Studies on preventive effects and mechanisms of garlic on dyslipidemia and gut microbiota dysbiosis

（ニンニクによる脂質異常症及び腸内細菌叢失調の予防効果及び作用機構に関する研究）

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2022

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Abbreviations

AFG	Alliinase free garlic
AMPK	AMP-activated protein kinase
ARE	Antioxidant response element
BCAAs	Branched-chain amino acids
BCFAs	Branched-chain fatty acids
BSH	Bile salt hydrolase
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CPT1A	Carnitine palmitoyl transferase 1A
CYP4A1	Cytochrome P450, family 4, subfamily A1
CYP7A1	Cytochrome P450 family 7 subfamily A member 1
DAS	Diallyl sulfide
DADS	Diallyl disulfide
DATS	Diallyl trisulfide
DCA	Deoxycholic acids
DPP-4	Dipeptidyl peptidase-4
ERK	Extracellular signal-regulated kinase
FFAR	Free fatty acid receptors
FMT	Fecal microbiota transplantation
FGFR4	Fibroblast growth factor receptor 4
FXR	Farnesoid X receptor
GSAC	γ-Glutamyl-S-allyl-L-cysteines
GOT	Glutamic-oxaloacetic transaminase
GPT	Glutamic-pyruvic transaminase
G6Pase	Glucose-6-phosphatase
HDL-c	High density lipoprotein cholesterol
HFD	High-fat diet
HOMA-IR	Homeostatic model assessment for insulin resistance
H&E	Hematoxylin-eosin
JNK	c-Jun N-terminal kinase
KEGG	Kyoto encyclopedia of genes and genomes
LCA	Lithocholic acids
LDL	Low-density lipoproteins
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
NAFLD	Non-alcoholic fatty liver disease
ND	Normal diet
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
Nrf2	The nuclear factor erythroid 2–related factor 2
PCR	Polymerase Chain Reaction
PD	Phylogenetic diversity
PEPCK	Phosphoenolpyruvate carboxy kinase
PPARα	Peroxisome proliferator-activated receptor alpha
ROS	Reactive oxygen species
SAC	S-ally-L-cysteine

SCFAs	Short-chain fatty acids
SHP	Small heterodimer partner
T-Cho	Total cholesterol
TG	Total triacylglycerol
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
WD	Western diet
OSCs	Organosulfur compounds
OTU	Operational taxonomic unit
ZO-1	Zonula occluden-1

Abstract

Garlic (*Allium sativum*) as a favorite health food has many functions. Especially, it has been reported that organosulfur compounds (OSCs) in garlic have the potential to improve lipid and glucose metabolism and regulate lifestyle-related diseases. However, the metabolic mechanisms on its function, and whether this function is related to gut microbiota remain unclear. In this study, the modes of animal experiment and bacterial culture in dyslipidemia and gut microbiota dysbiosis were used to investigate the effect and mechanism of garlic OSCs on gut microbiota, lipid and glucose metabolisms.

Firstly, the mice were fed with the high-fat diet (HFD) to induce dyslipidemia and gut microbiota dysbiosis, and HFD supplemented with 5% garlic for 12 weeks to investigate the effect. The result revealed that garlic supplementation attenuated HFD-enhanced ratio of serum GPT/GOT, the levels of total cholesterol and low-density lipoproteins. In addition, garlic supplementation was increased the relative abundance of f_*Lachnospiraceae*, while reduced the relative abundance of g_*Prevotella*. Therefore, garlic supplementation could meliorate the HFD-induced dyslipidemia and gut microbiota dysbiosis.

Secondly, we obtained alliinase-free garlic (AFG) with stable OSCs by inactivating the garlic alliinase. The effect of AFG on dyslipidemia and

gut microbiota dysbiosis were further investigated. The results revealed that AFG supplementation attenuated HFD-enhanced ratio of serum GPT/GOT. The ratio of p-*Firmicutes* to p-*Bacteroidetes* increased by aging and HFD was reduced by AFG. The AFG enhanced the f-*Lachnospiraceae*, g-*Akkermansia*, and g-*Lactobacillus* which were decreased by aging and HFD. AFG meliorated HFD-induced dyslipidemia and improve individual characteristic gut bacteria.

Thirdly, in order to clarify the effect and mechanism of AFG on the lipid and glucose metabolism, we used western diet (WD) containing high fat, high cholesterol and sugar. AFG with different OSCs concentrations was added to WD, with which the mice were fed for 12 weeks. Results revealed that garlic OSCs caused an increase in gut taurine and an inhibition of DPP-4, and ameliorated WD-induced disorders of lipid and glucose metabolism. Especially, the gut commensal *Bacteroides acidifaciens* (*B. acidifaciens*) was significantly increased by garlic OSCs. In *in vitro* culture, OSCs markedly increased the growth of *B. acidifaciens* growth in a dose-dependent manner. These data demonstrated that *B. acidifaciens*-taurine axis / DPP-4 axis was involved in the preventive effect of garlic OSCs on WD-induced metabolic disorder of lipid and glucose.

In conclusion, garlic OSCs has the preventive effects on metabolic syndrome and gut microbiota disorder induced by over nutrition diet.

Moreover, stable components and effects of garlic OSCs could be obtained by inactivating garlic alliinase. Furthermore, the mechanism of garlic OSCs on the regulation of lipid and glucose metabolism were clarified that OSCs enhanced the proliferation of the *B. acidifaciens* to produce taurine and inhibit DPP-4. These results will provide new insight for understanding the molecular mechanisms of garlic on the prevention of lifestyle-related diseases and gut microbiota disorder, and the scientific basis for the application of garlic in functional food.

要　旨

　ニンニク(*Allium sativum*) は健康食材として古くから愛用され、多くの機能性が報告されている。特に最近では、ニンニクの含硫黄成分が脂質と糖代謝を調節し、生活習慣病を改善する効果が報告されている。しかし、その作用機構、特に腸内細菌叢との関連性は不明のままである。本研究は、動物実験及び細菌培養手法を用いて、ニンニクによる脂質異常症や腸内細菌叢失調の予防効果及び作用機構を解析した。

　まず、高脂肪食（HFD）で脂質異常症及び腸内細菌叢失調のマウスを作成し、5%のニンニク含有飼料を 12 週間投与した。その結果、ニンニク投与はHFD で増加された血清肝臓逸脱酵素（GPT/GOT）、総コレステロール及び低密度リポタンパク質レベルを顕著に低減させた。また、ニンニク投与は腸内細菌である f_*Lachnospiraceae* の相対存在量を増加させ、g_*Prevotella* の相対存在量を減少させた。これにおりニンニク投与は、HFD で誘発された脂質異常症と腸内細菌叢失調を改善することを示した。

　次に、ニンニク含硫黄成分を安定化させるため、ニンニクのアリイナーゼを失活させ、アリイナーゼなしのニンニク(AFG)を調整し、その摂取による脂質異常症及び腸内細菌叢失調の改善効果を調べた。その結果、AFG は HFDで増加された血清肝臓逸脱酵素（GPT/GOT）を顕著に低減させた。また、腸内細菌 p-*Firmicutes* と p-*Bacteroidetes* の比率は加齢と HFD により増加し、AFGにより減少した。腸内細菌 f-*Lachnospiraceae*、g-*Akkermansia* 及び g-*Lactobacillus* は加齢と HFD により減少し、AFG により増加した。アリイナーゼなしのニンニクは、HFD で誘発された脂質異常症と特定の腸内細菌の改善

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効果を示した。

　脂質及び糖の代謝における AFG の効果と作用機構を明らかにするため、高脂肪食に高コレステロール及び高糖を加えた西洋食を用いて、異なる含硫黄成分濃度の AFG を 12 週間実験マウスに投与した。その結果、ニンニク含硫黄成分は腸内タウリンの増加及び肝臓ジペプチジルペプチダーゼ 4（DPP-4）の阻害を引き起こし、西洋食で誘発された脂質と糖代謝の失調を改善することを示した。特に、ニンニク含硫黄成分は腸内細菌 *Bacteroides acidifaciens* を有意に増加させ、体外培養でも *Bacteroides acidifaciens* 菌がニンニク含硫黄成分濃度依存的に増殖された。よって、ニンニク含硫黄成分は、*Bacteroides acidifaciens*/タウリン/DPP-4 の作用機構を通じ、西洋食で誘発された脂質と糖の代謝障害を改善することを示した。

　　　以上のように、ニンニクは過剰栄養食で誘発された生活習慣病と腸内細菌叢失調に対して改善効果を有し、その生理活性成分は含硫黄成分であった。含硫黄成分は、腸内細菌 *Bacteroides acidifaciens* を顕著に増殖させ、腸内タウリンの増加及び肝臓 DPP-4 の阻害を通じ、脂質と糖の代謝を改善することが明らかになった。さらに、ニンニクのアリイナーゼを失活させることにより、ニンニク含硫成分の分解及び揮発を防ぎ、安定なニンニク含硫成分及び効果を得ることができた。これらの知見は、生活習慣病と腸内細菌叢失調におけるニンニクの予防効果及びその作用機構を理解するうえに新しい科学知見を提供し、ニンニクの新たな機能性食材としての利用に繋がるものである。

Acknowledgement

First and foremost, I would like to express my sincere gratitude and appreciate to my chief advisor Prof. De-Xing Hou for providing me with a great opportunity, and for his patience and encouragement during my study at Kagoshima University. His enthusiasm, perseverance and excellence in conducting research have greatly inspired my life.

I would like to thank Prof. Hisham R. IBRAHIM, Kagoshima University, for his meticulous guidance and valuable suggestions. I would like to thank Prof. Md Amzad Hossain, Ryukyu University, for his cordial guidance. I would like to thank Dr. Kozue Sakao, Kagoshima University, for her patient guidance during my research.

I would like to express my sincere thanks to Yasushi Nakasone, KENKOUKAZOKU CO.,LTD, for provides samples and technical support during the past five year. I would also like to express my sincere appreciation to Rotary Yoneyama Memorial Foundation and all of the members of Kagoshima Central Rotary Club, for granted me a scholarship and meaningful experience during the past two year.

Finally, thanks to my cherished family and friends for their warm support. Thanks to my dear boyfriend Li Zhang for his everlasting support and companionship.

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Chapter 1   Introduction

1.1   Overview of garlic

Allium sativum, garlic has been used as a food and as a medicinal herb for thousands of years in worldwide, it is a valuable spice and a popular remedy for various ailments and physiological disorders. Based on current trends in economic value, garlic utilization can be expected to continue to expand. Aomori prefecture as the main garlic producing area in Japan, produces garlic with good quality, which is potential and suitable to be developed into a new functional food with added value in the future.

1.1.1   Origin and agronomy of garlic

Garlic is among the oldest known horticultural crops. In the Old World, garlic originated in Central Asia and the northeastern Iran, and was then introduced to China in the 1st century BC, to Japan and South Asia in the 9th century (Petrovska *et al.*, 2010). Garlic is most often planted with bulbs in the fall (between September and November), and is used to harvest in the summer (between May and July). Garlic has different temperature requirements at different stages of growth and development. After garlic planting, the differentiation of flower buds and squamous buds requires vernalization under low temperature stimulation below 10°C. The optimum temperature for garlic bulbs to mature was 20-25 °C, but it was not good

for bulb growth over 25℃. Garlic is a long-day plant, garlic does not form bulbs in 12 hours of sunlight in warm conditions, only suitable for leaf growth. (Brewster, J.L. *et al.*, 1990; Engeland *et al.*, 1991). However, the long sunshine and temperature difference in the Aomori Prefecture Japan is very good for garlic.

Fig.1-1 Garlic cultivation in Aomori prefecture
(2014-2018 Garlic Yoshidaya. https://www.yoshidaya-garlic.jp/?mode=f16)

Aomori Prefecture is the largest garlic producing area in Japan, accounting for about 70% of the domestic garlic production. Moreover, not only is the production volume high, but the garlic cultivated in Aomori Prefecture is large and white in color, and has a stronger sweetness than spiciness, thus has a reputation for being extremely delicious. As shown in fig.1-1, the severe winter cold and suitable soil that is unique to Aomori Prefecture, it becomes the Aomori garlic with a high sugar content and a rich flavour than other.

1.1.2 Economy and future of garlic

Today, garlic is an indispensable and important agricultural product in the world. Garlic production of World increased from 2.85 million tonnes in 1970 to 30.7 million tonnes in 2019 at an average annual rate of 5.12%, based on data from the Food and Agriculture Organization Corporate Statistical Database. Relatively, garlic is easy to grow and can be grown year-round in mild climates, and garlic plants are usually hardy and not affected by many pests or diseases (The Royal Horticultural Society, 2017). This makes garlic a less difficult crop to grow, which can achieve low cost and high harvest rate.

The agricultural value of garlic is mostly sold on fresh garlic, and there are few deep-processed garlic products, and the economic benefits now are relatively low. 70% to 80% of the total garlic is sold in supermarkets through refrigeration, and the remaining 20% small garlic and residual garlic are digested by processing garlic flakes and garlic powder and other garlic seasoning products (Yong-Jun Zheng *et al.*, 2012). Although the cost of growing garlic is very low, garlic is a very seasonal agricultural product. Although fresh garlic can be stored in cold storage to extend its freshness, the longest can only be postponed until the second year of new garlic on the market, otherwise it will lose its commercial value (Xiu-Li Dou *et al.*, 2013). Therefore, the development of secondary and

deep processed garlic products is of great significance, one is to reduce the waste of garlic, and the other is to increase the added value. Presently, the high-end products in the garlic intensive processing chain, such as natural allicin, alliin, black garlic fermented food, garlic egg yolk and other medical and health foods, are still in their infancy and have a lot of room for development.

In the future, in order to increase the added value of garlic and increase the economic efficiency of garlic, it should be implemented mainly through the following methods: 1. Intensive and deep processing transforms garlic into medicines and health products, 2. Extends the garlic industry chain, and transforms its resource advantages into industrial strength . The development of garlic health supplementation products has very important practical significance.
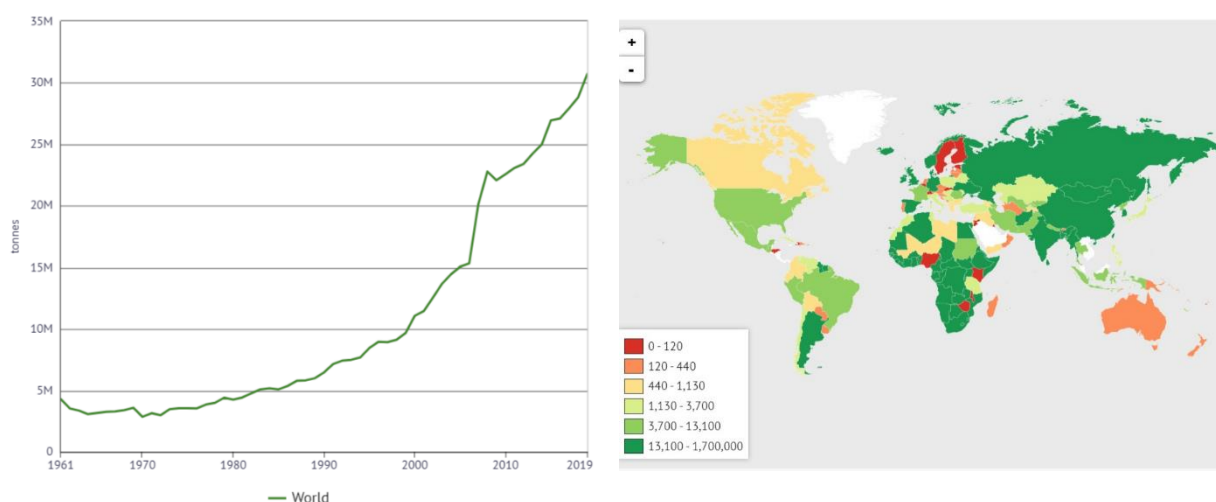
Fig.1-2 Garlic production of world statistics

1.2 Functional components of Garlic

The organosulfur compounds (OSCs) are organic compounds that contain sulfur, and OSCs are widely present in our bodies and the natural environment. In addition, a large number of studies in laboratories and animal models have shown that garlic has a wide range of biological activities, most of which are dependent on garlic's organic sulfur compounds. Natural organosulfur compounds are often found in vegetables of family *Brassicaceae* and family *Amaryllidaceae* such as broccoli and red cabbage, garlic, and onions. The organosulfur compound in garlic is the most studied natural OSC, and its types and transformation reactions are also very complicated, because cooked or processed garlic products showed different kinds of garlic OSCs, some of which are highly unstable and instantly decomposed (Phoebe Zapanta Trio *et al.*, 2014).

1.2.1 The organosulfur compounds

Organosulfur compounds (OSCs) are abundant in our bodies and the natural environment, and can be derived from both plant and animal sources. The most common source of sulfur for humans is a diet composed of broccoli, cauliflower, cabbage, garlic, onion, meat, eggs, and fish (Sener et al *et al.*, 2007; Vazquez-Prieto *et al.*, 2010). There are two principal

groups of vegetables that contain OSC with special properties. As shown in the figure 1-3, there are some common functional OSCs, mainly sulforaphane, raphanin, 6-MSITC contained in family *Brassicaceae* vegetables, and alliin, allicin and diallyl sulfide (DAS) in *Allium* genus (family *Amaryllidaceae*) vegetables (Ramesh C. Gupta *et al.*, 2021).
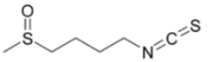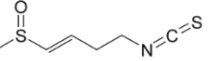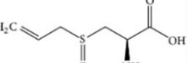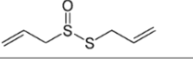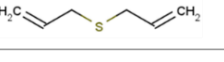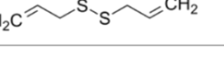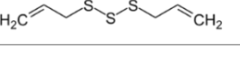
	Name	Chemical formula	Chemical structural formula	Main source
Family **Brassicaceae**	Sulforaphane	C6H11NOS2		Cruciferous vegetables (broccoli, brussels sprouts, cabbages)
	Raphanin	C6H9NOS2		Radish seeds of *Raphanus sativus*, broccoli and red cabbage
	6-MSITC 6-(Methylsulfinyl)hexyl isothiocyanate	C8H15NOS2		Cruciferous *Eutrema* vegetables (wasabi)
Family **Amaryllidaceae**	Alliin	C6H11NO3S		Garlic, Onion
	Allicin	C6H10OS2		Garlic, Onion
	Diallyl sulfide (DAS)	C6H10S		Garlic
	Diallyl disulfide (DADS)	C6H10S2		Garlic
	Diallyl trisulfide (DATS, Allitridin)	C6H10S3		Garlic

Fig.1-3 Main organosulfur compounds and their sources

Some classify organosulfur compounds according to the functional groups to which the sulfur is attached. The allium genus of flowering plants, which includes garlic and onions, contains important compounds such as cysteine sulfoxides and γ-glutamylcysteines. The hydrolysis of cysteine sulfoxides accounts for the flavor and pungency of garlic and onions (Vazquez-Prieto *et al.*, 2010). As shown in Fig.1-4, the major cysteine sulfoxides are of four types: *S*-allylcysteine sulfoxide (alliin), *S*-1-

propenylcysteine sulfoxide (isoallin), *S-n*-propylcysteine sulfoxide (propiin) and *S*-methylcysteine sulfoxide (Methiin). Onions are especially rich in isoallin, whereas garlic is rich in alliin (Naoko Yoshimoto *et al.*, 2019).

major *S*-alk(en)ylcysteine sulfoxides

(+)-alliin   (+)-isoalliin   (+)-propiin   (+)-methiin

minor *S*-alk(en)ylcysteine sulfoxides

(−)-*allo* alliin   (+)-ethiin   (+)-butiin
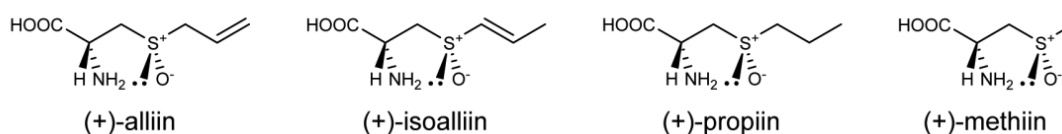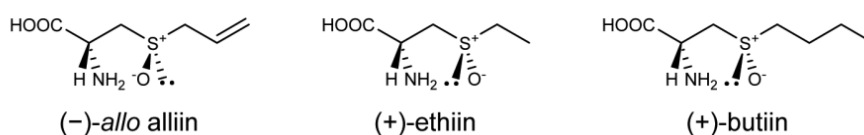
Fig.1-4 Chemical structures of main *S*-alk(en)ylcysteine sulfoxides in the genus *Allium*.

(Naoko Yoshimoto *et al.*, 2019)

1.2.2   The garlic organosulfur compounds

As far as this, organosulfur compounds in garlic have been reported and studied more than any other plant, garlic contains 1.1–3.5% organosulfur compounds (Matsuura. H *et al.*, 1997), and their types and transformation reactions are also very complicated. Since the OSCs in garlic will produce different OSCs due to different processing and cooking methods, thus the functionality as a food supplement will vary.
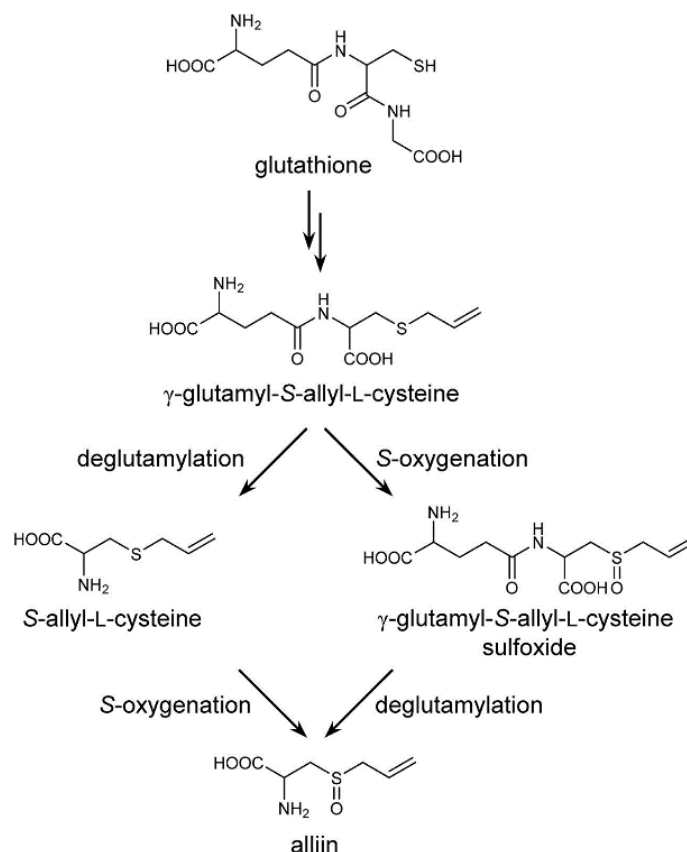
Fig.1-5 Proposed biosynthetic pathway for alliin in garlic.

