Staphylococcaceae* and family *Erysipelotrichaceae*, which both belong to the phylum *Firmicutes*, occupied more than 25% in the composition of the fecal microbiota of mice in HFD-C and HFD-H_2 groups at week 10 (Fig. 4-6, A and B). Hence, at the family level, the relative abundance of the phylum *Firmicutes* significantly increased in the HFD-C and HFD-H_2 mainly resulted from significantly increased in the relative abundance of the *Staphylococcaceae* and *Erysipelotrichaceae*. At the genus level, this mainly due to the significant increase of *Allobaculum* (family *Erysipelotrichaceae*) and *Staphylococcus* (family *Staphylococcaceae*) (Fig. 4-8, A, B and C).

There was no significant difference ($p \geq 0.05$) among HFD groups at the relative abundance of identified families or genera that belong to the phylum *Bacteroidetes* at week 10. Why the relative abundance of phylum *Bacteroidetes* in the HFD-C group was significantly lower (Fig. 4-5C) than that in the HFD-N_2 group? At week 10, the main family that belongs to the phylum *Bacteroidetes* was family S24-7 (Fig. 4-6A). A significantly decreased ($p < 0.05$) abundance of family S24-7 in HFD-C and HFD-H_2 groups were observed from week 0 to week 10 ((56 ± 11.3) % to (30.7 ± 7.8) % and (56.9 ± 5.8) % to (41.5 ± 8.9) %, respectively). However, the relative abundance of family S24-7 in the HFD-N_2 group was almost the same ((51 ± 19.5) % at week 0 to (51.2 ± 23.4) % at week 10, Fig. 4-7). Although there was no significant difference in the relative abundance of S24-7 between groups at week 10, a significant decrease abundance of it was one crucial factor resulted in the decrease of phylum *Bacteroidetes* in HFD-C and HFD-H_2 groups. Besides, the markedly increase in the relative abundance of other phyla (e.g. *Actinobacteria* and *Proteobacteria*) from week 0 to week 10 might also decrease the proportion of phylum *Bacteroidetes* in the gut microbiota (Fig. 4-5A). Therefore, the significant decrease abundance of family S24-7 and the increase abundance of other phyla from week 0 to week 10, together resulted in the significantly decreased abundance of phylum *Bacteroidetes* in HFD-C and HFD-H_2 groups at week 10. In summary, our results suggested that supplementation with N_2-NBW to mice under HFD feeding had the potential to alter the composition of gut microbiota in mice, especially

inhibited the relative abundance of S24-7 decrease at the family level and suppressed the relative abundance of *Allobaculum* and *Staphylococcus* increase at the genus level. The predicted function of fecal microbiota indicated these changes would significantly influence the functions of substances transport and metabolism (Fig. 4-9). However, supplementation with H_2-NBW to mice under HFD feeding has fewer effects on the composition of gut microbiota.

The increased ratio of *Firmicutes* to *Bacteroidetes* (F/B) in the gut microbiota of mice will improve the capacity for energy harvest from the diet [42]. In this study, the significantly increased F/B ratio in HFD-C and HFD-H_2 groups suggested that the mice in these two groups could assimilate more energy from food under the same diet intake (Fig. 4-5D). Besides, the energy intake also is influenced by the concentration of SCFAs in the intestinal. SCFAs are used as a source of energy [227] and are a major player in the energy metabolism of the host [228]. The significantly lower concentration of AA, PA and BA in the HFD-N_2 group might reduce the energy supplement for the host (Fig. 4-4). Gut microbes could affect the production of SCFAs in mice [229]. In the present study, the alteration of gut microbes at the genus level has a closed relationship with the concentration of AA, PA and BA according to the Spearman's correlation analysis. Thus, the NBW could influence the energy intake of mice through modulation of gut microbes.

HFD induced obesity in mice mainly related to dyslipidemia [230] which is characterized by the elevated concentration of serum lipids. In this study, results of serum lipids indicated that supplementation of N_2-NBW has significantly alleviated ($p < 0.05$) dyslipidemia under HFD feeding (Fig. 4-2A). The most important organ be responsible for regulating the metabolism of lipid is liver.[231] The elevated activity of ALT and AST in the serum will reflect the damage of liver cells. Compared with the HFD-C group, the significant lower activity of ALT and the decreased trend of the activity of AST in the mice serum of HFD-N_2 group suggested a better liver function of mice under HFD feeding with N_2-NBW (Fig. 4-2B). Hence, supplementation of N_2-NBW with mice could significantly protect the liver function to promote the ability of lipid metabolism under HFD feeding.