(Naoko Yoshimoto, 2015)

In raw garlic bulbs, *S*-allyl-L-cysteine sulfoxide (alliin) accounts for approximately 80% of cysteine sulfoxides, which is biosynthesized by γ-glutamyl-*S*-allyl-L-cysteine (GSAC) and *S*-ally-L-cysteine (SAC), are major natural OSCs (Lawson LD, 1998). As shown in Fig.1-5, Biosynthesis of *S*-allyl-L-cysteine sulfoxides in garlic has previously been proposed to proceed via glutathione *S*-conjugates, according to the results of precursor feeding and pulse radiolabelling experiments. In the proposed pathway, glutathione is *S*-alk(en)ylated at the cysteine residue, followed by the removal of a glycyl group to form a biosynthetic intermediate, γ-

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glutamyl-*S*-alk(en)yl-L-cysteine (GSAC). This γ-glutamylated sulfide compound is further de-glutamylated and *S*-oxygenated to yield *S*-alk(en)yl-L-cysteine sulfoxide (Naoko Yoshimoto *et al.*, 2015). At present, most of these OSCs such as alliin, G-SAC, SAC in whole garlic bulbs is characterized by high stability and water solubility, and they do not have strong bactericidal ability.
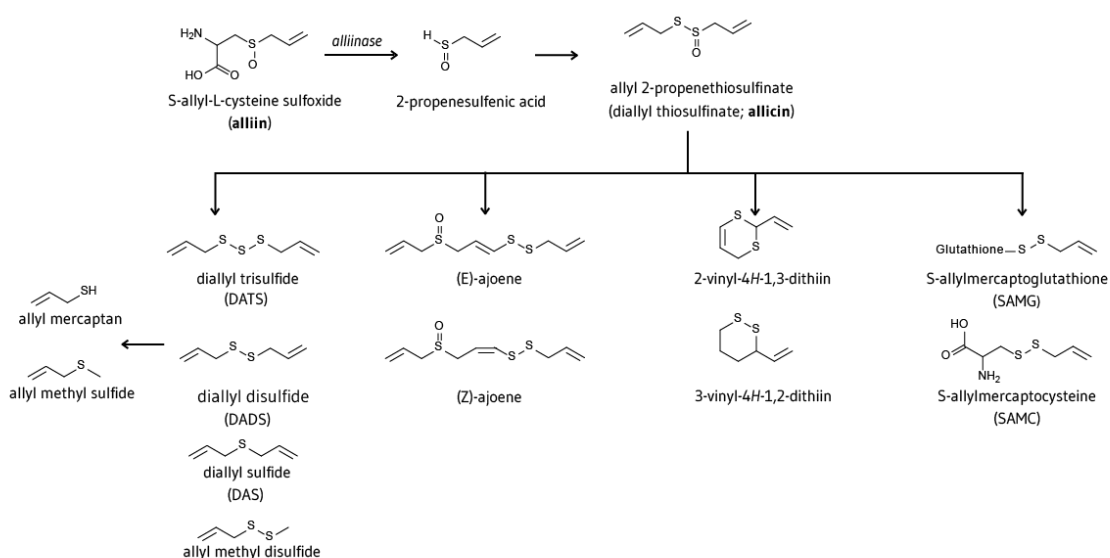
Fig.1-6 Biotransformation processes of alliin from garlic into different garlic OSCs.

(Phoebe Zapanta Trio *et al.*, 2014)

As shown in Fig.1-6, after crushing, cutting, and grinding of raw garlic bulb, alliin forms complexes with released alliinase to generate allicin. Allicin is an unstable and highly reactive molecule that transforms into a variety of fat-soluble organosulfur compounds, such as diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), ajoenes *in vitro*

(Phoebe Zapanta Trio *et al.*, 2014, Amagase H *et al.*, 2006). Allicin and its derivatives are mainly characterized by low stability, soluble in organic solvents and difficult to dissolve in water, and strong antioxidant and antibacterial ability.

In studies conducted in rodents, orally administrated alliin was found to be absorbed intact and to reach plasma and liver without being converted to allicin. There are no thiosulfates (like allicin) in intact garlic, and none can be generated in the stomach because alliinase would be irreversibly inhibited under acidic conditions (Amagase H *et al.*, 2006). Therefore, alliinase acts as a bridge in this series of reactions. As long as garlic remains intact and not damaged, alliinase will not be released, and then allicin will not be catalysed.

1.2.3 Overview of fructans

Fructan is a polymer of fructose molecules. Fructan as a soluble storage carbohydrate is present in over 12% of angiosperms (Hendry *et al.*, 1987) and is found mainly in flowering plants of the order Astrologes, Poales and Liliaceae. Vegetables with the highest quantity of fructans included garlic (17.4g/100g), Jerusalem artichoke (12.2g/100g), shallots(8.9g/100g), leek bulb(7.1g/100g), and spring onion bulb (6.3g/100g) (Jane G Muir *et al.*, 2007). Fructans are also designated as low-

calorie sweeteners, fat replacers, and prebiotics in the human diet.

As shown in fig.1-7, based on the glycosidic linkage, fructans are categorized into five groups: (1) Inulin type: linear inulin consists of β-1,2-linked fructose residues attached to a sucrose core and is found in members of the Asterales family (e.g., chicory). (2) Levan (or phlein) type: levan are found in grasses and consist of a β-2,6-linked fructose chain attached to sucrose. (3) Graminin type: having inulin or levan backbone with at least 1 short branch, also found in grasses. (4) Inulin neo-series type: like inulin but one glucose unit between two fructose moieties, possesses two β-1,2-linked fructose chains attached to the sucrose core and is found in members of the Liliacae family (e.g., onions). (5) Levan neo-series type: like levan but one glucose unit between two fructose moieties (RN Chibbar *et al.*, 2015; Robert V. Stick *et al.*, 2009).
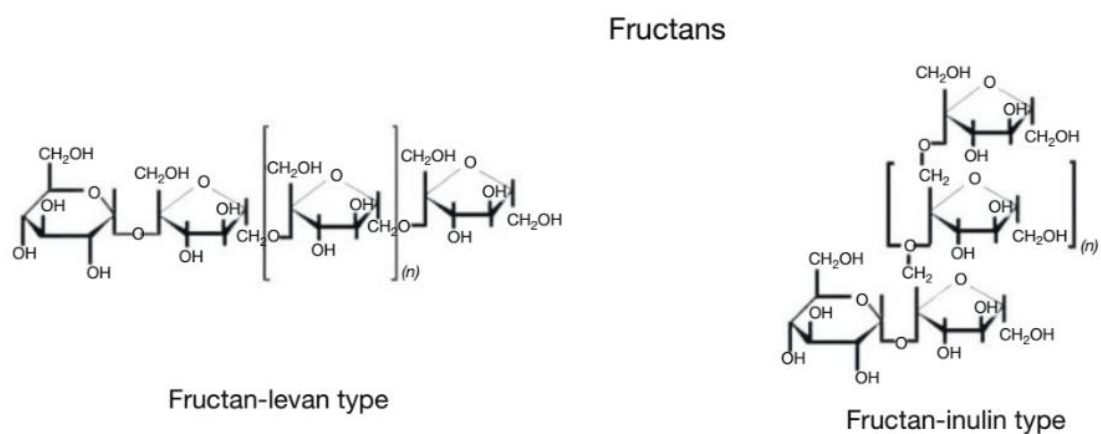
Fig.1-7 Main fructans type.

(R.N.Chibbar, 2015)

1.2.4 The garlic fructans

Garlic was found to contain fructans 12.5% to 23.5% on a fresh weight basis, and more than 75% on a dry weight basis. As shown in fig.1-8, garlic fructan is an inulin neo-series type fructan that has a (2→1)-linked β-d-Fru f backbone with a (2→6)-linked β-d-Fru f side chains. A structural model for high molecular weight garlic fructan estimated a degree of polymerization (DP) of about 58 based on its molecular weight and fructose: glucose ratio about 15:1 (S. Baumgartner *et al.*, 2000; J.N. Losso *et al.*, 1997).
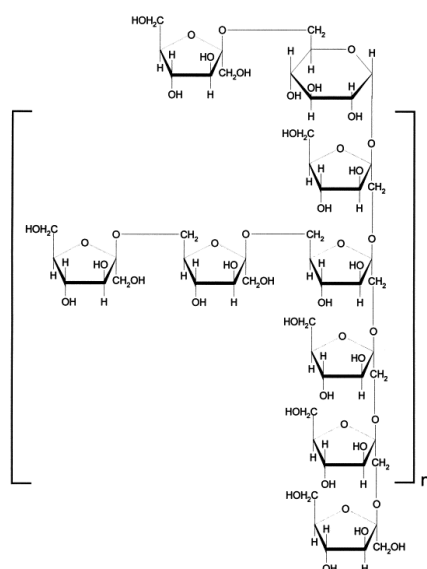
Fig.1-8 Suggested structure for the garlic fructan; n=9 for a dp of 58.

(S. Baumgartner *et al.*, 2000)

1.3 Biological activity of garlic

Garlic as a traditional medicine, which has been widely used since ancient times as an antiseptic for skin, a food preservative, a remedy for indigestion and fever, and a remedy for respiratory diseases (R. S. Rivlin, J *et al.*, 2001; L. Shamseer *et al.*, 2006). Garlic has been reported to possess several biological properties including antioxidant, anti-inflammation, antibacterial, antifungal, anticarcinogenic, anti-obesity, antidiabetic, anti-atherosclerotic and antihypertensive activities.

1.3.1 Antioxidant

As shown in figure3-1, A large number of studies in laboratories and animal models have shown that garlic has antioxidant activity, the mechanism of which mainly depends on garlic's organic sulfur compounds. The mechanisms at least involve OSCs reacting with intracellular glutathione to produce the thiol derivative, since allicin could easily penetrate the cellular membrane and it readily reacts with the most abundant non-protein thiol in the mammalian system (a). OSCs modulate Nrf2-ARE pathway to enhance the expressions of antioxidative enzymes or protein genes (b), and also downregulate ROS-induced NF-κB and MAPK signaling to exert the crosstalk with anti-inflammatory activity (c) (Phoebe Zapanta Trio *et al.*, 2014).

In addition, fructans may play an antioxidant role as ROS scavenger (Elena Franco-Robles *et al.*, 2015). Recently reports have suggested that fructans possess antioxidant activity in *in vivo* models. The addition of fructans to the diet may provide an early defense against oxidative stress and may act before the activation of the endogenous ROS detoxification systems (Jérôme Busserolles *et al.*, 2003).
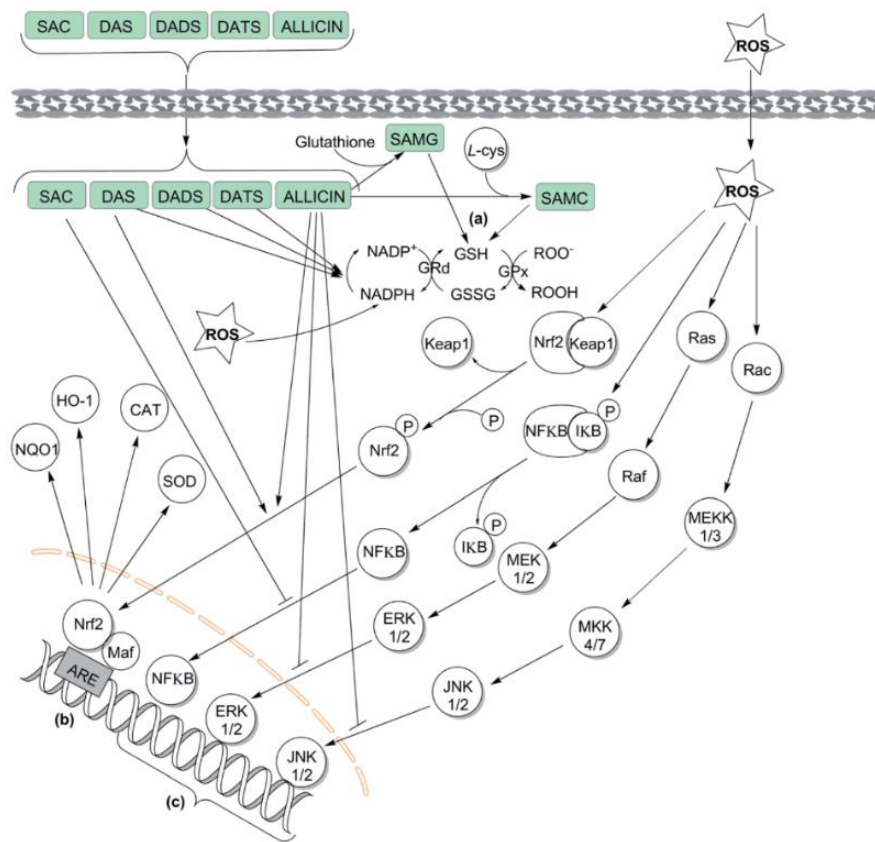
Fig.1-9 The schematic mechanisms of the antioxidative activities of garlic OSCs.

(Phoebe Zapanta Trio *et al.*, 2014)

1.3.2 Anti-obesity

Garlic has been repeatedly reported to have anti-obesity function, not only various extracts or processed products such as black garlic, aged garlic extract, garlic aqueous extract, garlic oil, garlic powder, but also including individual substances in garlic, such as alliin and allicin (Tanvir Ahmed *et al.*, 2021; Ao Shang *et al.*, 2019; Chao Yang *et al.*, 2018; Baiqiang Zhai *et al.*, 2018; Xin'e Shi *et al.*, 2019;). Obesity is the most common health problems that may lead to many ailments like hypertension, dyslipidemia, cardiovascular disorders, and metabolic syndrome. It has been reported that the anti-obesity activity of garlic is mainly reflected in dyslipidemia regulation, such as reducing body weight, adipose tissue mass, serum triglyceride, total cholesterol, low-density lipoprotein, and plasma malondialdehyde in mice with high-fat-diet-induced obesity (Gaber El-Saber Batiha *et al.*, 2020).

However, the direct effects of garlic OSCs on obesity have not been well clarified due to its complex and varied ingredients. It has revealed that the anti-obesity effect of garlic extracts attributed to stimulation of AMP-activated protein kinase (AMPK) as well as increased thermogenesis and decreased multiple genes expression that is included in adipogenesis (Mak-Soon Lee *et al.*, 2011). Moreover, a recent study investigated the effects and possible mechanisms of alliin on 3T3-L1 cells adipocyte

differentiation, and suggested that alliin exhibits anti-obesity activity by downregulating major adipogenic differentiation-related genes and Akt/PI3K expression (Ni Li *et al.*, 2021). In addition, there are also many animals' experimental reports that alliin's anti-obesity function is related to gut bacteria (Baiqiang Zhai *et al.*, 2018).

1.3.3 Anti-diabetes

A lager of animal model and human studies reported the antidiabetic effect of garlic extracts and its derived bioactive molecules (Ashraf R *et al.*, 2005; Juan Wang *et al.*, 2017; Baiqiang Zhai *et al.*, 2018). Although garlic's anti-diabetes function has been beyond doubt, its anti-diabetes mechanism still has many possibilities to explore. Garlic OSCs is widely believed to play an anti-diabetic role as insulin secretor or insulin stimulator. Alliin possibly accomplished its insulin-independent therapeutic effect by amplifying the antioxidant activity and lipolytic enzymes, and alliin as an insulin secretagogue could stimulate insulin secretion from β-cells in in vitro experiment. (K. T. Augusti *et al.*, 1996; Phoebe Zapanta Trio *et al.*, 2014). Recently, studies have also shown that garlic extract could as an DPP-4 (dipeptidyl peptidase-4) inhibitors to reduce diabetes (Poonam Kalhotra *et al.*, 2020).

1.4 Overview of gut microbiota

In recent years, progressively attention has been paid to the impact of gut microbiota on the host, and it has been found that there is an important link between gut microbiota and the host's intestinal, liver, brain and other organs and their diseases. Since 2013, the National Institutes of Health Mutual Fund has initiated and supported the Integrated Human Microbiome Project (iHMP). The overall mission of the HMP is to generate resources to facilitate characterization of the human microbiota to further our understanding of how the microbiome impacts human health and disease. With the advancement of technology, the analysis of intestinal bacteria has also improved, not only enriching the bacterial database, but also facilitating the analysis of technology.

1.4.1 Research status of gut microbiota

As published in Nature Outlook in 2020, the recent advances highlights the followings on gut microbiota: (1) The gut's link to mental health: strengthened the link of gut-bacterial, showed around 50 routes can produce neuroactive metabolites, and found bacteria that produce a metabolite of the neurotransmitter dopamine (M.Valles-Colomer *et al.*, 2019); (2) The key to fecal transplantation: A clinical trial of fecal microbiota transplantation (FMT) for ulcerative colitis might have

identified bacterial species that could help to treat this form of inflammatory bowel disease (S. Paramsothy *et al.*, 2019); (3) Microbe boosts metabolic health: According to a clinical trial, a daily dose of the designated bacteria might treat metabolic syndrome including type 2 diabetes and serious cardiovascular disease, and that is marked by obesity, high blood pressure, and raised levels of blood sugar, fats, and cholesterol (C. Depommier *et al.*, 2019); (4) Microbe boosts metabolic health: The genes associated with both microbiome structure and insulin responses influence gut microbiomes, which in turn disrupt insulin signaling (S. Sanna *et al.*, 2019).

1.4.2 Recent studies on the mechanisms of gut microbiota

As shown in fig.1-10, the relationship between gut bacteria and host metabolic syndrome on fat and sugar metabolism has been mainly based on the classical theory of gut bacteria - bile acid axis. Bile acid feedback regulation of bile acid synthesis via enterohepatic circulation of bile acids (John Y.L.Chiang *et al.*, 2020). Bile acids can be divided into unconjugated bile acids and conjugated bile acids according to their structure. The unconjugated bile acids include primary bile acids which are synthesized in the liver: cholic acid (CA) and chenodeoxycholic acid (CDCA), and secondary bile acids which are transformed by the intestinal microbiota including, deoxycholic and lithocholic acids (DCA and LCA). CA and

CDCA are the two major primary bile acids, whereas CA and α-muricholic acid (α-MCA) and β-muricholic acid (β-MCA) are the major primary bile acids in rodents. The primary bile acids CA and CDCA are conjugated to taurine (T) or glycine (G) in the liver, to form a total of 8 possible conjugated bile acids which also are often referred to as bile salts (T/GCA, T/GCDCA).

The gut microbiota has been suggested to be critically important for bile acid metabolism because of its expression of bile salt hydrolase (CA), which converts conjugated bile acids to unconjugated bile acids, therefore greatly affecting bile acids compositions. As reported, the BSH activity was mainly attributed to *Lactobacilli* and *Bifidobacteria, Streptococcus* and *Lactococcus* genera (Stefano Fiorucci *et al.*, 2015; Annika Wahlström *et al.*, 2016; Fengjie Huang *et al.*, 2019; Suocheng Hui *et al.*, 2019). However, different bile acids have different effects on farnesoid X receptor (FXR), either activating or antagonizing. This depends on the ratio of conjugated bile acids pools and unconjugated bile acids pools in the gut, in turn depending on the proportion of BSH-producing bacteria in the gut.

Activation of FXR in ileal enterocytes releases Fgf15 (FGF19 is the human ortholog), which reaches hepatocytes through the portal circulation, binds to Fibroblast Growth Factor Receptor 4 (FGFR4) and complex activates JNK/ERK signaling that inhibits expression of CYP7A1, thus

repressing bile acid synthesis by hepatocytes. Targeting FXR signaling by specific agonists and/or antagonists, probiotics and/or diet components is a promising approach for the treatment of metabolic disorders, diabetes, and obesity (Stefano Fiorucci *et al.*, 2015; AnnikaWahlström *et al.*, 2016).
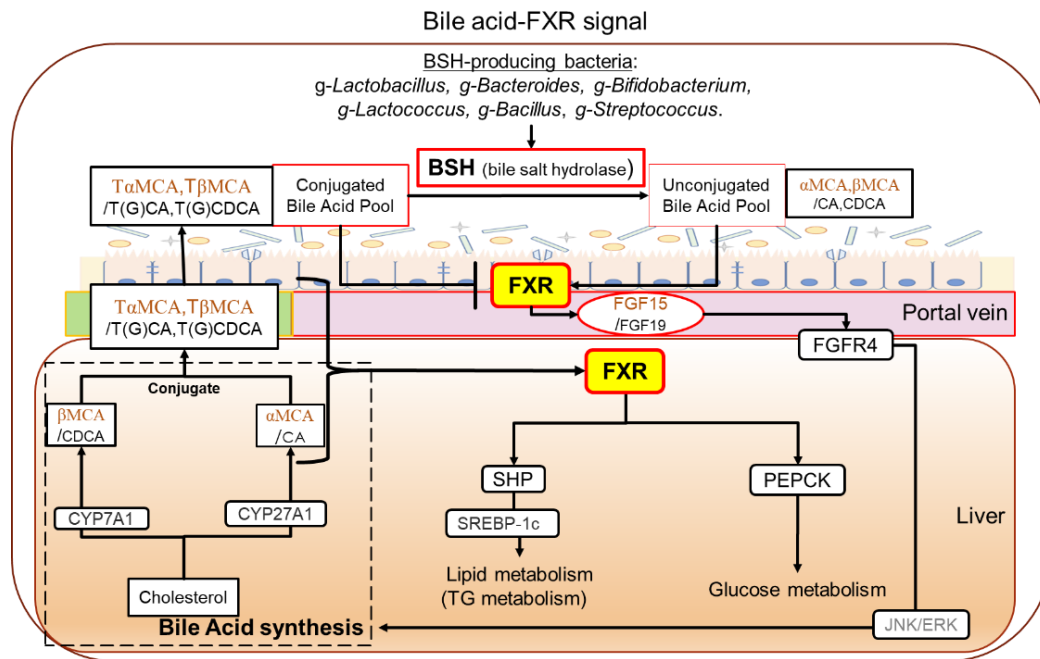
Fig.1-10 The metabolic mechanism of gut bacteria-bile acid-FXR signal.

As shown in fig.1-11, The relationship between gut bacteria and host enteritis or other inflammation-related diseases has been widely based on the theory of gut bacteria – Lipopolysaccharide (LPS) axis. The epithelial cell layer and the outer/inner mucin layer constitute the physical barrier and are often referred to as the gut barrier (S. S. Ghosh *et al.*, 2020). The endotoxin lipopolysaccharide (LPS) is produced by most Gram-negative bacteria, which induces local inflammation and active LPS into the

systemic circulation. To develop targeted therapies for improving intestinal barrier function, it is imperative to have a deeper understanding of the intestinal barrier itself, the mechanisms underlying the development of diseases due to barrier dysfunction (high circulating LPS levels). The gut mucosa forms a protective barrier for epithelial cells which are sealed by tight junction proteins such as Occludin, Claudin, and ZO-1, preventing paracellular transport (C. Belzer *et al.*, 2021). Recent evidence suggests that probiotics can restore the mucosal barrier integrity, ameliorate inflammation, such as *Akkermansia muciniphila*, *Lachnospiraceae, Bifidobacterium* (F. Ashrafian, 2019; Reeves, A.E, 2012). Also, there are some typical LPS-producing bacteria such as *Desulfovibrio*, *Escherichia coli* (N. Fuke *et al.*, 2019; T. L. Lin *et al.*, 2020)
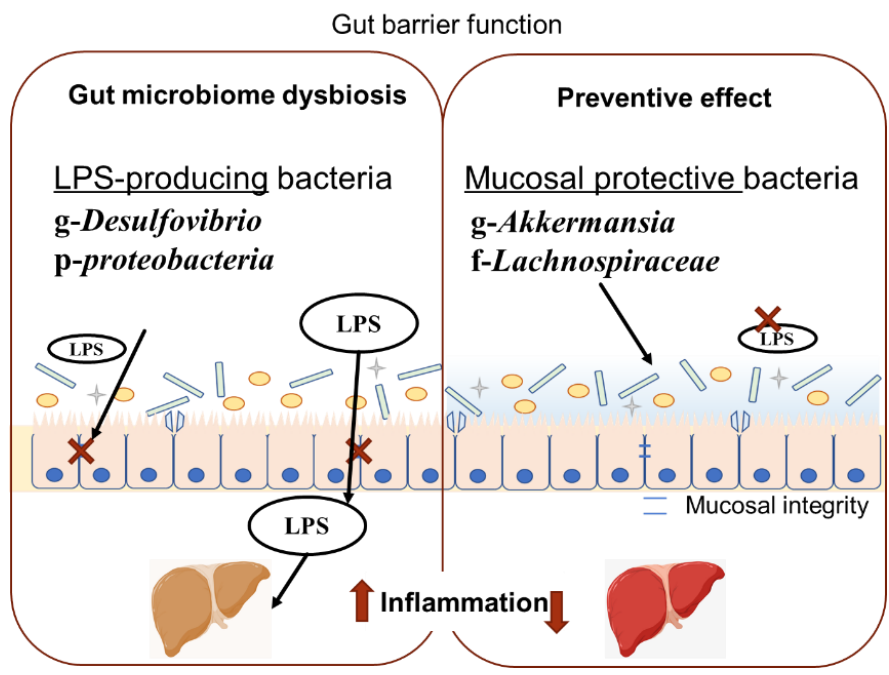
Fig.1-11 The metabolic mechanism of gut barrier function.

1.4.3 Garlic and gut microbiota

Foods that affect gut bacteria have been significantly reported, fermented foods, dietary fiber and polyphenols were reported more frequently. In addition, vegetables of family *Brassicaceae* and *Amaryllidaceae* including garlic are rarely reported, which may be due to the misunderstanding caused by the antibacterial effect of OSCs. However, the beneficial effects of garlic on the gut microbiota have been reported recently.

More widely report was on garlic fructans, which has the potential not only to bind as ligands to TLR2 and TLR4 to improve immunomodulatory properties, but also to be metabolized by gut bacteria as prebiotics (D. Peshev *et al.*, 2014). Fructans and their fermentation products (SCFAs, H_2) may act as signalling compounds, differentially affecting different cell types by influencing AMPK and/or NF-κB signaling pathways (D. Peshev *et al.*, 2014). In *vitro* culture experiments, some members of the fecal bacteria utilized garlic fructans to grow resulting in a decrease in the pH of the cultures, *Bifidobacterial* were stimulated while *Clostridia* were inhibited by the garlic fructans (Ning Zhang *et al.,* 2013). In addition, studies on garlic essential oil or garlic powder containing OSCs crude extracts indicated that their inhibited pathogens such as *streptococci* (Ankri *et al.,*1999; Groppo *et al.*, 2007), and some beneficial bacterial species in

the gut was increased after a long-time *in vitro* culture, such as *Lactobacilli* (A. Filocamo *et al.*,2012). However, due to the complexity of garlic samples and the difficulty in the control and analysis of gut bacteria, the mechanism of garlic regulating gut microbiota is still not clear.

To this end, the high-fat model was established by high-fat diet or western diet in mice, and the correlation between garlic regulation of fat or glucose metabolism and gut microbiota was examined. My research in divided into three steps to explore: 1. explore the effects of natural garlic powder on gut microbiota in mice; 2. according to the composition characteristics of garlic, alliinase free garlic will be developed and its effect on gut microbiota in mice will be explore; 3. according to the results of the previous two studies, specific gut bacteria that garlic has the potential to regulate were searched, and the mechanism of regulating host fat and glucose metabolism will be exploring.

Chapter 2 Preventive effects and mechanisms of garlic on dyslipidemia and gut microbiome dysbiosis

2.1 Introduction

Garlic (*Allium sativum* L.) has long been used for both culinary and medicinal purposes by many cultures. Based on fresh weight, garlic contains water (62–68%), carbohydrates (26–30%), proteins (1.5–2.1%), amino acids (1.0–0.5%), organosulfur compounds (1.1–3.5%), and fiber (1.5%). Carbohydrates are the most abundant class of compounds present in garlic bulbs and account for about 77% of the dry weight. The majority of the carbohydrate material in garlic consists of water-soluble fructose polymers called fructans (Koch *et al*., 1996), accounting for approximately 65% of the dry weight (Lawson *et al*., 1995). Garlic fructans are polymerized polysaccharides with high molecular weight ranging from <1000–6800 Da, corresponding to the degree of polymerization (Baumgartner *et al*., 2000). The biological activities of fructans have been intensely investigated as non-digestible polysaccharides or dietary fiber (Kelly, G.S *et al*., 1999; Paulsen, B.S *et al*., 2001; Block, K.I *et al*., 2003), especially their use as selective substrates to stimulate probiotic bacterial growth and immunomodulation (Tsai, C.C *et al*., 2013; Vogt, L *et al*., 2014; Xu, Q *et al*., 2006). Additionally, direct interaction between fructans

and intestinal immune cells has been recently suggested (Franco-Robles *et al.*, 2015).

On the other hand, garlic contains 1.1–3.5% organosulfur compounds, which is much higher than that in other plant food. In intact garlic, the primary organosulfur compounds (OSCs) are γ-glutamyl-*S*-allyl-L-cysteines (GSAC), which are hydrolyzed and oxidized to yield S-allyl-l-cysteine sulfoxides (alliin) during storage (Matsuura, H *et al.*, 1997). Crushing or chopping or chewing garlic releases alliinase, which catalyzes alliin to allicin and other thiosulfates (Amagase, H *et al.*, 2006). Allicin is considered to be responsible for most of the pharmacological activity of crushed raw garlic cloves (Larry, D *et al.*, 2018). These OSCs have been thought to be the bioactive principles for numerous health benefits (Gardner, C.D *et al.*, 2007), especially for defense components with broad antimicrobial activity.

Intestinal microbes play an important role in maintaining a healthy body (Chu, F *et al.*, 2018). Dietary supplementation with rice bran and navy bean (Sheflin, A.M *et al.*, 2017), dendrobium polyphenols (Li, X.W *et al.*, 2018), and propolis (Wang, K *et al.*, 2018) has been shown to impact the composition and activities of gut microbiota (Fontana, L *et al.*, 2015). High-fat diet (HFD) feeding modulates the gut microbiome composition by decreasing the prevalence of specific gut barrier-protecting bacteria and

increasing the prevalence of opportunistic pathogens that can release free antigens such as lipopolysaccharides. This imbalance may be associated with higher gut permeability, leading to higher plasma levels of endotoxin and inflammation factors and eventually the development of metabolic disorders (Cani, P.D *et al*., 2007; Zhang, C *et al*., 2010).

The complex ingredients of garlic seem to have paradoxical results on the gut microbiome. Experiments with separated compounds showed that fructans work as prebiotics for the gut microbiome (Peshev, D *et al*., 2014), while garlic OSCs, such as allicin, thiosulfinates, and ajoene, act as antibacterial agents (Leontiev, R *et al*., 2018; Jakobsen, T.H *et al*., 2012). Therefore, it is necessary to clarify the influence of whole garlic intake in daily life on the gut microbiome. In this study, we used a mouse model with normal diet (ND) and HFD to investigate the influence and mechanisms of whole garlic on the gut microbiome. Dextrin was used as positive control because dextrin is a polysaccharide (Slavin, J., 2013), similar to fructan, and can stimulate the growth of probiotic strains such as *Actinobacteria* and *Bacteroidetes* (Barczynska, R *et al*., 2016), and reduced numbers of pathogenic bacteria (Elli, M *et al*., 2010).

2.2 Materials and methods

2.2.1 Chemicals and reagents

Garlic was harvested from Aomori Prefecture, Japan. After hot air drying (moisture content 60%), garlic was stored at −2 °C for 10 months, and then pulverized as garlic crude powder (moisture content 4.8%). The amounts of OSCs and fructan in garlic powder were determined by HPLC or fructan assay kit (Biocon Ltd, Nagoya, Japan), respectively (Table2-1 Nutrient analysis).

Table 2-1 Nutrients in garlic

G: Garlic Supplementation		
Nutrients	**Value**	**Method**
Calorie	370 kcal/100 g	Calorie = Protein × 4 + Lipid × 9 + Carbohydrates × 4
Protein	16.2 g/100 g	Kaida's method
Lipid	1.1 g/100 g	Acid decomposition method
Carbohydrates	73.9 g/100 g	100 − (protein + lipid + moisture + ash)
Salt	0.02 g/100 g	Sodium conversion
Sodium	7 mg/100 g	Atomic absorption spectroscopy
Moisture	4.8 g/100 g	High pressure drying
Ash	4.0 g/100 g	Ashing method
Fructan	54.8 g/100 g	Fructan analysis kit
G-SAC	3.385 mg/g	HPLC
Alliin	13.261 mg/g	HPLC
SAC	1.867 mg/g	HPLC
Allicin	4.714 mg/g (wet)	HPLC

Indigestible dextrin was obtained from natural corn starch with 95% dextrin and 5% water. Lard oil was obtained from Sigma-Aldrich Japan (Tokyo, Japan). The nutrient composition of the diets is shown in Table2-2. ND contained 21% protein, 6% fat, 54% carbohydrate, 4% cellulose,

and about 370 kcal/100 g total calories. HFD contained 21% protein, 40% fat, 10% carbohydrate, 4% cellulose, and about 570 kcal/100 g total calories.

Table 2-2 Dietary compositions of each group

Components (%)	ND	NDG	NDD	HFD	HFDG	HFDD
Lard	6.0	5.9	6.0	40.0	39.9	40.0
Corn starch	54.0	50.2	54.0	20.0	16.2	20.0
Casein	21.0	20.2	21.0	21.0	20.2	21.0
Sucrose	10.0	9.8	10.0	10.0	9.8	10.0
Cellulose	4.0	3.9	0.0	4.0	3.9	0.0
Mineral mix	3.5	3.5	3.5	3.5	3.5	3.5
Vitamin mix	1.0	1.0	1.0	1.0	1.0	1.0
Choline chloride	0.2	0.2	0.2	0.2	0.2	0.2
Methionine	0.3	0.3	0.3	0.3	0.3	0.3
G (Garlic)	—	5.0	—	—	5.0	—
D (Dextrin)	—	—	4.0	—	—	4.0
Total calories (kcal/100 g)	367.8	368.5	367.8	567.4	567.9	568.1

2.2.2 Mouse model

The animal experimental protocol was drafted according to the guidelines of the Animal Care and Use Committee of Kagoshima University (Permission NO. A12005). Male C57BL/6N mice (5 weeks of age) from Japan SLC Inc. (Shizuoka, Japan) were housed separately in cages with wood shavings bedding under controlled light (12-h light/days) and temperature (25 °C), and free access to water and feed. Mice body weight was weighed once a week. After acclimatization for 7 day (6 weeks of age), the mice were randomly divided into six groups ($n = 5$) and fed with ND, NDG (5% garlic in ND), NDD (4% dextrin in ND), HFD, HFDG (5% garlic in HFD), or HFDD (4% dextrin in HFD). After 12 weeks

feeding (18 weeks of age), mice were sacrificed after overnight fasting. The fresh feces were collected at the beginning (6 weeks of age) and the end of the experiment (18 weeks of age) for investigating the gut microbiome associated with different ages or diets.

2.2.3 Measurement of serum biochemical indicators

Blood sera were obtained from mice eyeballs and collected in a tube with coagulant (Separable microtubes, FUCHIGAMI, 170720, Kyoto, Japan) for 30 min at room temperature to coagulate properly and were acquired by centrifuging at 3000 rpm for 5 min and stored at −80 °C until use. The serum levels of glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), gamma-glutamyl transferase (GGT), total cholesterol (T-Cho), total triacylglycerol (TG), high density lipoprotein cholesterol (HDL-c), and glucose were measured with an automated analyzer for clinical chemistry (SPOTCHEM EZ SP-4430, Arkray, Kyoto, Japan). The level of LDLs (low-density lipoproteins) was calculated using the Friedewald equation (LDL = T-Cho – HDL-c − TG/5) (Warnick, G.R *et al*., 1990). The insulin concentration in serum was measured with an ELISA kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) according to the manufacturer's instructions. The index of homeostatic model assessment for insulin resistance (HOMA-IR) was

calculated with the function of fasting glucose × fasting insulin/405 (Turner, R.C *et al*., 1979).

2.2.4 Histomorphology

Mice ileum tissue was sliced with a freezing microtome system (Yamato, Saitama, Japan) according to the manufacturer's instructions. The slice (7 μm) obtained was then stained with hematoxylin-eosin (H&E) staining, and observed under a fluorescence microscope (Keyence, Tokyo, Japan).

2.2.5 Cecal organic acid analyses

Cecum and cecum contents were isolated and weighed. Each 0.3 g sample of cecum content was transferred into 0.6 ml of distilled water, and stood on ice for 10 min after adding 0.09 mL of 12% peroxide acid. The supernatant was filtered after centrifugation with 15000 g at 4 °C for 10 min, and then used for organic acid analysis using ion-exclusion high-performance liquid chromatography with LC-10AD pump (Shimadzu, Kyoto, Japan) and Electrical conductivity meter (Waters431, Kyoto, Japan). Component identification was performed by CBM-20A data module (Shimadzu, Kyoto, Japan) (Tsukahara, T *et al*., 2014).

2.2.6 Characterization of the gut microbiome by 16S rRNA gene sequencing

Mice feces were collected from mice housed in different cages at 6- and 18-week age, and stored at −80 °C until use. The fecal genomic DNA was extracted with the Fast DNA spin kit for feces (MP BIOMEDICALS) according to the manufacturer's instructions, and used for analyzing the composition of gut bacterial communities by sequencing 16S rRNA genes, as described in our previous paper (Wu, S *et al*., 2018).

2.2.7 Statistical analysis

Results were expressed as mean ± SD or median and range. All of the data were first evaluated with Shapiro-Wilk test to assess the normality of the distribution. The data satisfying the normality were further evaluated using Levene's test for equal variances to test the equality of variances between populations or factor levels. The data of equal variances were analyzed by one-way analysis of variance (ANOVA) tests, followed by Duncan's multiple range tests with the SPSS statistical program (version 19.0, IBM Corp., Armonk, NY, USA). A probability of $p < 0.05$ was considered significant.

2.3 Result

2.3.1 Body weight and index of liver injury

The final body weight of mice fed with HFD at 18 weeks was significantly higher than the mice fed with ND (ND: 36.5 ± 3.34 g, HFD: 47.0 ± 2.19 g, $p < 0.05$, Fig.2-1) although there was no difference in initial body weight and in daily food intake throughout the 12-week intervention period. Supplementation with garlic had no significant effect on body weight in both the ND and HFD groups, while supplementation with dextrin increased the body weight in the ND group.
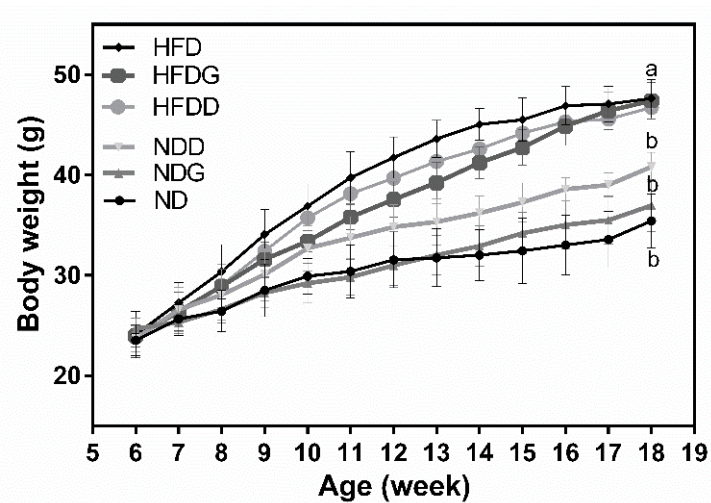
Fig.2-1 Effects of garlic supplementation on mice body weight. Results are expressed as the mean $\pm$ SD for each group of rats (n = 5). The body weights at 18 weeks with different letters significantly differ (p < 0.05). HFD: high-fat diet; HFDG: 5% garlic in HFD; HFDD: 4% dextrin in HFD; ND: normal diet; NDG: 5% garlic in normal diet; NDD: 4% dextrin in normal diet.

Moreover, the serum levels of GOT and GPT were significantly increased ($p < 0.05$ in Fig.2-2) in the HFD group, and they were significantly reduced ($p < 0.05$) by garlic supplementation. These data indicated the dose of garlic used in this experiment did not result in any damage to the liver and might attenuate the HFD-induced burden of liver, since GPT and the GOT enzymes usually leak out into the general circulation when liver cells are injured. Dextrin, as a polysaccharide control, also showed similar effects on these markers, suggesting that the dose of dextrin used in this study did not result in any damage to the liver.
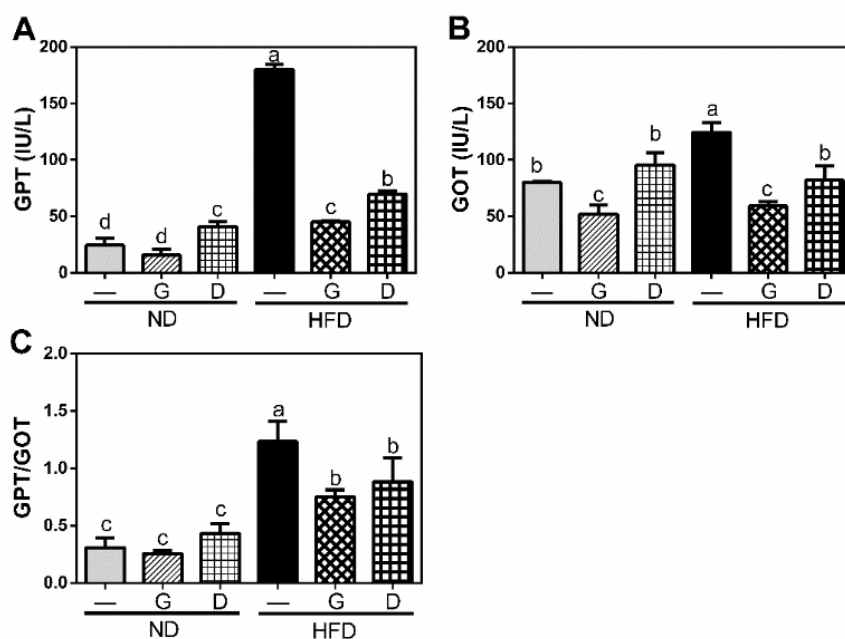
Fig.2-2 Effects of garlic supplementation on serum level of glutamic-pyruvic transaminase (GPT) (A), glutamic-oxaloacetic transaminase (GOT) (B), and the ratio of GPT/GOT (C). The data represent the mean ± SD of five mice. G: garlic; D: Dextrin. Columns with different letters significantly differ ($p < 0.05$).

2.3.2 Effect of garlic on metabolism of lipid and glucose

To clarify the effect of garlic on metabolism of lipid and glucose, we measured the serum levels of lipid and glucose metabolism markers at the final day of experiment after 12 h fasting. The serum levels of T-Cho, TG, and LDL were significantly increased in HFD group ($p < 0.05$), and they were significantly reduced ($p < 0.05$) by garlic supplementation ($p < 0.05$) (Fig.2-3). Moreover, the serum concentration of insulin was also increased in HFD group, and reduced by garlic supplementation ($p < 0.05$) (Fig.2-4). Although there was no significant difference in serum level of glucose between all groups, the ratio of HOMA-IR, an indicator of insulin resistance, was significantly increased in HFD group, and it was then reduced by garlic supplementation ($p < 0.05$) (HFDG in Figure 4). Dextrin as polysaccharide control showed similar effect on these markers. These data indicated that garlic supplementation attenuated HFD-induced dyslipidemia.
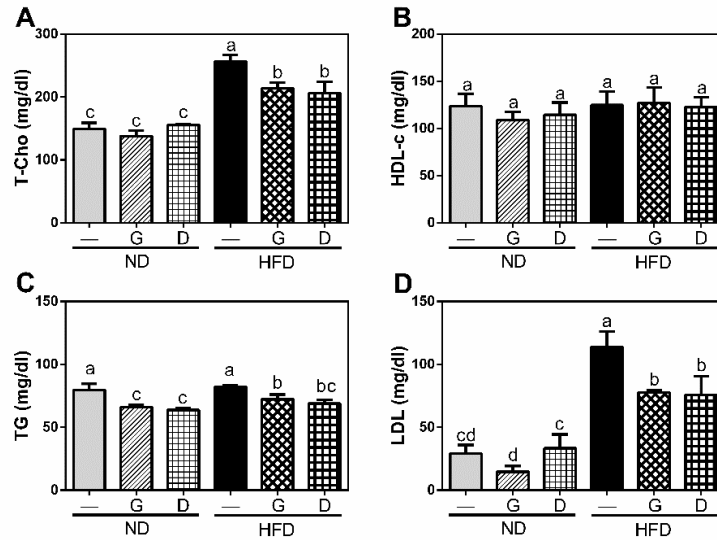
Fig.2-3 Influence of garlic supplementation on serum level of lipid profiles including total cholesterol (T-Cho) (A), high density lipoprotein cholesterol (HDL-c) (B), total triacylglycerol (TG) (C), and low-density lipoproteins (LDLs) (D). The data represent the mean ± SD of five mice for each group. Columns with different letters significantly differ ($p < 0.05$).
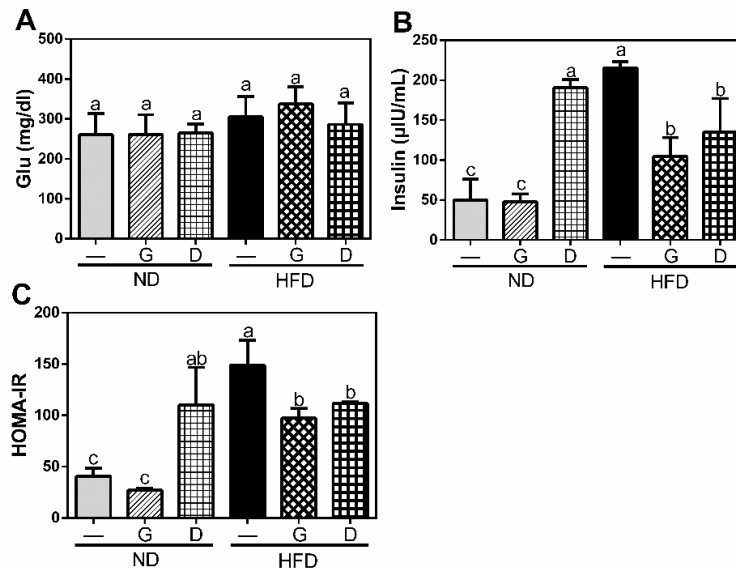
Fig.2-4 Effects of garlic supplementation on serum level of glucose (A) and insulin (B) and homeostatic model assessment for insulin resistance (HOMA-IR) (C). The data represent the mean ± SD of five mice. Columns with different letters significantly differ ($p < 0.05$).