A high-fat diet will increase the concentration of LPS in the blood [224]. In this study, supplementation with NBW to mice under HFD feeding effectively decreased ($p < 0.05$) the concentration of LPS in serum (Fig. 4-2C). Obesity has long been recognized as a low-grade systemic and adipose tissue inflammation. LPS could act as a triggering factor for obesity by directly or indirectly involved in the inflammatory reaction in adipose tissue [232]. Hence, the

lower concentration of LPS in the serum could retard the process of obesity. LPS are found on the outer membrane of Gram-negative bacteria. The concentration of it is significantly influenced by the alteration of gut microbes. Therefore, the NBW could impact the concentration of LPS through modulation of gut microbes.

4.5 Summary

In this study, supplementation with N_2-NBW to mice under HFD feeding effectively alleviated the development of obesity, while supplementation of H_2-NBW has no obvious effects. This effect is mainly achieved by altering the composition of gut microbiota to impact the energy intake, lipid metabolism and LPS concentration in mice. This study supplies a new view to ameliorate obesity induced by HFD and enlarges the application fields of NBW.

Chapter 5 Conclusions and future perspectives

As a kind of physiologically active and environmentally friendly substance, NBW has been applied in many fields. According to the literature review, this is the first study focus on investigating the effects of NBW on the growth and metabolism of intestinal microorganisms and on the modulation of gut microbiota in mice. The following conclusions were obtained.

5.1 NBW has beneficial effects on gut microbes.

1) Except CO_2-NBW, other three kinds of NBW (N_2/H_2/Air-NBW) promoted the growth of strain LA1028 in a volume percentage dependent manner. More specifically, this promotion effect occurred at the lag and logarithmic phases.
2) Supplementation with NBW influenced the species diversity of the gut microbiota in the standard diet fed mice. For α-diversity, NBW has no effects on the species richness (Observed species and Chao-1) when compared with the SD-C group, however, the species diversity (Shannon and Simpson) was increased in the NBW groups. For β-diversity, NBW groups have a significant difference with the SD-C group based on the principle coordinate analysis (PCoA).
3) Supplementation with NBW alleviated the obesity-associated markers in the HFD fed mice through modulation of gut microbiota. There was a reduction trend of body weight in NBW groups. The Spearman's correlation analysis indicated that the changes of obesity-associated markers have a closed relationship with the alteration of gut microbiota in mice.

NBW has helpful effects on the growth of LA 1028 in the MRS medium and supplementation with NBW might alleviate the development of the HFD induced obesity. An implication of this is the possibility that NBW could be applied in the probiotics production and functional food and drug development fields.

5.2 Comparison between N_2-NBW and H_2-NBW.

1) N_2-NBW had a better performance than H_2-NBW on the growth of LA 1028 under the same condition. In the N_2-NBW group, the highest increase rate was achieved to 51.1% after 6 h cultivation when compared with the DW group, while the H_2-NBW obtained 34.2%.

2) The species diversity in the SD-N_2 group was significantly increased when compared with that in the SD-C group, however, there was no differences between the SD-H_2 group and the SD-C group.
3) Compared with the SD-C group, the relative abundance of two beneficial genera (*Clostridium* and *Coprococcus*) in the gut microbiota of mice were significantly increased in the SD-N_2 group, while two pathogenic genera (*Mucispirillum* and *Helicobacter*) abundance were reduced in the SD-H_2 group.
4) Supplementation with N_2-NBW to the HFD fed mice effectively retarded the body weight increase rate, while supplementation with the H_2-NBW has no obvious effects.
5) The energy metabolism, lipid metabolism, and serum LPS level were ameliorated after the HFD fed mice were supplied with N_2-NBW. The HFD fed mice were supplied with H2-NBW only had reduced the serum LPS level. The ratio of *Firmicutes* to *Bacteroidetes* (F/B) had no changes in the HFD-N_2 group from week 0 to week 10 ((51 ± 19.5) % to (51.2 ± 23.4) %), while the F/B ratio in the HFD-H_2 group was significantly decreased from (56.9 ± 5.8) % to (41.5 ± 8.9) %.

The N_2-NBW has a better performance than H_2-NBW on the gut microbes at the *in vitro* and *in vivo* tests. The properties of NBW suggested that the higher absolute value of N_2-NBW at zeta potential and water mobility than those in H_2-NBW might be responsible for the different performance.