2.3.3 Effect of garlic on terminal ileum histomorphology and the concentration of organic acids on cecum

The ratio of villus height/crypt depth is an important indicator for reflecting the digestive and absorptive functions of the small intestine. As shown in Figure 2-5, HFD decreased the ratio of villus height/crypt depth compared to that in ND ($p < 0.05$). Supplementation with garlic, but not dextrin, significantly recovered the ratio ($p < 0.05$).
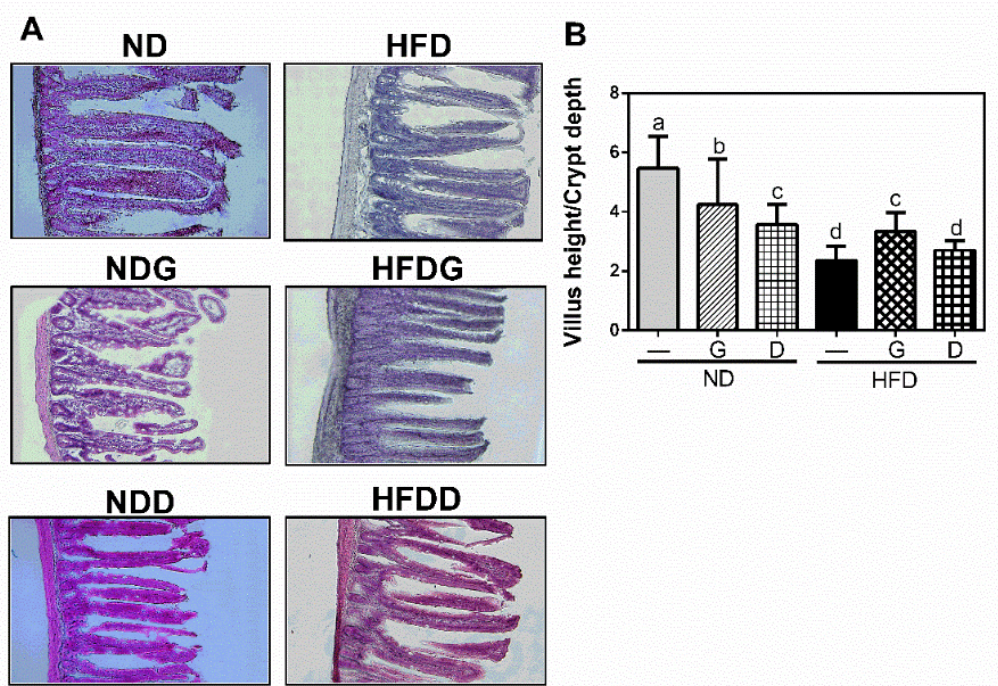
Fig.2-5 Morphology of terminal ileum (A) and the ratio of villus height to crypt depth (B). Values are presented as means ± SD of 16 histomorphological points of each group. Columns with different letters significantly differ ($p < 0.05$).

Moreover, the cecum weight in the HFD group was significantly lower than that in the ND group, and was recovered by garlic or dextrin supplementation (Figure 2-6A). To further elucidate the effect on the concentration of organic acids in caeca, we measured short-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs). The results revealed that the BCFAs, including *iso*-butyric acid, were increased in the HFD group and were reduced by garlic or dextrin supplementation. Acetic acid, propionic acid, n-butyric acid, succinic acid, lactic acid, and formic acid are SCFAs. Of these, the concentrations of butyrate acid and acetate acid were increased in the HFD group and attenuated by garlic or dextrin supplementation. These data indicated that garlic supplementation could attenuate both HFD-induced damage of small intestine morphology and HFD-induced higher concentrations of *iso*-butyric acid, n-butyrate acid, and acetate acid.
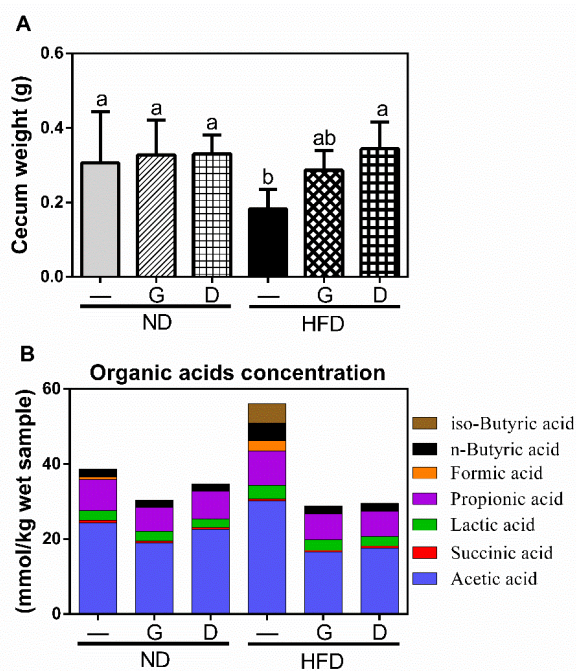
Fig.2-6 The effects of garlic supplementation on cecum weight (A). The cecum weight including cecum and cecum content was measured after the mice were sacrificed. The data represent the mean ± SD of five mice for each group. Columns with different letters significantly differ ($p < 0.05$). The effects of garlic supplementation on organic acid concentration in cecum content (B).

50

2.3.4 Modulation of the gut microbiome by garlic

To further understand the effects of garlic on gut bacteria, the composition and relative abundance of microbiota were determined using high throughput 16 rRNA gene sequencing. The diversity of gut microbiota is shown in Figure 2-7; supplementation with garlic increased the chao1 value (A), observed species (B), phylogenetic diversity (PD) whole tree index (C), and shannon value (D) from 6 to 12 weeks in both the ND and HFD groups. As a control polysaccharide, dextrin decreased all of these four values in the HFD group.

Furthermore, we used principal coordinate analysis (PCoA) plots (β-diversity: between-habitat diversity) based on unweighted UniFrac distance matrices to investigate the similarities in gut microbial community structure among the different groups. The percent of dataset variability explained by each principal coordinate is shown in the axis's titles (PC1: 15.79%, PC2: 11.60%, PC3: 7.56%). PC1 and PC2 were the two principal coordinate components. PC1 represents the principal coordinate component that can explain the changes in data as much as possible; PC2 represents the principal coordinate component that accounts for the largest proportion of the remaining changes (and so on for PC3). The PCoA plot indicated that the structure of the gut microbiota in the ND group experienced no obvious change as a result of the age of the mice, but it was
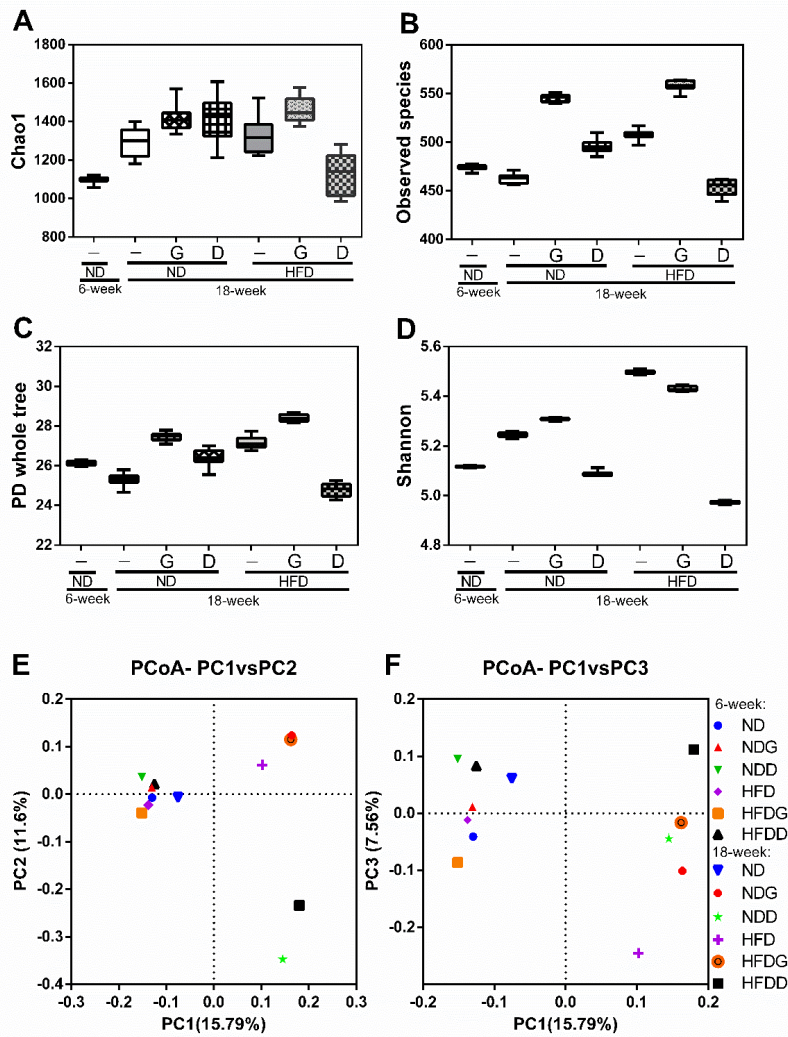
Fig.2-7 Effects of garlic supplementation on the gut microbiome. The taxa richness of the gut microbiome assessed by α-diversity analyses using Chao1 value (A), Observed species index (B), PD whole tree index (C), and Shannon index (D). The data represent the median and range of ten alpha rarefaction values. The species compositions of the gut microbiomes were assessed by β-diversity analyses using principal coordinate analysis (PCoA) of the unweighted UniFrac distance matrices, which is showed in PC1 vs. PC2 (E) and PC2 vs. PC3 (F). Each dot in (E) and (F) represents the beginning (6 weeks of age) or ending point (18 weeks of age) of the experiment for each group (n = 8).

altered by HFD. Shifts in the microbial structure were also observed for both garlic and dextrin supplementation, however, there was no significant clustering according to anatomical location. The data suggest that the

mechanisms for the regulation of the gut microbiome by garlic and dextrin are different.

Therefore, we further investigated the changes of individual microbial species at the phylum level. The ratio of p_*Firmicutes*/p_*Bacteroidetes* was increased by aging from 6 weeks to 18 weeks in the ND group, and it was attenuated in the NDG and NDD groups (Fig.2-8). Furthermore, supplementation with garlic increased the relative abundance of f_*Lachnospiraceae,* and decreased the relative abundance of g_*Prevotella* at the genus level of species. In addition, supplementation with dextrin increased the relative abundance of g_*Parabacteroides*, g_*Sutterella*, and f_*Rikenellaceae* (Fig.2-9).
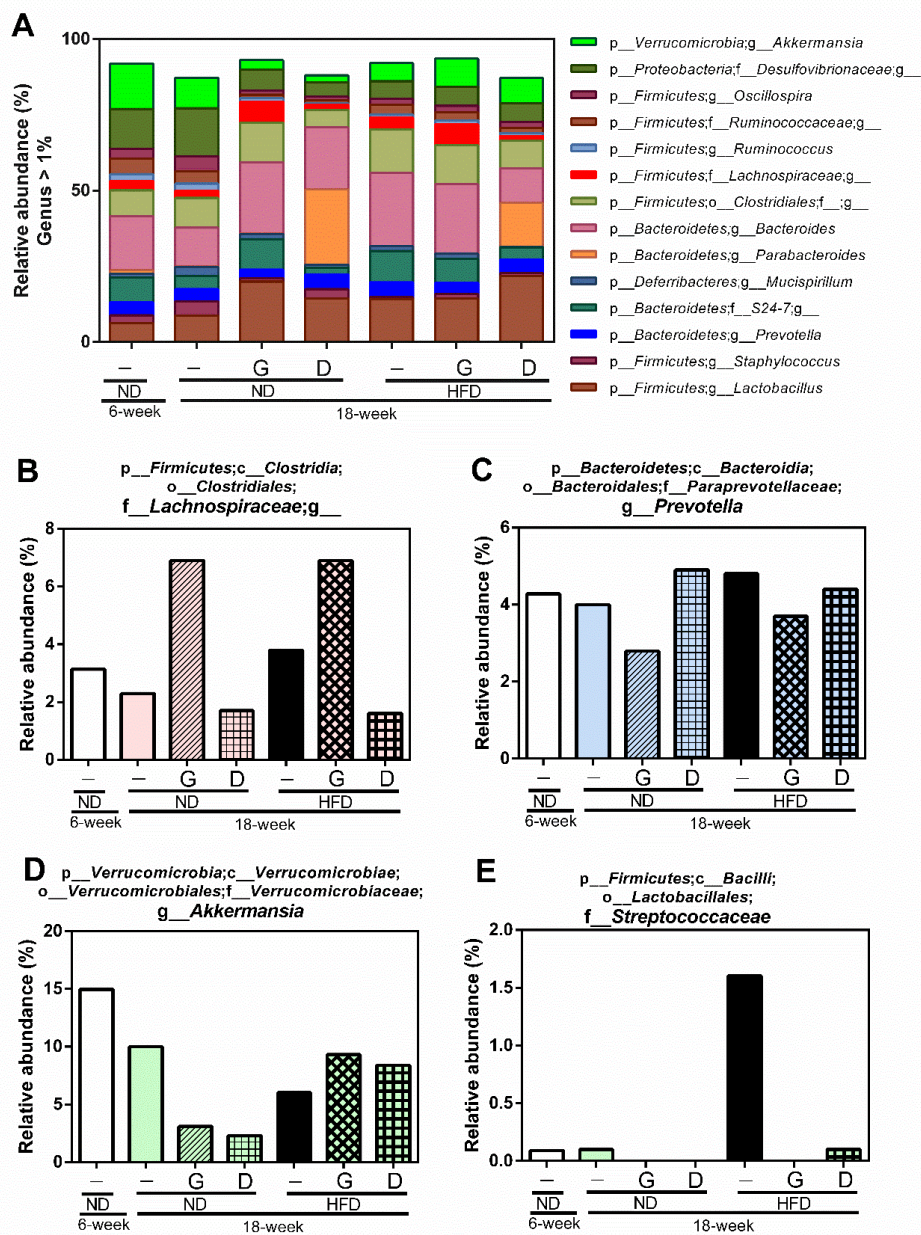
Fig.2-8 Modulation of the gut microbiome at the phylum level. The gut microbiota was characterized by 16S rRNA gene sequencing. (A) The relative abundance of bacteria at the phylum level. (B) The ratio of p_*Firmicutes* to p_*Bacteroidetes* based on their relative abundance.
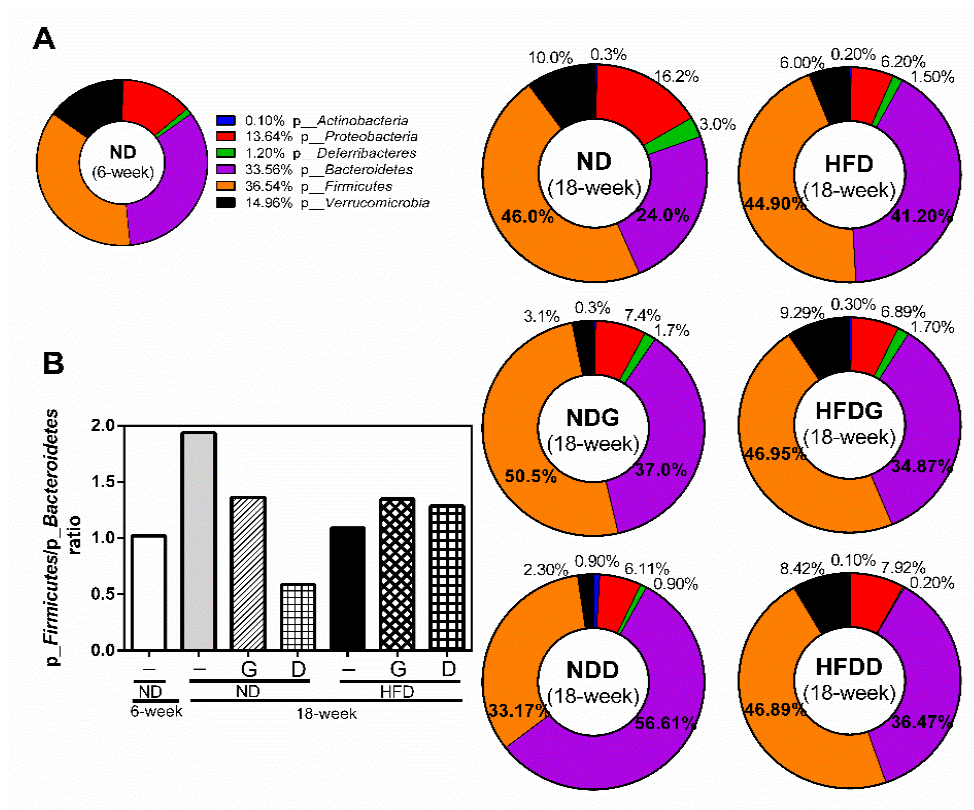
Fig.2-9 Modulation of the gut microbiome at the genus level. The gut microbiome was characterized by 16S rRNA gene sequencing, and the data represent the relative abundance of each bacterial genus. p_, c_, o_, f_, and g_ represent phylum, class, order, family, and genus, respectively, and a blank after the letter means undefined. (A) The relative abundance of more than 1% of bacteria at the genus level. Specifically, four kinds of bacteria (f_*Lachnospiraceae* (B), g_*Prevotella* (C), g_*Akkermansia* (D), f_ *Streptococcaceae* (E)) were regulated by garlic supplementation.

2.4 Discussion

In this study, garlic supplementation revealed the preventive effects on HFD-induced metabolic disorders and dyslipidemia. These effects included that garlic attenuated HFD-induced increases in LDL serum level, insulin resistance, liver injury, and the concentration of total organic acids in caeca. It has previously been reported that HFD-enhanced the concentration of *n*-butyrate acid, and acetic acid in caecum is associated with the gut microbiota for energy gain in obese mouse (Turnbaugh, P.J *et al*., 2006). Butyrate is regarded as the primary energy source for colonic epithelial cells, and propionate and acetate are largely utilized by the liver. Both of them are necessary for lipogenesis and gluconeogenesis (Turnbaugh, P.J *et al*., 2006; Lin, H.V *et al*., 2012). Molecular data showed that acetate, propionate, and butyrate can preferentially activate a series of free fatty acid receptors (FFARs) to increase energy harvest and triglyceride storage in adipose tissue (Brown, A.J *et al*., 2003; Le Poul, E *et al*., 2003; Shen, J *et al*., 2013). In addition, we also observed that *iso*-butyrate acid was increased in the HFD group. A raise in *iso*-butyric acid was previously observed in hyperlipidemia, which was positively correlated with an unfavorable lipid metabolism (Granado-Serrano, A. B *et al*., 2019). Therefore, the attenuation of HFD-induced levels of n-butyrate acid, acetic acid, and *iso*-butyrate acid by garlic supplementation

might play an important role in the prevention of HFD-induced metabolic disorders and dyslipidemia.

Several lines of studies have stated that moderate consumption of garlic enhanced some gastro-intestinal function, and revealed the protective effect for mucosal defense against *Helicobacter pylori* activity and ulcers development (Ghosh, S *et al*., 2003; Munday, R *et al*., 1999). In this study, the jejunal crypt depth was significantly deepened in the HFD group, and garlic supplementation alleviated this situation by promoting epithelial cell renewal and the maturing rate of enterocytes. It is reported that *L-*Glutamate supplementation decreased HFD-deepened crypt depth, and enhanced the cell maturing rate and the secretory function of epithelial cells (Lin, M *et al*., 2014). On the other hand, excessive consumption of garlic could result in the loss of intestinal epithelial cells (Hoshino, T *et al*., 2001), leading to the inhibition of intestinal absorption of glutamic acid, sucrose, and glucose (Sood, D.R *et al*., 2003), which might be the reason why garlic shortens the height of villi in a normal diet.

To understand the effects and mechanisms of whole garlic extract on the gut microbiota, we used a mouse model and compared the data with dextrin, a positive polysaccharide. For this, we further used four different indexes to comprehensively analyze the α-diversity of the gut microbiota. The Chao1 index is a community richness estimator for estimating the

number of OTUs (operational taxonomic units) in the sample. The observed species index is a biological species quantitative index, which is calculated according to the number of confirmed OTUs (Chao, A *et al*., 1984; N. Balakrishnan *et al*., 2005). The PD whole tree is a phylogenetic diversity index based on the values of PD. The values of PD are defined as the minimum total length of all the phylogenetic branches on the phylogenetic tree (Forest, F *et al*., 2007). The Shannon index is a more comprehensive presentation of diversity; it is calculated by the scaled OTUs, which is based on community evenness (Shannon, C.E, 1948). The results revealed that the β-diversity cluster of microbial communities in the mice supplemented with garlic or dextrin were clustered to different locations, and the supplementation of garlic could enhance the species richness and species evenness of the gut microbiota more than dextrin.

The ratio of p_*Firmicutes* and p_*Bacteroidetes* (F/B) was increased with aging and was reduced in the NDG and NDD groups. Furthermore, the relative abundance of p_*Bacteroidetes; g_Prevotella* was increased in the HFD group, but was reduced by garlic supplementation. g_*Prevotella* is reported to induce insulin resistance, aggravate glucose intolerance, and augment the circulating levels of branched-chain amino acids (BCAAs) (Pedersen, H.K *et al*., 2016). The metabolites of BCAAs can further cause insulin resistance by facilitating the transportation of vascular fatty acids

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(Jang, C *et al*., 2016). It is noteworthy that the relative abundance of f_*Lachnospiraceae* was upregulated by garlic supplementation in this study. Recent studies demonstrated that f_*Lachnospiraceae* plays an essential role in the maintenance of gut immune homeostasis as the inducer of colonic regulatory T cells (Atarashi, K *et al*., 2011). The abundance of f_*Lachnospiraceae* was possibly associated with anti-inflammatory activity (Reeves, A.E *et al*., 2012), and is closely related to host mucosal integrity, bile acid metabolism, polysaccharides decomposition, and protection from colon cancer (Lin, Z *et al*., 2018; Cho, I *et al*., 2012). Moreover, the level of f_*Lachnospiraceae* is correlated negatively with the consumption of energy and positively with the level of leptin cells (Méndez-Salazar, E.O *et al*., 2018). Fructan from garlic has been reported to increase the abundance of f_ *Lachnospiraceae* (Carrillo-Navarrete, F *et al*., 2018; Wang, Y *et al*., 2018). On the other hand, alliin extracted from garlic was found to decrease the abundance of f_*Lachnospiraceae* (Zhai, B *et al*., 2018). These data clearly reveal that fructan and organosulfur derivatives in garlic have opposite effects on the abundance of f_ *Lachnospiraceae* when their intakes are isolated from each other. In this study, supplementation with whole garlic, which contained fructan (548 mg/g), alliin (7 mg/g), and other organosulfur derivatives, including allicin (5 mg/g), G-SAC (4 mg/g), and *S*-allylcysteines (SAC, 2 mg/g), could

upregulate the abundance of f_*Lachnospiraceae*. Our data revealed that whole garlic could increase the abundance of f_*Lachnospiraceae*, suggesting that the ratio of fructan and organosulfur derivatives is important for gut microbiota. The relative abundance of BCAA-producing bacteria f_ *Streptococcaceae* (Yue, S.-J *et al*., 2019), which is significantly increased in patients with cirrhosis (Chen, Y *et al*., 2011), was also dramatically increased in the HFD group and was noticeably decreased by garlic and dextrin supplementation. The relative abundance of g_*Akkermansia* was decreased with aging and HFD, and was restored by garlic supplementation. It has been reported that the abundance of g_*Akkermansia* has a negative correlation with the value of body mass index (BMI) (Tilg, H *et al*., 2014) and that g_*Akkermansia* has a protective effect for the mucus layer in pro-inflammation (Ganesh, B.P *et al*., 2013).

We also observed different effects on some typical bacteria of the gut microbiota from garlic and dextrin supplementation, although both contain polysaccharides. The abundance of g_*Parabacteroides*, g_*Sutterella*, and f_*Rikenellaceae* were increased by dextrin supplementation. The number of g_*Parabacteroides* is enriched by increases in dietary fiber (Holscher, H. D, 2017) and g_*Parabacteroides* can digest starch that has been chemically modified (Gamage, H.K.A.H *et al*., 2018). The g_*Parabacteroides* and f_*Rikenellaceae* are reported to be associated with

food allergy mice (Xiao, L *et al*., 2016; Noval Rivas, M *et al*., 2013). The g_*Sutterella* are widely prevalent commensals with intestinal epithelial cell adhesion and mild pro-inflammatory capacities (Hiippala, K *et al*., 2016). These changes in gut microbiota resulting from dextrin supplementation were different than with garlic, and they might be due to the chemical properties of dextrin.

In summary, whole garlic supplementation could attenuate the HFD-induced dyslipidemia and disturbance of the gut microbiota. The data revealed that whole garlic may be a potential prebiotic that is able to prevent against HFD-induced disturbance of the gut microbiota.

2.5 Abstract

Garlic (*Allium sativum L.*) contains prebiotic components, fructans, antibacterial compounds, and organosulfur compounds. The complex ingredients of garlic seem to impart a paradoxical result on the gut microbiota. In this study, we used a mouse model to clarify the effects of whole garlic on the gut microbiota. C57BL/6N male mice were fed with or without whole garlic in normal diet (ND) or in high-fat diet (HFD) for 12 weeks. Supplementation with whole garlic attenuated HFD-enhanced ratio of serum GPT/GOT (glutamic-pyruvic transaminase/glutamic-oxaloacetic transaminase), levels of T-Cho (total cholesterol) and LDLs (low-density lipoproteins), and index of homeostatic model assessment for insulin resistance (HOMA-IR), but had no significant effect in the levels of serum HDL-c (high density lipoprotein cholesterol), TG (total triacylglycerol), and glucose. Moreover, garlic supplementation meliorated the HFD-reduced ratio of villus height/crypt depth, cecum weight, and the concentration of cecal organic acids. Finally, gut microbiota characterization by high throughput 16S rRNA gene sequencing revealed that whole garlic supplementation increased the α-diversity of the gut microbiota, especially increasing the relative abundance of *f_Lachnospiraceae* and reducing the relative abundance of *g_Prevotella*. Taken together, our data demonstrated that whole garlic supplementation

could meliorate the HFD-induced dyslipidemia and disturbance of gut microbiota.
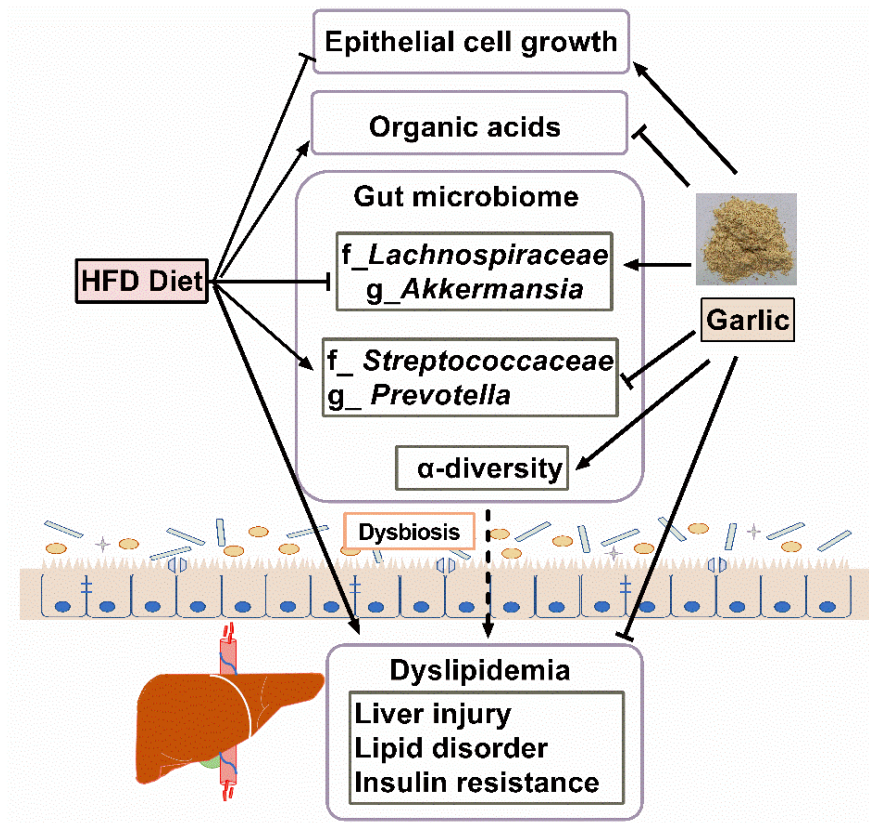
Fig.2-10 Graphic abstract. In conclusion, garlic supplementation improved epithelial cell growth, inhibited organic acids production, and alleviated gut dysbiosis, thereby reducing dyslipidemia induced by high-fat diet.

Chapter 3 Modulation of alliinase free garlic on gut microbiota

3.1 Introduction

Gut microbiota plays an important role in maintaining a healthy body (Chu, F *et al*., 2018). Diet has been shown to impact the composition and activity of gut microbiota (Fontana, L *et al*., 2015). A high-fat diet (HFD) modulates the gut microbiota composition by decreasing the prevalence of specific gut barrier-protecting bacteria and increasing the prevalence of opportunistic pathogens that can release free antigens such as lipopolysaccharides. This imbalance may be associated with higher gut permeability, leading to higher plasma levels of endotoxin and inflammation factors, and eventual development of metabolic disorders (Cani, P.D *et al*., 2007; Zhang, C *et al*., 2010). Simultaneously, fluctuation of gut microbiota was also affected by aging. For example, the ratio of Firmicutes/Bacteroidetes and the abundance of g-*Akkermansia*, which is considered to maintain the integrity of the intestinal barrier, were changed along with aging (Hoffman, J.D *et al*., 2017; Tachon, S *et al*., 2013; Reunanen, J *et al*., 2015).

Garlic (Allium sativum L.) has long been used in food and medicine. Most of the carbohydrates in garlic are composed of a water-soluble

fructose polymer called fructan (Koch, H.P *et al*., 1996), accounting for approximately 65% of the dry weight (Lawson, L.D *et al*., 1995). On the other hand, garlic contains 1.1–3.5% organosulfur compounds (OSCs), which is far higher than that in other plant food. The primary OSCs are γ-glutamyl-*S*-allyl-*L*-cysteines (GSAC), which are hydrolyzed and oxidized to yield S-allyl-L-cysteine sulfoxides (alliin) during storage (Matsuura, H, 1997). The alliin is a major *S*-alk(en)ylcysteine sulfoxide compound which is stored in the mesophyll cells of garlic. When garlic bulbs are crushed, cut, or ground, alliinase is released from the bundle sheath cells to catalyze alliin into a reactive intermediate, sulfenic acid, pyruvic acid, and ammonia (Manabe, T *et al*., 1998). Sulfenic acid undergoes self-condensation to produce allicin, which is then decomposed into other many OSCs, such as diallyl sulfide series, thiosulfates, and ajoene (Trio, P.Z *et al*., 2014).

The experiments with separated garlic compounds revealed that fructans work as prebiotics for gut microbiota (Peshev, D *et al*., 2014), while garlic OSCs, such as allicin, thiosulfates, and ajoene, act as antibacterial effects (Leontiev, R *et al*., 2018; Jakobsen, T.H *et al*., 2012). The complicated ingredients of garlic seem to give paradoxical results for the gut microbiota. To develop garlic supplements that are beneficial to the gut microbiota, we made alliinase free garlic (AFG) extract by heating a garlic bulb at 80°C to inactivate alliinase for blocking allicin production, and then

investigated its effect on the gut microbiota in a mouse model fed with normal diet (ND) or HFD.

3.2 Materials and methods

3.2.1 Chemicals and reagents

Garlic was harvested from Aomori Prefecture, Japan. To inactivate alliinase and prevent the formation of allicin, raw garlic was heated at 80 °C for 1 h in a water ratio of 1:5. After removing water, heated garlic was homogenized and then centrifuged 4 times at 5000 rpm for 30 min. The supernatant was collected and freeze-dried to garlic powder, which was further washed with ethanol and dried again at 60 °C for 40 min to obtain AFG powder. The recovery rate was 14%. The amount of OSCs and fructans on garlic powder was determined by high performance liquid chromatography (HPLC) or fructans assay kit (Biocon Ltd., Nagoya, Japan), respectively (Table 3-1).

The nutrient composition of the diets is shown in Appendix Table S2. All ND contained 21% protein, 6% fat and 54% carbohydrate, 4% cellulose and about 370 kcal/100 g total calories. All HFD contained 21% protein, 40% fat and 10% carbohydrate, 4% cellulose and about 570 kcal/100 g total calories. Lard oil was obtained from Sigma–Aldrich Japan (Tokyo, Japan).

Table 3-1 Nutrient analysis

Nutrients	AFG	Method
Calorie (kcal/100g)	357.5	Calorie=Protein*4+Lipid*9+Carbohydrates*4
Protein (g/100g)	13.05	Kaida's method
Lipid (g/100g)	1.4	Acid decomposition method
Carbohydrates (g/100g)	73.2	100-(protein+lipid+moisture+ash)
Salt (g/100g)	0.02	Sodium conversion
Sodium (mg/100g)	8.5	Atomic absorption spectroscopy
Moisture (g/100g)	8.9	High pressure drying
Ash (g/100g)	3.75	Ashing method
Fructan (mg/g)	468.8	Fructan analysis kit
G-SAC (mg/g)	23.675	HPLC
Alliin (mg/g)	17.415	HPLC
SAC (mg/g)	0.785	HPLC
Allicin (mg/g)	0	HPLC
Organosulfide (mg/g)	41.875	
Fructan/Sulfide	11.195	

3.2.2 Mouse model

The animal experimental protocol was drafted according to the guidelines of the Animal Care and Use Committee of Kagoshima University (Permission NO. A12005). Male C57 BL/6N mice (5 weeks of age) from Japan SLC Inc. (Shizuoka, Japan) were housed separately in cages with wood shavings bedding under controlled light (12-h light/day) and temperature (25 °C), where they had free access to water and feed. Mice body weight was weighed once a week. After 14 day of acclimatization (7 weeks of age), the mice were randomly divided into six groups ($n = 4$) and fed with ND, ND + AFG (alliinase free garlic supplement) with different concentration of 1% or 5%, HFD (40% of fat), HFD + AFG with different concentration of 1% or 5% (Table 3-2). After

11 weeks of feeding (18 weeks of age), mice were sacrificed after overnight fasting. The fresh feces were collected at the beginning (7 weeks of age) and the end of the experiment (18 weeks of age).

Table 3-2 Dietary compositions of each group

Components (%)	ND	ND 1AFG	ND 5AFG	HFD	HFD 1AFG	HFD 5AFG
Lard	6.0	6.0	6.0	40.0	40.0	39.8
Corn starch	54.0	54.0	49.0	20.0	20.0	15.2
Casein	21.0	21.0	21.0	21.0	21.0	21.0
Sucrose	10.0	9.0	10.0	10.0	9.0	10.0
Cellulose	4.0	4.0	4.0	4.0	4.0	4.0
Mineral mix	3.5	3.5	3.5	3.5	3.5	3.5
Vitamin mix	1.0	1.0	1.0	1.0	1.0	1.0
Choline chloride	0.2	0.2	0.2	0.2	0.2	0.2
Methionine	0.3	0.3	0.3	0.3	0.3	0.3
AFG	——	1.0	5.0	——	1.0	5
Total calories (kcal/100g)	367.8	365.1	369.1	567.4	564.7	567.6

3.2.3 Measurement of serum biochemical indicators

Blood was obtained from mice eyeballs and collected in the tube with a coagulant (Separable microtubes, FUCHIGAMI,170720, Kyoto, Japan) for 30 min at room temperature to coagulate properly and acquired by centrifuging at 3000 rpm for 5 min and stored at −80 °C until use. The serum levels of glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), gamma-glutamyl transferase (GGT), total

cholesterol (T-Cho), total triacylglycerol (TG), high-density lipoprotein cholesterol (HDL-c) and glucose were measured with an automated analyzer for clinical chemistry (SPOTCHEM EZ SP-4430, Arkray, Kyoto, Japan). Using the Friedewald equation that LDL = Tcho-HDLc-TG/5 to calculate the level of LDL.

3.2.4 Characterization of gut microbiota by 16S rRNA gene sequencing

Mice feces were collected from mice housed in different cages at 7- and 18-weeks age, and soon stored at −80 °C until use. The feces genomic DNA was extracted with the Fast DNA spin kit (MP BIOMEDICALS, Kyoto, Japan) according to the manufacturer's manual, for analyzing the composition of gut bacterial communities by sequencing 16S rRNA genes as described in our previous paper.

3.2.5 Statistical analysis

Results were expressed as mean ± SD. The significant differences between the groups were analyzed by one-way analysis of variance (ANOVA) tests, followed by Duncan's multiple range tests with the SPSS statistical program (version 19.0, IBM Corp., Armonk, NY, USA). A probability of $p < 0.05$ was considered significant.

3.3 Result

3.3.1 Body weight and index of liver injury

There was no difference in initial body weight and in daily food intake among the groups. The final body weight of mice fed with HFD was significantly higher than the mice fed with ND (ND: 35.9 ± 3.34 g, HFD: 43.2 ± 0.67 g, $p < 0.05$). AFG supplementation had no significant effect on body weight in both ND and HFD groups (Fig.3-1A). Moreover, the ratio of GPT to GOT in serum increased significantly ($p < 0.05$) in HFD group and reduced significantly ($p < 0.05$) by AFG supplementation (Fig.3-1B). These data indicated that the dose of garlic used in this experiment has no
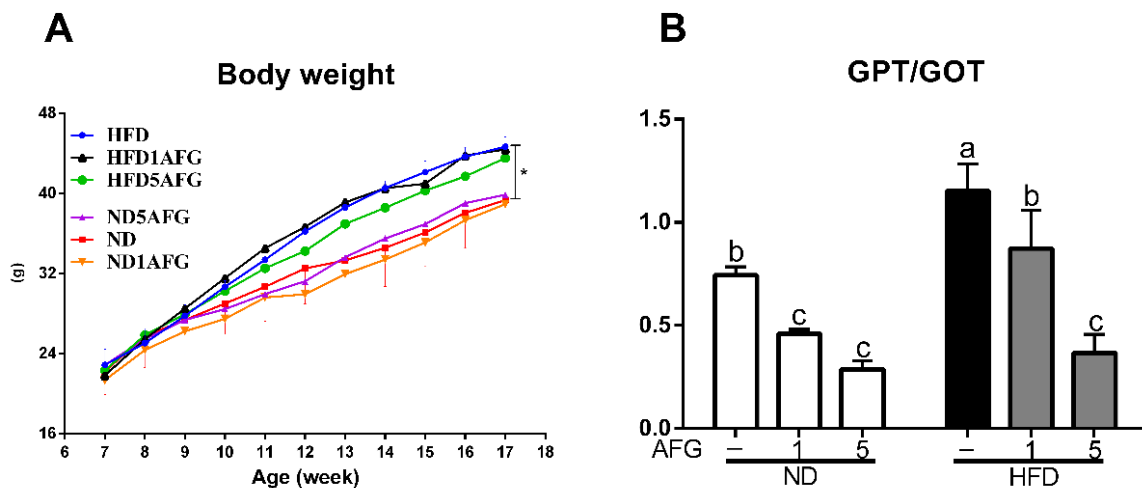
Fig.3-1 Body weight and index of liver injury. (**A**) The changes in body weight of mice fed with different diets from 7 to 18 weeks. Results are expressed as the mean ± SD for each group of mice (n = 4). The asterisk indicates a significant difference in weight at 18 weeks ($p < 0.05$). HFD: high-fat diet, HFD1AFG: HFD plus 1% AFG, HFD5AFG: HFD plus 5% AFG, ND: normal diet, ND1AFG: ND plus 1% AFG, ND5AFG: ND plus 5% AFG. (**B**) The ratio of serum GPT/GOT. The data represent the mean ± SD of four mice. The different letter indicates a significant difference at $p < 0.05$.

damage to liver and might attenuate the HFD-induced burden of liver since GPT and GOT enzymes usually leak into the systemic circulation when liver cells are injured.

3.3.2 Effect of AFG on lipid metabolism

To clarify the effect of AFG on lipid metabolism, we measured the serum levels of lipid and glucose metabolism markers at the final day of the experiment after 12 h fasting. As shown in Figure3-2, the serum levels of triacylglycerol (TG), total cholesterol (T-Cho), and low-density
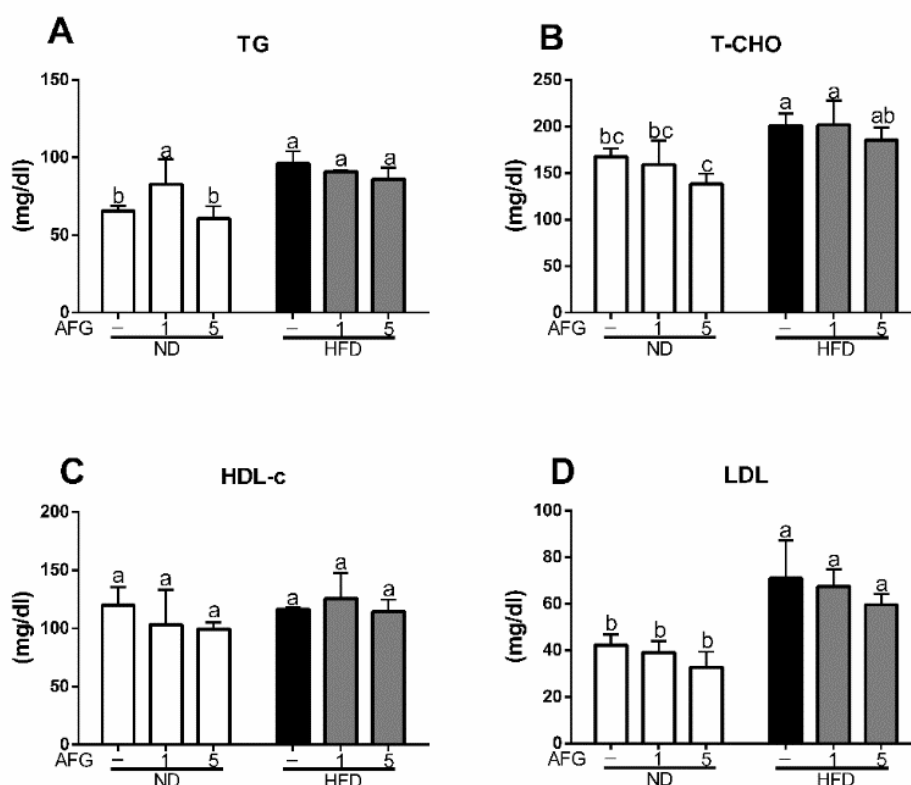
Fig.3-2 Effect of alliinase free garlic on lipid metabolism. (A–D) Influence of alliinase free garlic supplementation on serum level of total triacylglycerol (TG), total cholesterol (T-Cho), high-density lipoprotein cholesterol (HDL-c) and low-density lipoproteins (LDL). The data represent the mean ± SD of four mice for each group. Columns with different letters significantly differ ($p < 0.05$).

lipoproteins (LDL) increased significantly in HFD group ($p < 0.05$), and decreased slightly by AFG.

3.3.3 Effect of AFG on the diversity of gut microbiota

To further understand the effects of AFG on gut bacteria, the composition and relative abundance of microbiota were determined, using high throughput 16S rRNA gene sequencing. The taxa richness of the gut microbiota was assessed by α-diversity analyses using Chao1 value, observed species index, PD whole tree index, and Shannon index. As shown in Fig.3-3 A–D, the α-diversity of gut microbiota increased by aging and HFD diet, and restored by AFG supplementation. Moreover, we used principle coordinate analyses (PCoA) plot (β-diversity: between-habitat diversity) based on unweighted UniFrac distance matrices to investigate the similarities in gut microbial community structure among different groups. Percent of dataset variability explained by each principal coordinate is shown in the axis's titles (PC1:11.24%, PC2:9.95%,

PC3:7.32%). The PCoA plot indicated that the structure of gut microbiota

in the ND group was changed along with aging (Fig.3-3 E,F).
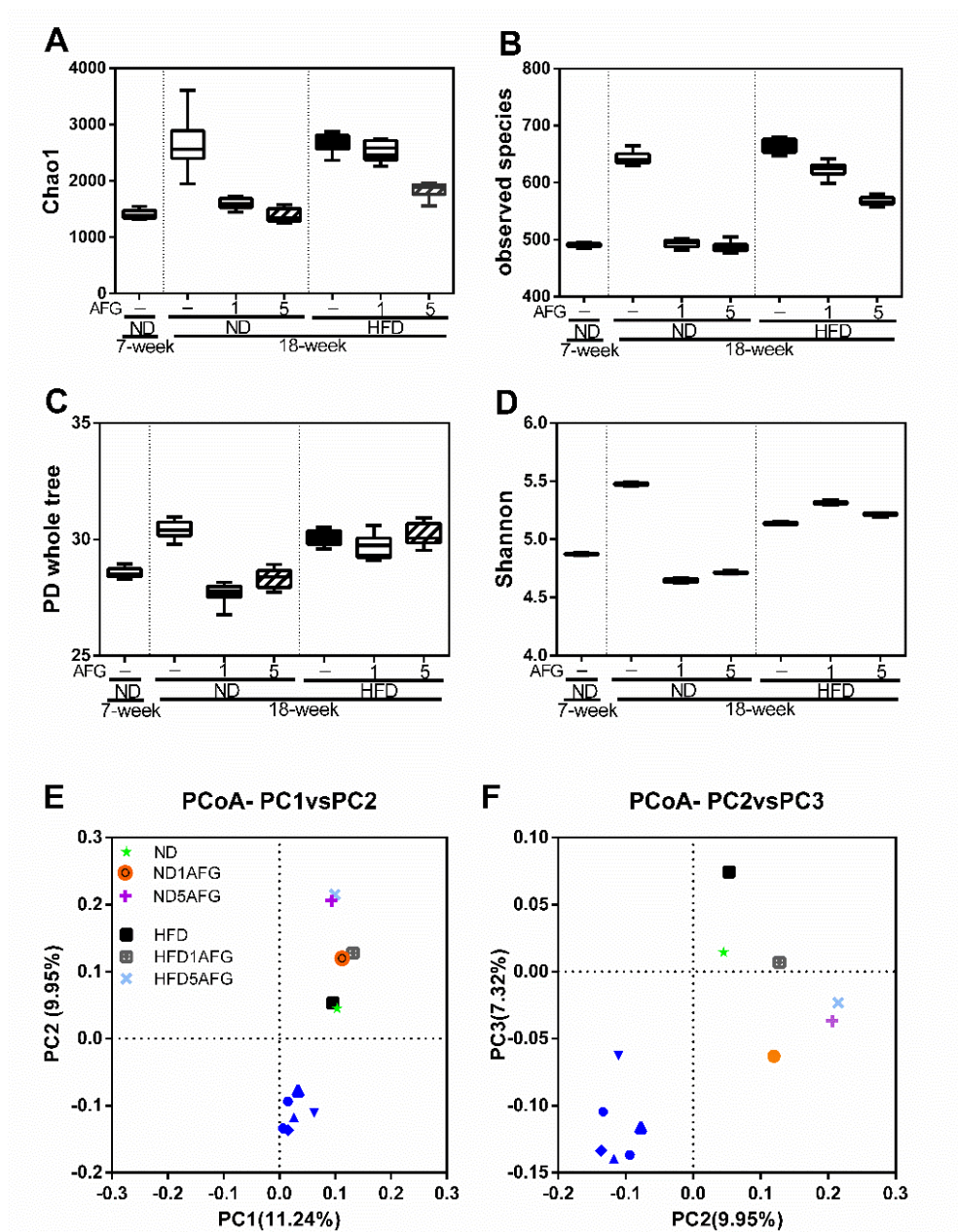
Fig.3-3 The diversity of gut microbiome. (A–D) The taxa richness of the gut microbiome assessed by α-diversity analyses using Chao1 value, observed species index, PD whole tree index, and Shannon index. The data represent the median and range of ten alpha rarefaction values. (E–F) The species compositions of the gut microbiomes were assessed by β-diversity analyses using principle coordinate analysis (PCoA) of the unweighted UniFrac distance matrices, which is showed in PC1 vs. PC2 and PC2 vs. PC3. Each dot represents the beginning (7-week) or ending point (18-week) of the experiment for eight rarefaction values in each group.