5.3 Preliminary mechanism analysis.

1) The negatively surface charged NBs exist in the water might offer a new way for the substance transport. All of the four kinds of NBW had negative zeta potential, in which the N_2-NBW had the highest absolute value ((-25.3±5.43) mV) while the CO_2-NBW had the lowest ((-6.96±2.36) mV). The NBs might attract the positively charged substances (i.e. metal ions) in the medium and transport them to the bacteria surface. However, the zeta potential of NBs can be strongly affected with the increase of hydrogen ions. That's might be the reason for there were no effects of NBW on the growth of LA 1028 at the stationary phase.
2) Water mobility might influence the food properties (i.e. starch) through affecting the molecule structure. Changes in food properties will directly affect the composition of

intestinal microorganisms. Moreover, the mass transport rate would improve with the mobility of water increase. The water mobility of N_2-NBW and H_2-NBW ((3200.0 ± 139.6) ms and (2957.1 ± 44.1) ms, separately) was higher than that of DW ((2497.9 ± 42.1) ms). Therefore, the effects of NBW on the gut microbes in mice might be achieved by the alteration in the food properties and in the mass transport rate.

3) Although NB has a variety of unique properties, its physiological activity might depend on the density of NB. All kinds of NBW in this study have more than 1.0×10^7 particles per milliliter. The existence of a high density of NBs creates the necessary condition for stably play its physiological activity on the gut microbes.

The effects of NBW on gut microbes might be due to the combined action of NB's zeta potential, water mobility and density.

5.4 Future perspectives

In this study, the effects of NBW on the gut microbes have been investigate, results suggested the NBW could affect the growth and metabolism of model strain LA 1028 and alter the gut microbiota in mice. However, the exact mechanism should be further investigated and the application of NBW in food and medicine fields should be expanded. To provide the theoretical and factual basis for the application of NBW in the practical production. Future researches should focus on the following topics.

1) Investigate the mechanism of NBW to promote the growth and metabolism of bacteria through molecular biological methods and from NB properties view including the DO, pH zeta potential, and water mobility.
2) Study the alteration of NBs in the long-term (> 30 days) preserved NBW and its effects on the growth and metabolism of bacteria.
3) The effects of NBW on the development of obesity in HFD fed mice should be further confirmed by changing the strain of mice and determining the markers which are related to lipid metabolisms including TG and TC in the liver and adipose tissue.
4) Study the effects of NBW on the prevention and treatment of chronic metabolic diseases such as diabetes and cancers through the animal experiment and omics technology.

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Acknowledgement

Three years of doctoral life in University of Tsukuba passed in a flash. Looking back on the past years, not only adds knowledge and ability but also gains valuable life wealth. When the thesis is about to be finished, I have many feelings.

Firstly, I would like to express my sincerely gratitude to my supervisor, Professor Zhenya Zhang. During the three-year study, Professor Zhang devoted much attention to guide me from the design of the subject, the implementation of the experiments to the writing of the thesis. I have benefited a lot from Professor Zhang's profound knowledge, rigorous academic attitude, keen insight, and the unremitting spirit of exploration. Professor Zhang's guidance and encouragement will inspire me to make greater efforts and innovations in my future work and life. Besides, I would like to appreciate other academic advisors, Professor Motoo Utsumi, Professor Zhongfang Lei, Professor Hidehisa Shimizu, and Professor Kazuya Shimizu, for their numerous suggestions, support, and encouragement during the doctoral study and the writing of the thesis.

Secondly, I am very grateful to the students in the lab. The daily communication and discussion with you have provided a lot of help for the completion of my project. I would like to appreciate every friend I have met in the past three years, for their accompany has made the life of studying aboard more colorful.

Finally, I want to express the depth of my gratitude to my mother and wife. It is their selfless contribution and active support that enables me to face all kinds of difficulties in scientific research and life bravely. This dissertation would not have been completed without their love and support.

Appendix

Zitao Guo, Xuezhi Wang, Hanxiao Wang, Bo Hu, Zhongfang Lei, Motoyoshi Kobayashi, Yasuhisa Adachi, Kazuya Shimizu, Zhenya Zhang (2019) Effects of nanobubble water on the growth of *Lactobacillus acidophilus* 1028 and its lactic acid production. RSC Advances, 9, 30760-30767.

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