3.3.4 Modulation of gut microbiota by AFG

To know the modulation of AFG on gut microbiota, first we investigated the changes of individual microbial species at the phylum level. The phylum of *Verrucomicrobia* decreased by aging and HFD diet, and recovered by 5% concentration of AFG supplementation (Fig.3-4A). Moreover, the ratio of *p-Firmicutes* to *p-Bacteroidetes* in the ND group increased with aging from 7 weeks to 18 weeks, and decreased by 5% AFG supplementation. This ratio was also increased by HFD, and reduced by 5% AFG supplementation (Fig.3-4B).
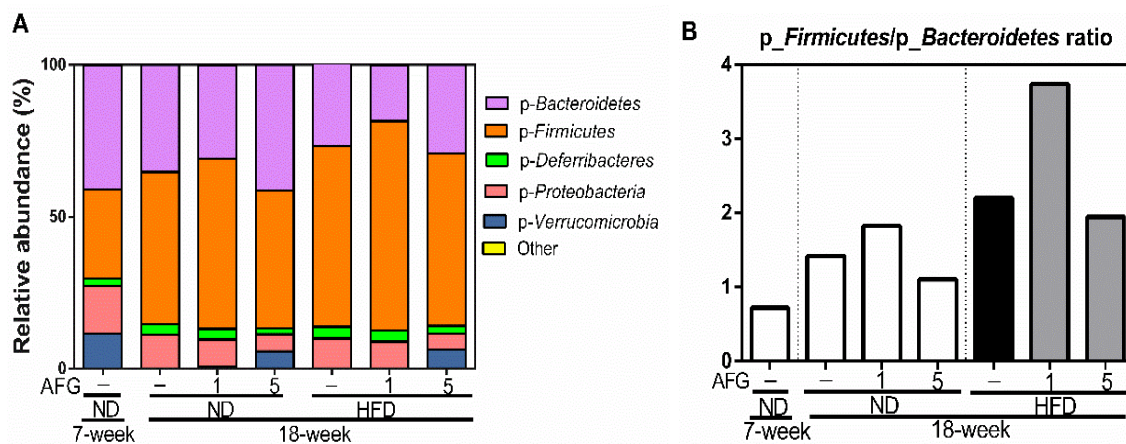
Fig.3-4. The composition of gut microbiome at phylum level. (A) The relative abundance of bacteria at phylum level. (B) The ratio of p-Firmicutes to p-Bacteroidetes based on their relative abundance.

Secondly, we analyzed the changes of individual microbial species at genus level (Fig.3-5). The results revealed that the relative abundance of g-*Akkermansia* belonging to the phylum of *Verrucomicrobia* decreased by aging and HFD diet, and recovered by AFG supplementation. Furthermore, the relative abundance of f-*Lachnospiraceae* was also decreased by aging and HFD diet, and increased by AFG supplementation.
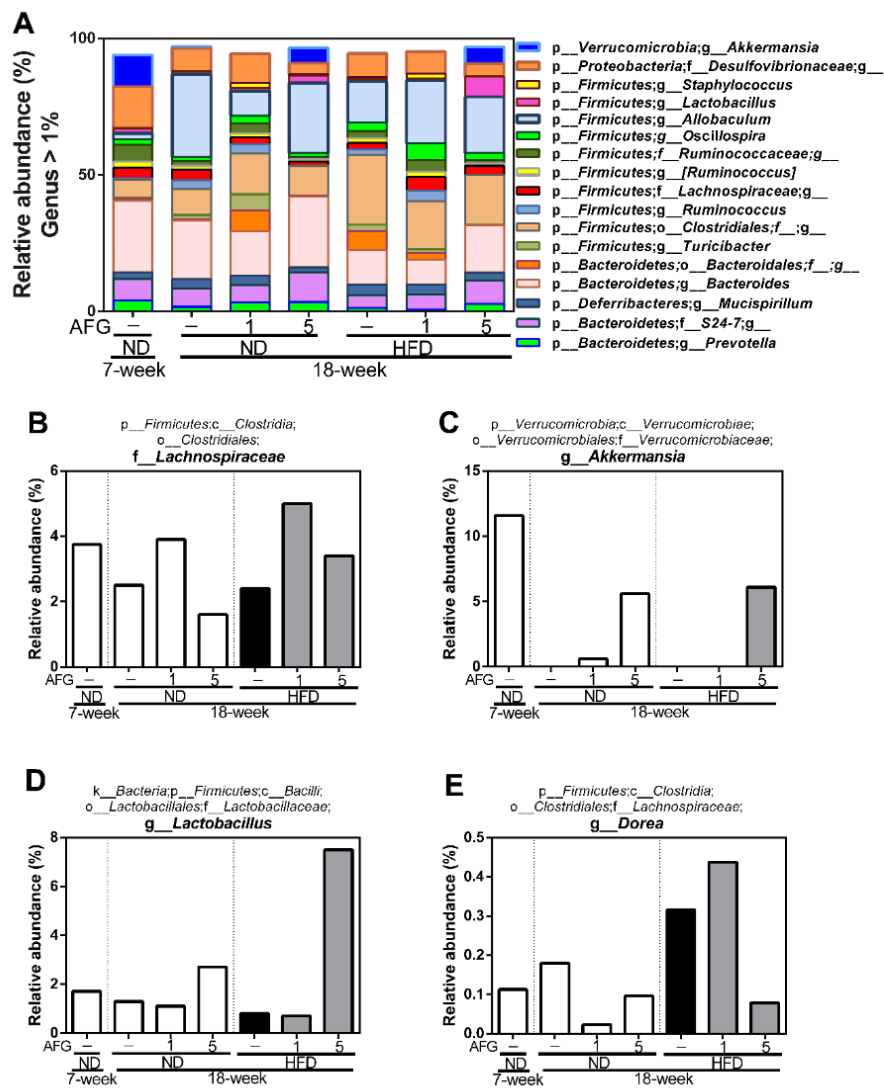
Fig.3-5 The composition of gut microbiome. (A) The relative abundance of more than 1% of bacteria at the genus level. (B–E) The relative abundance of f-*Lachnospiraceae*, g-*Akkermansia*, g-*Lactobacillus*, g-*Dorea*, respectively. p-, c-, o-, f-, and g- represent phylum, class, order, family, and genus, respectively.

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3.4 Discussion

This study revealed the preventive effects of AFG supplementation on the HFD-induced hepatocyte damage and the dysbiosis of the gut microbiota. Those effects were that AFG attenuated HFD-induced increases in the ratio of GPT/GOT and the ratio of p-*Firmicutes* to p-*Bacteroidetes*. The dysbiosis of gut microbiota is found in a lot of diseases; Non-alcoholic fatty liver disease (NAFLD) is one of them in humans. Thus, targeting microbiota and their metabolites have recently been developed to address this issue (Bashiardes, S *et al*., 2016). Obese people are found to have a higher ratio of *Firmicutes*/*Bacteroidetes*, compared to normal-weight people in the adult population (Koliada, A *et al*., 2017). Our data revealed that HFD diet increased the ratio of *Firmicutes*/*Bacteroidetes*, and this increase was significantly inhibited by 5% AFG.

Furthermore, the relative abundance of f-*Lachnospiraceae* was decreased by HFD, but increased by AFG. f-*Lachnospiraceae* has been reported to be associated with anti-inflammatory activity (Reeves, A.E *et al*., 2012), host mucosal integrity (Lin, Z *et al*., 2018), the consumption of energy and the level of leptin (Méndez-Salazar, E.O *et al*., 2018). Fructan (Carrillo-Navarrete, F *et al*., 2018; Wang, Y *et al*., 2018) and whole garlic (Chen, K *et al*., 2019) have been reported to increase t

The abundance of f-*Lachnospiraceae*, but alliin was found to decreased it (Zhai, B *et al*., 2018). In particular, the relative abundance of g-*Akkermansia* decreased by aging and HFD diet, and increased by 5% AFG supplementation. g-*Akkermansia* is associated with the reduction of gut leakiness and attenuation of low-grade inflammation (Anhê, F.F *et al*., 2015), and considered to be next-generation beneficial bacteria (Naito, Y *et al*., 2018; De Vos, W.M, 2017). Moreover, the relative abundance of f-*Lactobacillus* was enhanced by 5% AFG, and it has been reported that oral *Lactobacillus* tablets decreased the levels of GOT and GPT in patients with NAFLD in two double-blind randomized clinical trials (Aller, R *et al*., 2011; Famouri, F *et al*., 2017). Interestingly, the growth of f-*Lactobacillus* was mostly unaffected by the addition of raw garlic containing allicin compared with other gastrointestinal symbiotic bacteria in vitro culture experiments (Filocamo, A *et al*., 2012). However, it was enhanced by AFG in this study. Thus, it may be due to fructan effects, a major beneficial ingredient for gut bacterial growth.

To understand the effects of allicin on the gut microbiota, we compared the whole garlic extract from our previous experiments (Chen, K *et al*., 2019). We found that whole garlic and AFG had the same tendency to inhibit the ratio of GPT to GOT caused by HFD. Although we found that whole garlic inhibited the HFD-induced increases of TG and LDL, AFG

had no significant effect on lipid metabolism in this study. These data suggested that the garlic OSCs played an important role in improvement of HFD-induced dyslipidemia. On the other hand, although both of the whole garlic and AFG increased the relative abundance of f-*Lachnospiraceae*, g-*Akkermansia, and* g-*Lactobacillus*, AFG showed down-regulation of the diversity of gut bacteria, and whole garlic showed up-regulation of the diversity of gut bacteria. It is possible that this is due to the interaction effect of garlic OSCs and fructans on gut microbiota, although the reason is still unknown.

In summary, the supplementation with 1–5% of AFG in diet significantly decreased the ratio of GPT/GOT induced by HFD in serum. Fecal microbiota characterization by high throughput 16S rRNA gene sequencing demonstrated that AFG reduced the *Firmicutes/Bacteroidetes* ratio caused by aging and the ingestion of HFD. Moreover, the abundance of f-*Lachnospiraceae*, g-*Akkermansia,* and g-*Lactobacillus* were enhanced by AFG. Our data demonstrated that alliinase free garlic is better than whole garlic for the modulation of the gut microbiota.

3.5 Abstract

The allicin diallyldisulfid-*S*-oxide, a major garlic organosulfur compound (OSC) in crushed garlic (*Allium sativum* L.), possesses antibacterial effects, and influences gut bacteria. In this study, we made alliinase free garlic (AFG) extract and investigated its effects on gut microbiota. C57BL/6N male mice were randomly divided into 6 groups and fed normal diet (ND) and high-fat diet (HFD) supplemented with or without AFG in concentrations of 1% and 5% for 11 weeks. The genomic DNAs of feces were used to identify the gut microbiota by sequencing 16S rRNA genes. The results revealed that the ratio of p-*Firmicutes* to p-*Bacteroidetes* increased by aging and HFD was reduced by AFG. In particular, the f-*Lachnospiraceae*, g-*Akkermansia*, and g-*Lactobacillus* decreased by aging and HFD was enhanced by AFG. The g-*Dorea* increased by aging and HFD decreased by AFG. In addition, the ratio of glutamic-pyruvic transaminase to glutamic-oxaloacetic transaminase (GPT/GOT) in serum was significantly increased in the HFD group and decreased by AFG. In summary, our data demonstrated that dietary intervention with AFG is a potential way to balance the gut microbiota disturbed by a high-fat diet.

Chapter 4 Natural garlic organosulfur compounds prevent metabolic disorder of lipid and glucose by increasing gut commensal *Bacteroides acidifaciens*

4.1 Introduction

Garlic (*Allium sativum L.*) has long been used in food and medicine. The garlic organosulfur compounds (OSCs) are demonstrated as major components for garlic's beneficial functions. Accumulating data and our recent study have proven that garlic could regulate lipid and glucose metabolism in both human and mice, whereas the gut microbiota are suggested as a likely factor for these regulations (Eric Block, 2010; D. A. Locatelli *et al*., 2017; P. Liu *et al*., 2020; N. Yoshimoto *et al*., 2014; P. Z. Trio *et al*., 2014). However, the OSCs composition and function of garlic are unstable due to the various processing and cooking methods. There are 33 known OSCs that can be obtained from garlic, (D. A. Locatelli *et al*., 2015). *S*-allyl-*L*-cysteine sulfoxide (alliin) and *S*-ally-*L*-cysteine (SAC), γ-glutamyl-*S*-allyl-*L*-cysteine (G-SAC) are the major natural and stable OSCs in raw garlic bulbs. After crushing and cutting of the garlic bulb, alliin forms complexes with released alliinase to generate allicin, and allicin will transforms into a series of unstable OSCs, such as diallyl trisulfide (D. A. Locatelli *et al*., 2017). Thus, ensuring the stable OSCs in

garlic resource is necessary for maintaining the functions of garlic.

In order to obtain stable OSCs from garlic resource, we deactivated alliinase by boiling garlic bulb to maintain stable OSCs as stable alliin, GSAC and SAC in natural status (refer to "allicin-free garlic"). The allicin-free garlic has a more stable and reliable composition, not only allicin and other series of derivatives were not appeared, but also rodent's studies have found that oral allicin will be completely absorbed and cannot be converted to allicin in the stomach (D. A. Locatelli *et al.*, 2015). However, the mechanism of garlic OSCs to affect the gut microbiota and the relation with the preventive effects against the disorders of glucose and lipid metabolism remain unclear.

Dyslipidemias are characterized by abnormal levels of total cholesterol (T-Cho), triglycerides (TG) in the blood. (A. D. Mooradian, 2020). In the liver, the peroxisome proliferator-activated receptor alpha (PPARα) is a ligand-activated transcription factor that regulates fatty acid metabolism. Furthermore, PPARα can regulate the expression of carnitine palmitoyl transferase 1A (CPT1A), and the cytochrome P450, family 4, subfamily A1 (CYP4A1) which catalyzes the rate-limiting step in fatty acid β-oxidation. (A. Purushotham *et al.*, 2009; B. N. Finck *et al.*, 2005; J. M. Peters *et al.*, 2012). Thus, the PPARα is a key factor involved in fatty acid β-oxidation by regulating the expressions of CPT1A and CYP4A1

enzymes. Moreover, the homeostatic model assessment for insulin resistance (HOMA-IR) index is widely used as a clinical tool for assessing insulin resistance. The enteral inhibition of dipeptidyl peptidase-4 (DPP-4) enzyme potentiates incretin action, and it is widely used in the treatment of type 2 diabetes. Furthermore, Glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxy kinase (PEPCK) play a vital role in the control of hepatic gluconeogenesis. (G. Riccardi *et al*., 2000; D. M. Nathan *et al*., 2007; E. G. Beale *et al*., 2004). Thus, repressing enteral DPP-4 production and enhancing hepatic gluconeogenesis could be the potential strategies to improve glucose homeostasis and insulin sensitivity.

The *Bacteroides acidifaciens* sp. nov. (*B. acidifaciens*), isolated from the caecum of mice, was recently found to grow well in bile acid, and to enhance the glucose-6-phosphate dehydrogenase activity. (Y. Miyamoto *et al*., 2000). Furthermore, it was observed that the gut commensal *B. acidifaciens* improved insulin sensitivity, prevent obesity with negative correlation of liver triacylglycerol levels during the metabolic adaptation to high-fat diet in mice. (V. Blasco-Baque *et al*., 2017). Compared with the mice fed with PBS, taurine levels in feces of the mice fed with *B. acidifaciens* were significantly increased, and DPP-4 levels in the small intestine were inhibited. (J. Y. Yang *et al*., 2017). Therefore, gut commensal *B. acidifaciens* may be a potential target for regulating lipid

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and glucose metabolism.

The above information combined with our previous studies suggested that garlic OSCs have potential effects to prevent high fat-induced disorders, and which are possibly regulated by gut microbiota. In order to know how garlic OSCs work *in vivo* and *in vitro*, in this study, we chose allicin-free garlic as experiment materials, and prepared two concentrations of these OSCs to confirm the dose-dependence in bacteria culture of *B. acidifaciens*. Moreover, a mouse model fed with western diet (WD) containing high fat, sugar, and cholesterol, (K. Xie *et al*., 2020), or WD supplementing two doses of garlic OSCs, was used to investigate the changes on metabolism of lipid and glucose as well as gut microbiota, and further clarify their underlying relationships.

4.2 Material and methods

4.2.1 Chemicals and reagents

The HPLC standards including alliin (L-Alliin 98.07%, 3101151) and SAC (S-Allyl-L-cysteine 99.2%, 27319405) were offered by LKT Laboratories, INC. (Minnesota, U.S.). The G-SAC (Glu [Cys (All)] 97.1%, 590718) was provided by Peptide Institute, INC. (Osaka, Japan). The mice feed ingredients including lard oil (18-0260-5) and cellulose (435236) were obtained from Sigma-Aldrich (Tokyo, Japan). Soybean oil (25621-55), choline chloride (08809-45), cholesterol (08721-75), glucose (16806-25) and methionine (21718-25) were offered by Nacalai Tesque, INC. (Kyoto, Japan). Mineral mix (AIN93G) and vitamin mix (AIN93) were obtained from Oriental Yeast Co., LTD. (Tokyo, Japan). Sucrose was provided by Hayashi pure chemical IND., LTD. (Osaka, Japan). Corn starch was offered by Sanwa Cornstarch CO., LTD. (Nara, Japan). Casein (Edible acid casein 30) was obtained from Meggle GmbH & Co. (Germany). MCP-1 ELISA kits were provided by Sigma-Aldrich (Tokyo, Japan). The primary antibodies including anti-CPT1A (sc-393070), anti-CYP4A1 (sc-53248), anti-CYP4A1 (sc-53248) and anti-PPARα (sc-9000) were obtained from Santa Cruz Technology. Anti-PEPCK (D12F5) and β-actin were offered by Cell Signaling Technology, and anti-G-6-Pase (ab83690) was obtained from Abcam. Hematoxylin solution was provided

by Muto pure chemicals co., LTD. (Tokyo, Japan).

4.2.2 Preparation and quantification of garlic extract

Garlic (*Allium sativum*) harvested in Aomori Prefecture of Japan were boiled at 80 °C for 1 hour to inactivate alliinase, and pulverized as alliinase free garlic powder (K. Chen *et al*., 2020). To focus on the effect of OSCs as a factor on intestinal bacteria, we prepared two doses of alliinase free garlic powder containing a two-fold difference in OSCs concentration with the same concentration of fructan. In bacterial culture experiment, garlic powder was suspended with 5 times sterile water for 12 hours, and centrifuged at 2330g for 10 min, the supernatant was extracted and stored at -20°C until use.

Garlic OSCs were quantified by high performance liquid chromatography (HPLC, Prominence-I LC-2030C, Shimadzu Corporation, Japan), which is equipped with ultraviolet detector and data processor. Briefly, HPLC column was used the Intersil ODS-4 5μm (Inner diameter: 4.6mm x 150mm, GL Sciences, Japan), and detected with 205nm wavelength at 35°C. The mobile phase A were potassium dihydrogen phosphate aqueous solution (pH 2.6) 85%, the mobile phase B were methanol 15%. The contents of OSCs in garlic powder were quantified as 20.889mg/g (G1) and 43.869mg/g (G2), respectively. The contents of fructan in garlic powder were quantified by fructan assay kit (Biocon Ltd,

Japan) according to the manufacture's manual. Detailed methods and results of nutrient analysis of garlic samples are shown in Table4-1.

Table 4-1 Nutrient components of garlic powder

Nutrients	G1	G2	Method
Total OSCs(mg/g)	20.889	43.869	
G-SAC (mg/g)	4.227	20.518	HPLC
Alliin (mg/g)	10.779	22.158	HPLC
SAC (mg/g)	5.883	1.193	HPLC
Allicin (mg/g)	0	0	HPLC
Fructan (mg/g)	477.4	484.0	Fructan analysis kit
Carbohydrates (g/100g)	68.0	71.6	100-protein+lipid+moisture+ash)
Lipid (g/100g)	1.6	0.9	Acid decomposition method
Protein (g/100g)	19.8	18.1	Kaida's method
Ash (g/100g)	4.8	3.7	Ashing method
Salt (g/100g)	0.03	0.02	Sodium conversion
Calorie (kcal/100g)	366	367	Calorie=Protein*4+Lipid*9+Carbohyd

4.2.3 Bacterial strains and culture

Bacteroides acidifaciens (JCM10556) used in this study were offered by the Japan Collection of Microorganisms (JCM) at RIKEN Bio-Resource Center (Tokyo, Japan), and cultured on Brucella Broth medium (5215861, Becton, Dickinson and Company) with 7% Fetal Bovine Serum (FBS, S-001A-BR, Life Science Production, Brazil). The incubations were performed at 37°C in an anaerobic culture square jar or jar system with AnaeroPack (A-02, Mitsubishi Gas Chemical, Japan) as oxygen absorber-

CO2 generator. The bacteria growth was measured by a spectrophotometer (Nanodrop 2000c, Thermo) at 600nm, and the bacteria concentration was adjusted to OD_{600nm}=0.06, and then seeded 100µl to 96-well microtiter plate. Simultaneously, 100µl sample was also added to the 96-well plate, and the OD_{620nm} value was set as the 0h initial value. The growth change was calculated by subtracting OD values of 0 hour from OD values at 30 hours.

4.2.4 Dosage information

The animal diet was described previously (S. Wu *et al*., 2017; T. Ishimoto *et al*., 2013). Briefly, normal diet (ND) contained 6% fat, 43% carbohydrate, and about 3547 kcal/1kg total calories. Western diet (WD) contained 33% fat, 15% carbohydrate, 1% cholesterol, and about 5096 kcal/kg total calories. And two sample treatment groups: WD + a low OSCs concentration garlic (WG1), WD + a high OSCs concentration garlic (WG2). The dosage of 5g garlic sample per 100g of Western diet. The contents of protein, lipid and carbohydrate of the WG1 and WG2 were adjusted by casein, lard, and cornstarch, respectively. The nutritional composition of the diets was standardized according to the garlic nutrition (Table 4-1). In addition, the mice fed with WD, WG1, WG2 diet were also supplied by 4% syrup water containing 18.9g/L sucrose and 23.1g/L (R. Kohli *et al*., 2010). The nutrient composition of the diets is shown in Table

4-2.

Table 4-2 Dietary compositions of each group

Components (%)	ND	WD	WG1	WG2
Soybean Oil	3.00	3.00	3.00	3.00
Lard	3.00	30.00	29.90	29.90
Corn starch	43.00	15.00	10.60	10.60
Casein	21.00	21.00	20.50	20.50
Sucrose	20.00	20.00	20.00	20.00
Cellulose	5.00	5.00	5.00	5.00
Mineral mix	3.50	3.50	3.50	3.50
Vitamin mix	1.00	1.00	1.00	1.00
Cholesterol	0.00	1.00	1.00	1.00
Choline chloride	0.20	0.20	0.20	0.20
Methionine	0.30	0.30	0.30	0.30
G1	0.00	0.00	5.00	0.00
G2	0.00	0.00	0.00	5.00
Total calories (kcal/kg)	3547.39	5096.89	5095.82	5096.32

4.2.5 Mouse model design

The animal experimental protocol was drafted according to the guidelines of the Animal Care and Use Committee of Kagoshima University (Permission NO. A12005). Male C57BL/6N mice (5 weeks of age) from Japan SLC Inc. (Shizuoka, Japan) were housed separately in cages with wood shavings bedding under controlled light (12-h light/day) and temperature (23.5°C), and they were given free access to water and feed. Mice body weight was weighed once a week. After acclimatization for 1 week (6 weeks of age), the mice were randomly divided into four

groups (n=6) and fed with ND, WD, WG1, WG2 diet. After 12 weeks feeding (18 weeks of age), mice were sacrificed after overnight fasting. The fresh feces were collected at the beginning (6 weeks of age, SS) and the end of the experiment (18 weeks of age) and they were stored at −80 °C until required. At the end of experiment, overnight fasted mice were anesthetized by isoflurane. Epididymis fat, liver tissue, intestine samples, and cecal content were collected.

4.2.6 Measurement of serum biochemical indicators

Blood sera were obtained from mice eyeballs and collected in the tube with coagulant (Separable microtubes, FUCHIGAMI,170720, Japan) after 30min at 24°C to coagulate properly, and acquired by centrifuging at 3000rpm for 10min. The serum sample were stored at -80°C until use. The serum levels of glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), total cholesterol (T-Cho), triacylglycerol (TG), and glucose were measured with an automatic analyzer for clinical chemistry (SPOTCHEM EZ SP-4430, Arkray, Kyoto, Japan). The insulin concentration in serum was measured with an ELISA kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) according to the manufacturer's manual. The index of HOMA-IR was calculated by the formula: (fasting glucose× fasting insulin) /405 (R. C. Turner *et al*., 1979). Serum MCP-1

level was measure with ELISA kit according to the manufacturer's manual.

4.2.7 Analysis of histomorphology and liver fat rate

Hepatic histologic analysis was performed as described previously (K. Chen *et al*., 2019). Mice livers were collected and sliced using a freezing microtome system (Yamato Kohki Industrial Co., Ltd., REM-710, Saitama, Japan) according to the manufacturer's manual with a thickness of about 5µm. Liver sections were then stained with Hematoxylin and eosin stain, and observed under a fluorescence microscope (Keyence, Tokyo, Japan). In addition, the liver fat rate was calculated by simplified Soxhlet extraction method. Briefly, equal proportion of hexane was added to liver tissue and homogenized until the liver tissue was completely shattered, the supernatant was collected by centrifugation at 3000rpm for 10 minutes. After the extract supernatant was further evaporated, the remaining crude fat was weighed to calculate the fat rate of liver.

4.2.8 Taurine and DPP-4 quantitation

Quantities of taurine in mice feces were measured with a Taurine Assay kit (Cell Biolabs, Inc., San Diego, CA) according to the manufacturer's manual. Briefly, the fecal samples were homogenized in

cold Assay Buffer (Taurine Assay kit, MET-5071) at a ratio of 1:20, and centrifuged at 10000g for 10 minutes at 4°C. Sample taurine concentrations are determined by comparison with a known taurine standard, a detection sensitivity limit of 15.6 µM taurine, which was read at 405nm by microplate reader. Quantities of DPP-4 in mice serum were measured with a Mouse DPP-4 Elisa kit (Abcam, ab264630, U.K.) according to the manufacturer's manual. The serum samples were diluted by Sample Diluent NS Buffer (DPP-4 Elisa kit, ab193972) at a ratio of 1:50. Mouse serum DPP-4 concentrations are determined by comparison with a known DPP-4 standard from kit. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm.

4.2.9 Western blotting analysis

Equal amounts of liver tissues were homogenized in RIPA buffer (0.1 g/mL) with a Speed-Mill PLUS homogenizer (Analytik Jena, Jena, Germany) (S. Wu *et al*., 2017). The protein concentrations were determined by using a due-binding protein assay kit (Bio-Rad Hercules, CA, USA) according to the manufacturer's manual. The protein extracts were boiled for 7min with 6×SDS buffer, and equal amounts of protein were separated on SDS-PAGE gels (8–12%) and then transferred electrophoretic gels to PVDF membrane (GE Healthcare, Buckinghamshire, UK). The membrane was then incubated with specific

primary antibody and Horseradish peroxidase (HRP)-conjugated secondary antibody, following by detection with a LumiVision PRO system (TAITEC Co., Saitama, Japan).

4.2.10 Gut microbiota analysis and metabolic prediction

Mice feces were collected at 6-weeks and 18-weeks age, and soon stored at -80°C until use. The feces genomic DNA was extracted with the Fast DNA spin kit (MP BIOMEDICALS) according to the manufacturer's manual, and used for analyzing the composition of gut bacterial communities by sequencing 16S rRNA genes as described in our previous paper (K. Chen *et al*., 2020). Each DNA sequence was amplified by PCR using primers for the V3–V4 regions of the 16S rRNA gene, Operational taxonomic units (OTUs) were picked from the list of filtered sequences, which were consisted of sequences with 97% identity, by Qiime2 (ver. 2020.2) and Greengene (ver. 13_8). Metabolic prediction was performed by using *Piphillin* Server (S. Iwai *et al*., 2016). based on the KEGG database by the OTU Abundance and Representative Sequence.

4.2.11 Statistical analysis

Data were expressed as mean ± SD. The significant differences between groups were analyzed by one-way analysis of variance (ANOVA) tests, which is followed by Duncan's multiple range tests with the SPSS

statistical program (version 19.0, IBM Corp., Armonk, NY, USA). A

probability of $p<0.05$ was considered significant.

4.3 Result

4.3.1 The effects of garlic OSCs on the prevention of WD-induced dyslipidemia and fatty liver
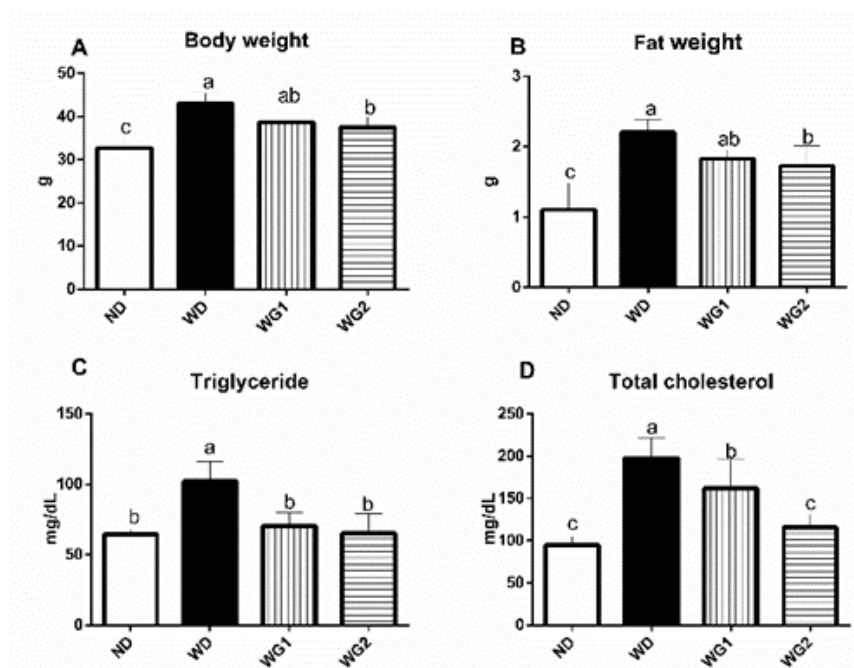
Fig. 4-1 The effects of garlic OSCs on the prevention of WD-induced dyslipidemia.
Effects of garlic supplementation on mice body weight (A), epididymal fat weight (B),
serum TG (C) and T-Cho (D). The data represent mean ± SD of six mice for each group.
Columns with different letters differ significantly ($p<0.05$).

To explore the preventive effect of garlic OSCs on WD-induced

obesity and dyslipidemia, we fed mice with two doses of garlic OSCs and

investigated some indexes related obesity and dyslipidemia. As shown in

Fig. 4-1, G2 remarkably reduce WD-induced mice body overweight and

epididymal fat overweight, while G1 only showed reduction trend without significance (Fig.4-1A, B). Both G1 and G2 significantly reduce WD-induced high levels of triglyceride and total cholesterol on serum (Fig.4-1C, D), and G2 was significantly stronger than G1 in the inhibitory effect of serum T-Cho (Fig. 4-1D).

Furthermore, we examined their preventive effect on fatty liver disease. As shown in Fig. 4-2, both G1 and G2 significantly reduce WD-induced increase in liver fat droplet accumulation (Fig.4-2A), liver weight (Fig.4-2B) and liver fat rate (Fig.4-2C), and G2 showed stronger inhibitory effect than G1. Additionally, there was no difference in initial body weight between the groups, neither in energy intake between WD and G1, G2 groups throughout the 12-week intervention period. These data demonstrated that garlic OSCs contributed to alleviate WD-induced dyslipidemia and fatty liver.
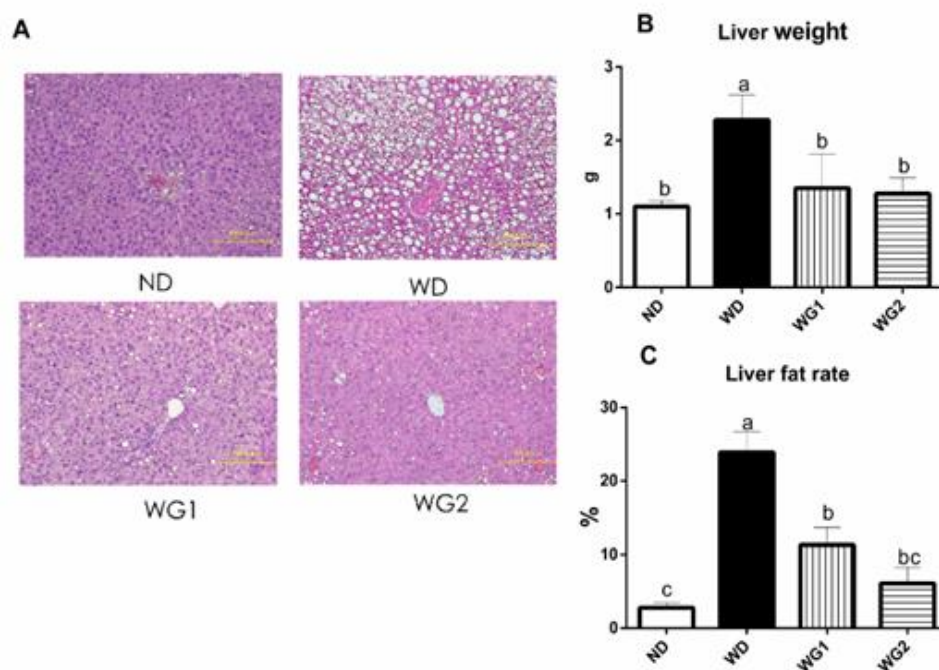
Fig. 4-2 The effects of garlic OSCs on the prevention of WD-induced fatty liver.
Effects of garlic supplementation on liver fatty droplet (A), liver weight (B), liver fat rate (C). Liver tissue was stained by HE stains. The data represent mean ± SD of six mice for each group. Columns with different letters differ significantly ($p<0.05$).

4.3.2 The effects of garlic OSCs on the prevention of WD-induced hyperglycaemia and insulin resistance

To confirm the preventive effect of garlic OSCs on WD-induced hyperglycemia and insulin resistance, we investigated the levels of fasting serum glucose and fasting serum insulin. As shown in Fig.4-3, both G1 and G2 could significantly decrease WD-induced high levels of serum glucose and insulin (Fig.4-3A, B) and G2 showed stronger inhibitory effect on serum glucose than G1 (Fig.4-3A). Moreover, both G1 and G2

significantly decreased the HOMA-IR index, and G2 was more effective than G1 (Fig.4-3C). These results revealed that garlic OSCs might contribute to protect WD-induced hyperglycemia and insulin resistance.
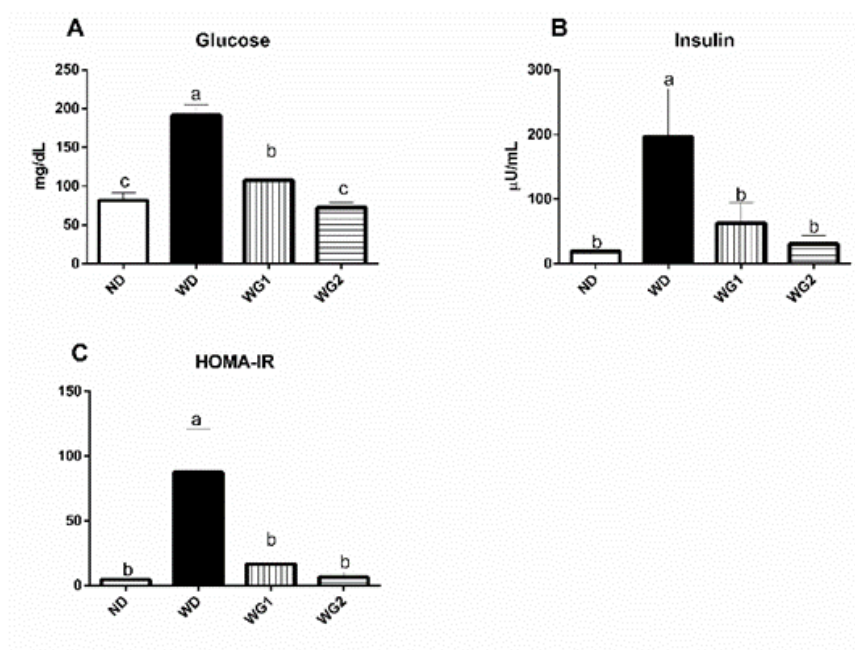
Fig. 4-3 The effects of garlic OSCs on the prevention of WD-induced hyperglycemia and insulin resistance. Effects of garlic supplementation on serum glucose (A), insulin (B) and HOMA-IR (C). The data represent mean ± SD of six mice. Columns with different letters differ significantly ($p<0.05$).

4.3.3 The effects of garlic OSCs on abundance of gut commensal *bacteroides acidifaciens*

In order to assess the effect of garlic on gut microbiota in mice, the fresh fecal samples were collected at the beginning (6 weeks of age, SS) and the end of the experiment (18 weeks of age). The compositions of

bacteria in feces identified by 16S rRNA sequencing are as shown in Fig.5. At the phylum level, the relative abundance of *Deferribacteres* was significantly decreased by G1 and G2, while the relative abundance of *Bacteroidetes* was significantly increased by G1 and G2 compared with WD group (Fig.4-4A). Although there were 19 species of bacteria were modified by G1 or G2 (Fig.4-4B) at the species level, it was interestingly noticed that both G1 and G2 noticeably increased the relative abundance of *B. acidifaciens*, and G2 was strikingly stronger than G1 (Fig.4-4C). Additionally, the metabolic prediction results based on KEGG database showed that G1 and G2 had a significant regulatory effect on fatty acid metabolism, cholesterol metabolism, glucagon signaling pathway and non-alcoholic fatty liver disease (NAFLD) (Fig.4-4D). These data indicated that natural garlic OSCs could significantly modify the compositions of gut microbiota, especially, increase the relative abundance of *B. acidifaciens* significantly. These results suggest that a link between growth stimulation of *B. acidifaciens* and metabolism regulation of lipid and glucose might present in garlic OSCs. These results indicate that the regulation of garlic OSCs on lipid and glucose metabolism may be related to the growth stimulation of *B. acidifaciens*.
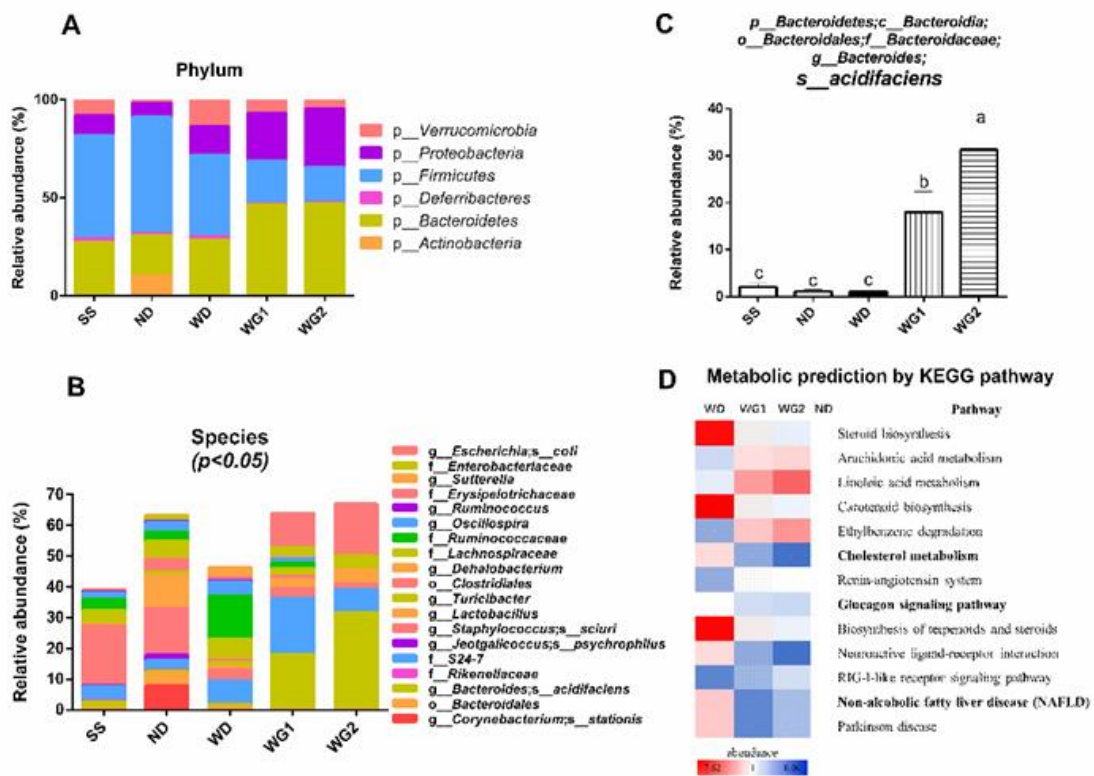
Fig. 4-4 The effects of garlic OSCs on the relative abundance of gut commensal *Bacteroides acidifaciens*. Fresh feces were collected at the beginning (6 weeks of age, SS) and the end of the experiment (18 weeks of age), and the gut microbiota in the feces was characterized by 16S rRNA gene sequencing. These data represent the relative abundance of each bacterial genus: p_, c_, o_, f_, and g_ represent phylum, class, order, family, and genus, respectively, and a blank after the letter means undefined bacterial genus. (A) The relative abundance of bacteria at the phylum level. (B) The relative abundance of bacteria at the species level. The relative abundance of *Bacteroides acidifaciens* (C). The data represent mean ± SD of three mice. Columns with different letters differ significantly ($p<0.05$). (D) 13 metabolic predictions and functional annotations based on the KEGG database. ND was set as control value 1, its positive effect is red, and its negative effect is blue.

4.3.4 The effects of garlic OSCs on the growth of *bacteroides acidifaciens in vitro*

To determine the direct effects of garlic on the growth of *B. acidifaciens*, we cultured *B. acidifaciens in vitro* anaerobic culture. As shown in Fig.4-5, both G1 and G2 significantly increased the growth of *B.*

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acidifaciens in a dose- and a time-dependent manner. After 30 hours culture, the *B. acidifaciens* was significantly grown in the concentration range from 1.25 mg/ml to 5.00 mg/ml of both G1 and G2 (Fig.4-5A). The *B. acidifaciens* was significantly grown from 18 hours in 5.00 mg/ml of both G1 and G2 (Fig.4-5B). Moreover, the growth effect of G2 was significantly higher than that of G1 (Fig.4-5A). These results suggest that garlic OSCs may directly promote the growth of *B. acidifaciens in vitro*.
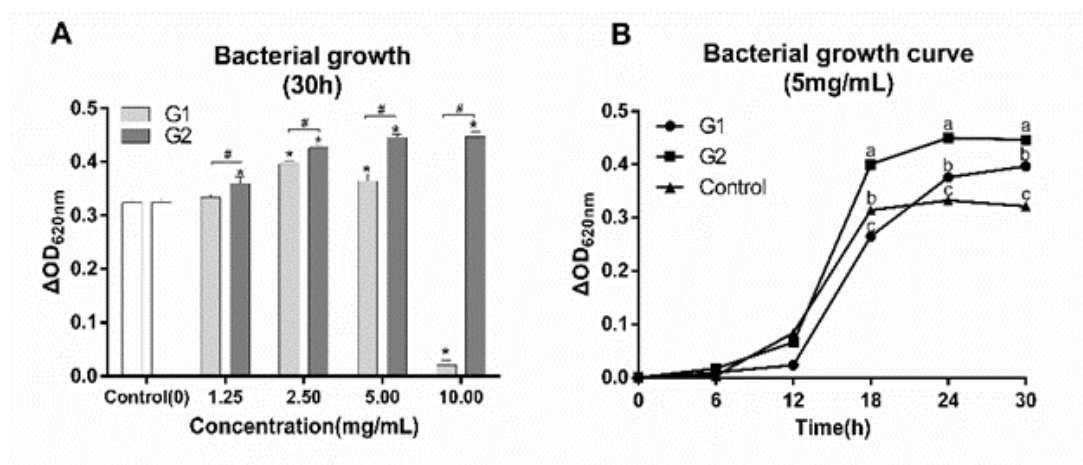
Fig. 4-5 The effects of garlic OSCs on the growth of *Bacteroides acidifaciens in vitro*. *Bacteroides acidifaciens* (JCM10556) were anaerobic cultured in Brucella Broth medium with 7% FBS at 37°C. (A) A dose-dependency experiments. *B.acidifaciens* was cultured in the concentration range of 1.25-10.00 mg/ml of both G1 and G2 for 30 hours. (B) A time-course experiments. *B.acidifaciens* was cultured with 5.00 mg/ml of both G1 and G2 from 0 hour to 30 hours. The bacterial growth was measured by turbidimetry at 620nm. The data represent mean ± SD of four repeat for each group. The "*" represents a significant difference from the control group ($p<0.05$), "#" represents a significant difference between the two sample treatment groups ($p<0.05$).

4.3.5 The effects of garlic OSCs on taurine production and PPARα activation

In order to explore the metabolic mechanism of *B. acidifaciens* increased by garlic OSCs on the effect of lipid profiles, we quantified taurine in feces. As shown in Fig.4-6, both G1 and G2 significantly increased the level of taurine, and G2 was significantly better than G1 (Fig.4-6A). Furthermore, G2 significantly enhance the WD-induced downregulation of PPARα (Fig.4-6B), CPT1A (Fig.4-6C) and CYP4A1 (Fig.4-6D) protein expression in liver, and G1 also showed significant upregulation in CYP4A1 expression, and upregulation trend in other factors. These results indicated that garlic OSCs might promote fatty acid β-oxidation through taurine accumulation and PPARα activation.
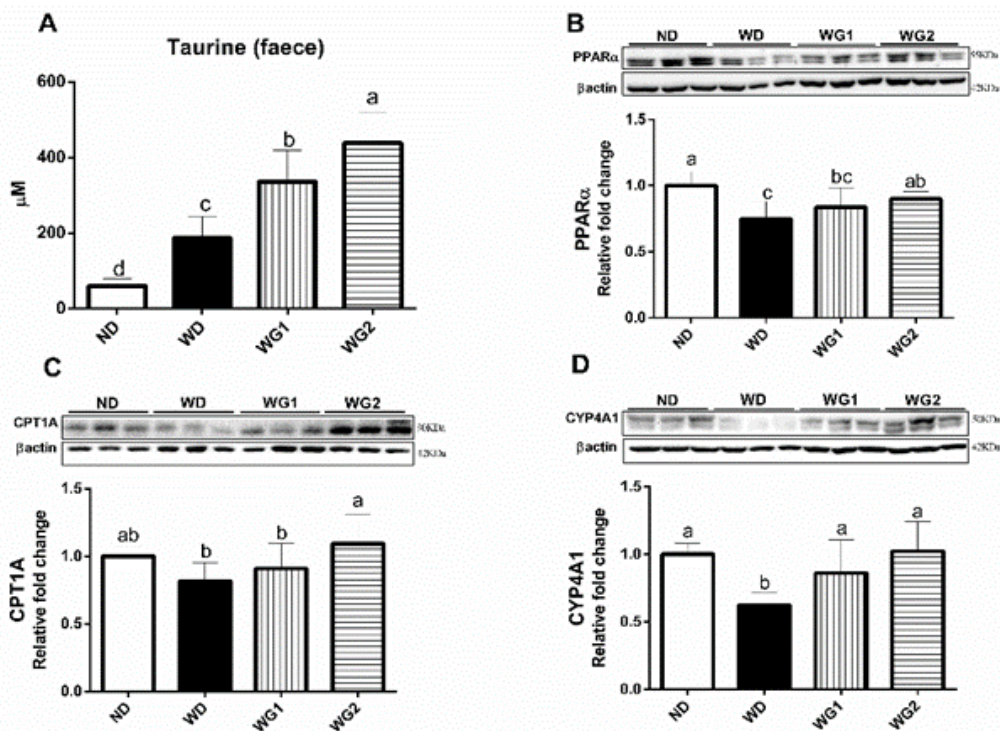
Fig. 4-6 The effects of garlic OSCs on taurine production and PPARα activation. Effects of garlic supplementation on fecal taurine (A), and the protein expression of hepatic PPARα (B), CPT1A (C), CYP4A1 (D). The fecal taurine was measured with a Taurine Assay kit. The proteins were detected by Western blotting. The induction folds of the proteins were calculated as the intensity of the treatment relative to that of control normalized to β-actin by densitometry. The data represent mean ± SD of six mice. Columns with different letters differ significantly (p<0.05).

4.3.6 The effects of garlic OSCs on the DPP-4 activity and gluconeogenesis

To further clarify the metabolic mechanism of garlic OSCs on the glucose homeostasis in mice. We examined the concentration of serum DPP-4 which was negatively correlated with the level of *B. acidifaciens*, and the protein expression of hepatic G6Pase and PEPCK involved in hepatic gluconeogenesis. As shown in Fig. 4-7, both G1 and G2 significantly inhibit the WD-induced high DPP-4 level, and G2 was

notedly more effective than G1(Fig. 4-7A). In addition, G2 could downregulate the WD-induced increase of hepatic G6Pase (Fig. 4-7B) and PEPCK (Fig. 4-7C), while G1 only showed significant downregulation in G6Pase expression. These findings suggest that garlic OSCs might improve glucose homeostasis by inhibiting DPP-4 and by reducing hepatic gluconeogenesis.
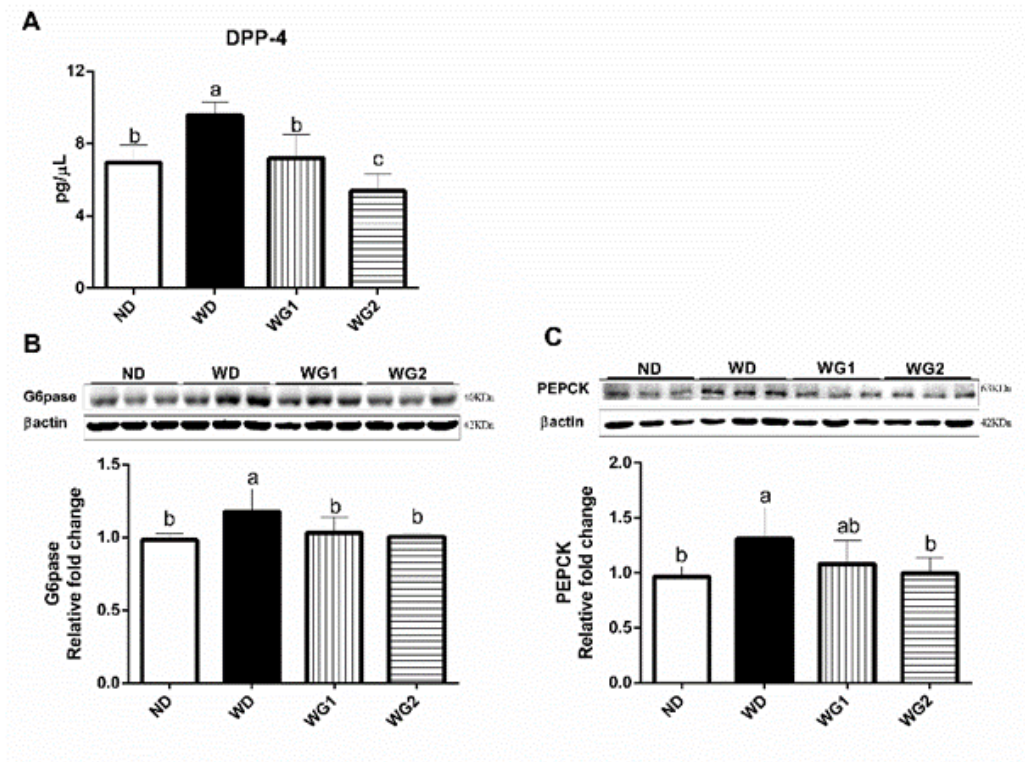
Fig. 4-7 The effects of garlic OSCs on DPP-4 activity and hepatic gluconeogenesis.
Effects of garlic supplementation on serum DPP-4 (A), and the protein expression of hepatic G6Pase (B) and PEPCK (C). The proteins were detected by Western blotting. The induction folds of the proteins were calculated as the intensity of the treatment relative to that of control normalized to β-actin by densitometry. The data represent mean ± SD of six mice. Columns with different letters differ significantly ($p<0.05$).

4.4 Discussion

In animal experiments, both G1 and G2 could specifically increase the relative abundance of *B. acidifaciens* in the mice gut, and G2 was significantly stronger than G1 (Fig.4-4C). To determine whether OSCs directly affected its growth, we cultured *B. acidifaciens* bacteria in an anaerobic environment. Result revealed that garlic OSCs markedly increased the growth of *B. acidifaciens* in a dose-dependent and a time-dependent manner (Fig.4-5). Based on our knowledge, this is the first report that garlic could increase the growth of gut commensal *B. acidifaciens* in vitro cultures.

In lipid metabolism, our data revealed that garlic attenuated WD-induced dyslipidemia and NAFLD. Moreover, garlic significantly reduced WD-induced the high level of serum GPT/GOT rate (Fig. 4-8) and hepatic MCP-1, indicating that garlic could relieve WD-induced mild steatohepatitis. Furthermore, the supplementation of garlic OSCs observably increased the levels of taurine in the mice feces. We also have found that the protein expressions of hepatic PPARα, CPT1A and CYP4A1 were activated by garlic. Taurine supplementation has been observed to prevent obesity and promote fatty acid β-oxidation by upregulating PPARα in mice (S. Murakami, 2015; N. Tsuboyama-Kasaoka *et al.*, 2006). It is consistently reported that garlic essential oil dose-dependently upregulated

the expression levels of hepatic PPARα and CPT1A in HFD-fed mice (Y. S. Lai *et al*., 2014). Therefore, the *B. acidifaciens*-Taurine-PPARα axis possibly plays a crucial role in the lipid metabolism regulated by garlic OSCs.
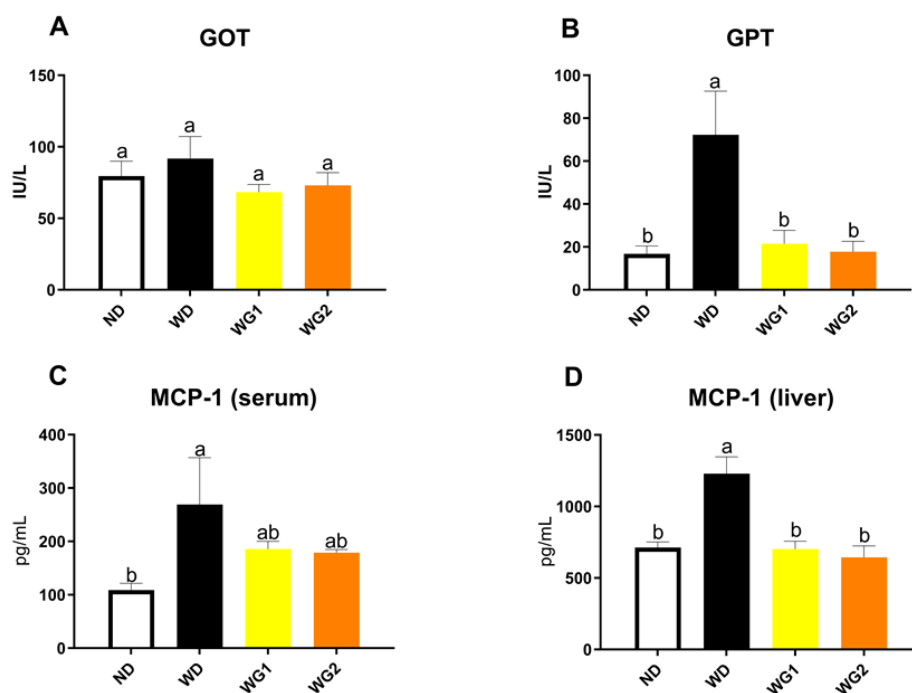
Fig. 4-8 The effects of garlic OSCs on the prevention of WD-induced hepatitis. (A) The serum GOT. (B) The serum GPT. (C) The concentration of monocytes chemotactic protein-1 (MCP-1) on serum. (D) The concentration of MCP-1 in liver. The data represent mean ± SEM of six mice for each group. Columns with different letters differ significantly (p<0.05).

In glucose metabolism, we have found that garlic significantly decreased WD-induced insulin resistance. Consistently, garlic extract has been reported to improve type 2 diabetes, (T. Maeda *et al*., 2019; S. Miki *et al*., 2017; S. L. Zheng *et al*., 2018), and the gut microbiota profile in a mouse model of insulin resistance, even inhibited 50% DPP-4 activity *in*

vitro (P. Kalhotra *et al*., 2020).　It has been determined that DPP-4 inhibitors were a class of oral hypoglycemics, and the commensal bacteria *B. acidifaciens* was also reported to reduce DPP-4 enzyme activity (J. Y. Yang *et al*., 2017). Our data revealed that garlic supplementation increased the abundance of *B. acidifaciens* and reduced serum DPP-4. Consequently, we speculated that the inhibitory effect of garlic OSCs on DPP-4 was not only the direct effect of garlic, but also the effect of *B. acidifaciens* enhanced by garlic OSCs. Furthermore, the hepatic of G6Pase and PEPCK were the key protein to observe and detect hepatic gluconeogenesis and glycogen synthesis rate (Q. Yang *et al*., 2018; M. C. Petersen *et al*., 2017). We found that G2 could restore the WD-induced upregulation of G6Pase and PEPCK hepatic protein expression. Therefore, garlic OSCs could possibly improve glucose homeostasis by increasing *B. acidifaciens* to inactivates DPP-4 and subsequently decrease the protein expression of hepatic G6Pase and PEPCK.

In addition, the metabolic prediction results also indicated that the gut microbiota composition altered by garlic has an impact on lipid and glucose metabolism. Several lines of animal studies have demonstrated that the relative abundance of *B. acidifaciens* was markedly increased by high-fiber diet and acetate (F. Z. Marques *et al*., 2017), (Q. Zhang *et al*., 2019), chondroitin sulfate (F. Liu *et al*., 2017). Recent research has demonstrated

the effect of *B. acidifaciens* as a probiotic, which is mainly reflected in the regulation of fat and glucose metabolism, as well as immunity. For instance, oral administration of live *B. acidifaciens* can prevent obesity and improve insulin sensitivity, (J. Y. Yang *et al*., 2017), and promote IgA production (T. Yanagibashi *et al*., 2013; A. Nakajima *et al*., 2020). Therefore, garlic OSCs might ameliorate WD-induced metabolic disorder by modulating the growth of *B. acidifaciens*, while the metabolic mechanism of garlic OSCs promoting the *B. acidifaciens* proliferation is still unclear. Taurine is an essential amino sulfonic acid, usually ingested from seafood, eggs, meat, and milk, but not from plant-originated foods (A. Milito *et al*., 2019). However, high levels of taurine were observed in the supplementation of garlic OSCs mice. Moreover, it has reported that taurine was found to be one of the metabolites produced by *B. acidifaciens* in the intestines of mice fed with *B. acidifaciens* (A. Nakajima *et al*., 2020), and taurine was also increased in the feces of *B. acidifaciens* -colonized mice (J. Y. Yang *et al*., 2017). Consequently, we speculated that OSCs in garlic might provide the raw material for *B. acidifaciens* to produce taurine. Meanwhile, other gut microbiota converts taurine to sulphide to inhibit pathogen aerobically respiration (A. Stacy *et al*., 2021), and promote the anaerobic bacteria *B. acidifaciens* proliferation.

In summary, we presented evidence that garlic OSCs supplementation

could improve lipid metabolism and glucose homeostasis in WD-fed mice by enhancing fatty acid β-oxidation and reducing hepatic gluconeogenesis. Gut microbiota data revealed that these effects were associated with the remodeling of the gut microbiota, especially, the significant increase in gut commensal *B. acidifaciens*. The *B. acidifaciens*-taurine axis and *B. acidifaciens*-DPP-4 axis might contribute to the preventive effect of garlic OSCs on WD-induced lipid and glucose metabolic disease.

4.5 Abstract

There are quite different reports in the effects of garlic and gut microbiota due to unstable garlic organosulfur compounds (OSCs) produced by alliinase during garlic preparation. In this study, garlic alliinase was deactivated to obtain stable garlic OSCs. The results from C57BL/6J mice experiment revealed that stable garlic OSCs prevented the disorder of Western diet (WD)-induced lipid and glucose metabolism by increasing the relative abundance of gut *bacteroides acidifaciens*. Molecular data showed that garlic OSCs inhibited dyslipidemia and fatty liver by increasing taurine, sequentially promoting hepatic fatty acid β-oxidation. Meanwhile, garlic OSCs meliorated glucose homeostasis by inhibiting dipeptidyl peptidase-4 (DPP-4) and hepatic gluconeogenesis. *In vitro* culture results demonstrated that garlic OSCs directly increased the growth of gut *bacteroides acidifaciens*. Taken together, this study demonstrated that *bacteroides acidifaciens*-taurine /DPP-4 axis was involved in the preventive effect of garlic OSCs on metabolic disorder of WD-induced lipid and glucose.
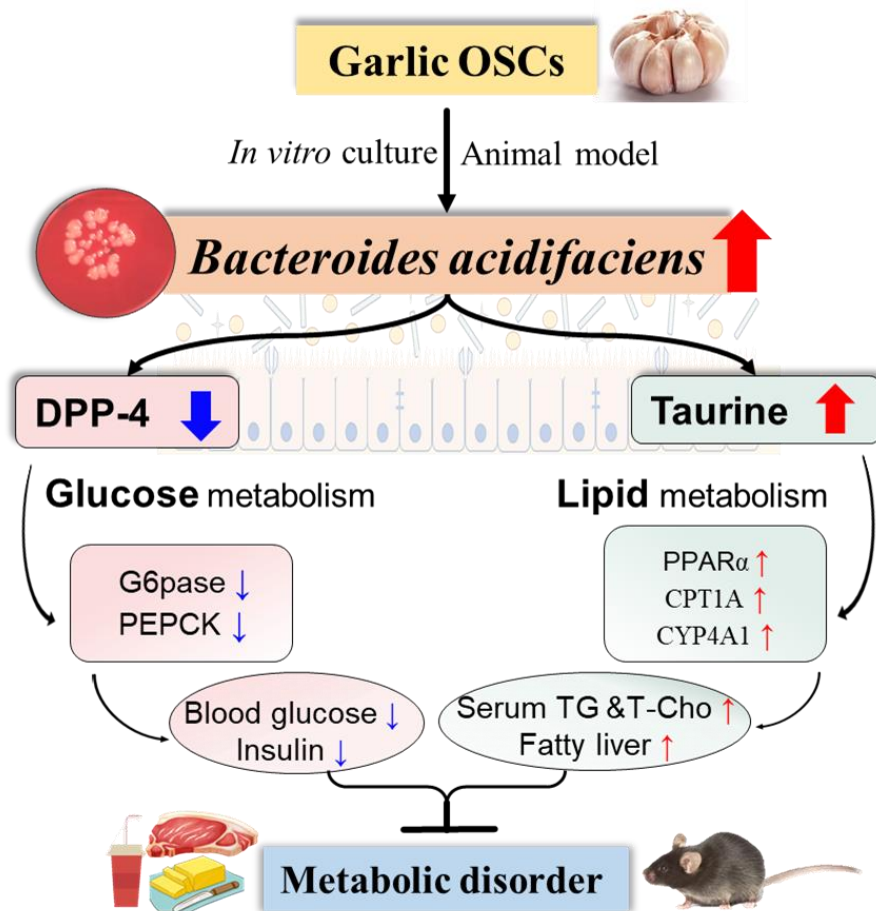
Fig. 4-9 Graphical abstract: Summary diagram the *B. acidifaciens*-taurine axis and *B. acidifaciens*-DPP-4 axis might contribute to the preventive effect of garlic organosulfur compounds on WD-induced lipid and glucose metabolic disorder. Animal data revealed that garlic organosulfur compounds significantly inhibited WD-induced high total cholesterol (T-Cho), liver fat by increasing gut taurine, thereby promote hepatic fatty acid β-oxidation. Meanwhile, garlic OSCs meliorated glucose homeostasis by inhibiting gut dipeptidyl peptidase-4 (DPP-4), followed by the downregulation of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxy kinase (PEPCK) expressions.

Chapter 5 General discussion and conclusion

5.1 General discussion

In the present study, the preventive effect of garlic on metabolic syndrome were characterized at molecular levels, focusing on the lipid and glucose metabolism and gut microbiota modification. The most important result is that *B. acidifaciens*-taurine axis and -DPP-4 axis were involved in the preventive effect of garlic OSCs on metabolic disorder of lipid and glucose.

Firstly, Alliin, SAC and GSAC are the main natural OSCs in raw garlic, and have almost no bactericidal ability. After garlic was crushed, alliinase flowed out of garlic cell vacuoles, and catalyse alliin to produce allicin. Allicin is an unstable molecule that can be transforms into a series of OSCs with strong bactericidal ability, including DADS, DATS, ajoenes (Phoebe Zapanta Trio *et al*., 2014). On the other hand, garlic is one of the vegetables with the highest fructan content and has the potential to be a prebiotic (Jane G Muir *et al.*, 2007). Combined with previous reports on garlic's various functions, we predicted that garlic might improve the gut bacteria and thus the host's metabolism.

In the chapter 2 section, we used a mouse model with normal diet and high-fat diet to investigate the influence and mechanisms of unprocessed

whole garlic on the gut microbiota, and use dextrin as positive control. From this experiment, I mainly discovered the following information: 1. garlic at 5% concentration has no side effects. 2. garlic has better preventive effect on lipid metabolism disorder induced by HFD model than dextrin. 3. preliminary tests show that garlic has a great effect on gut microbiota and their metabolites. However, Because of the complexity of garlic ingredients, we have to enhance extraction techniques, try to simplify the garlic ingredients, for analyse the mechanism of its effects better accurately.

Hence, in the chapter 3, administration of 1% and 5% AFG were performed in the same mouse model to investigate the influence on gut microbiota. The data mainly showed the following results: 1. 5% AFG has better preventive effect on lipid metabolism disorder than 1% AFG. 2. The significant modification on gut microbiota focusing on the p-*Bacteroidetes* was increased by AFG. 3. The probiotics including the g-*Akkermansia*, and g-*Lactobacillus* were enhanced by AFG. These results further prove that garlic can improve gut microbiota, and AFG seems to increase probiotics more effectively than whole garlic. However, available data are still insufficient to screen out individual characteristic bacteria and analyse their molecular mechanisms. In addition, even though AFG has been extracted, the OSCs and fructans, the two most promising components in AFG, still

do not control for a single variable.

Thus, in the chapter 4, we enhanced the mouse metabolic syndrome model, using WD diets and WD adding two types of AFG with different OSCs concentrations and the same fructan concentration. The experimental data mainly gave the following findings: 1. the garlic OSCs significantly inhibited WD-induced dyslipidemia and NAFLD, insulin resistance. 2. the gut commensal *B. acidifaciens* were notably increased by garlic OSCs. 3. the garlic OSCs significantly increased the levels of taurine on gut and DPP-4 on serum. Meanwhile, it has been reported that mice fed with *B. acidifaciens* can produce taurine in gut and inhibit DPP-4 in the small intestine (J. Y. Yang *et al*., 2017; A. Nakajima *et al*., 2020). Furthermore, we have found that the OSCs significantly increased the growth of *B. acidifaciens* in vitro culture. Therefore, we have demonstrated that *B. acidifaciens*-taurine axis and -DPP-4 axis were involved in the preventive effect of garlic OSCs on metabolic disorder of lipid and glucose.

However, existing garlic samples are still mixtures, and it is impossible to determine which single substance is responsible. In terms of direct interaction between *B. acidifaciens* and garlic, it is necessary to extract purer samples such as fructan, alliin, and detected their effect with *B. acidifaciens* in *in vitro* culture. In addition, it is still difficult to simulate

the real intestinal anaerobic environment *in vitro* culture of *B. acidifaciens* bacteria. Therefore, it is necessary to design an animal experiment with the purer substance samples.

5.2   General conclusion

In conclusion, the study demonstrates that garlic had the preventive effects on diet induced metabolic syndrome and gut microbiota disorder. The natural garlic OSCs could remodel gut microbiota, and increase the growth of *B. acidifaciens*. The gut commensal *B. acidifaciens* produce taurine and inhibit DPP-4 to mediate lipid and glucose metabolism. These findings provide new insight for understanding the molecular mechanisms of garlic on the prevention of metabolic syndrome and gut microbiota disorder.

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Chapter 1 Introduction

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