Doctoral Dissertation Summary
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Examining preventive effect of natural product for lifestyle diseases and analyzing its molecular mechanism

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Abstract

Lifestyle diseases including obesity, fatty liver diseases, and type 2 diabetes, which has imposed big health challenges for societies worldwide. Diet intervention is an important key factor to regulate lifestyle diseases. Functional food (containing natural product) is also an effective way to control our daily diet for its continuously. In this study, we aimed to investigate the preventive effect for lifestyle diseases by three natural products and analyze its mechanism. Firstly, we found that asperuloside which is one of glycoside, derived from Eucommia leaf is effective for obesity and type 2 diabetes. Asperuloside prevented diet-induced metabolic disorders in several organs. One of the mechanisms is mediated by gut microbiota and gut microbiota-derived metabolite. Secondly, we revealed that asperuloside prevents non-alcoholic fatty liver disease (NAFLD) and its progressive disease, non-alcoholic steatohepatitis (NASH). Asperuloside changed liver lipid metabolism and prevented mitochondria dysfunction. Thirdly, antioxidant natural compound Melon GliSODin prevented NAFLD/NASH onset, and the mechanism was explained by activated antioxidant pathway and improved lipid metabolism in the adipocyte. Finally, we used *Agaricus brasiliensis KA21* which is known to have an anti-inflammation effect. *Agaricus brasiliensis KA21* prevented NAFLD/NASH onset and changed NADPH pathway which induce oxidative stress. As a result, asperuloside, Melon GliSODin, and *Agaricus brasiliensis KA21* could be effective natural products for preventing lifestyle diseases.

Keywords: Obesity, Type 2 diabetes, NAFLD, NASH, Natural products

Chapter 1
Metabolic disorders in lifestyle diseases

With the development of a variety of diets, such as westernized diet and the rapid progress of an aging society, the prevalence of lifestyle diseases such as obesity and diabetes patients are increasing not only in developed countries (Bhurosy and Jeewon, 2014) but also in many developing countries. The medication is under seeking by many pharmaceutical companies and researchers. However, most of these medications is palliative. These medications are therapeutic drugs that can be taken only after receiving a prescription from a doctor after being diagnosed with a disease. In addition, lifestyle diseases have weak symptoms, so that patients do not notice until they become serious. It is necessary and important to pay attention to the daily habit and prevent the progression of the lifestyle disease, as much as possible before the disease becomes serious, not only after it have developed.

Lifestyle diseases, including obesity, type 2 diabetes, hyperlipidemia, high blood pressure, colon cancer, squamous cell lung cancer, chronic bronchitis, emphysema, and periodontal disease. This factor is based on irregular life habits such as overeating, lack of physical activity, smoking and drinking alcohol (Figure 1). In Japan, around 60 % of total deaths are lifestyle diseases and large number of patients, are closely linked to the development of more serious diseases such as arteriosclerosis and arteriosclerosis which significantly impair our quality of life (QOL).

Dietary intervention is easy way to control our health and effective way to prevent the development of lifestyle disease (Fock and Khoo, 2013; Sackner-Bernstein et al., 2015). There are many dietary methods such as macrobiotic diet, low carbohydrate diet and many other methods. Functional food is also paid attention by many people for its continuity.

To demonstrate the effectiveness of functional food (natural product), we investigated the health benefit and underlying molecular mechanisms by natural products.

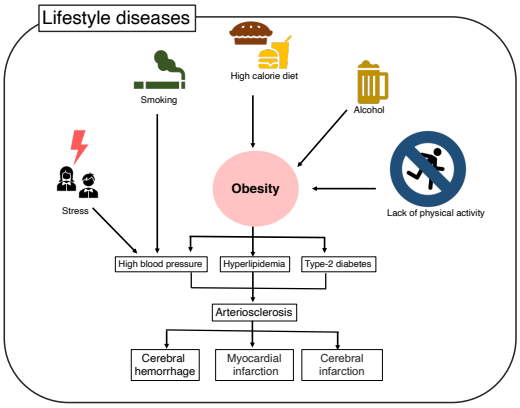

Figure 1 Factor of lifestyle diseases and its process
Lifestyle disease including obesity, diabetes, hyperlipidemia and high blood pressure has a development risk to arteriosclerosis and more serious diseases. Controlling our life habits is important to prevention of lifestyle diseases.

Chapter 2
Anti-metabolic syndrome compound "Asperuloside"

In Chapter 2, we have evaluated that asperuloside (ASP), a kind of iridoid glycoside, extraction from eucommia leaf has anti-obesity and anti-type 2 diabetic effect and analyzing its underlying molecular mechanism. Our study showed that in high-fat diet (HFD) fed mice, ASP prevented body weight gain, dyslipidemia, glucose intolerance and insulin resistance which is commonly phenomenon of metabolic syndrome. And, the mechanism for anti-obesity and anti-diabetic effect is mediated by gut microbiota and gut microbiota-derived metabolite (Figure 2). Recent study growing the evidence which suggests that gut microbiota contribute to metabolic disorders through an axis of communication (Cani and Knauf, 2016). And the abundance of specific bacteria is important to contribute metabolism. Virtually, ASP increased beneficial bacteria i.e. *Parabacteroides* and *Akkermansia*. And ASP changed gut microbial structure in phylum level. *Parabacteroides* has been reported as anti-inflammatory symbionts, produce succinate and improve glucose homeostasis via regulating gluconeogenesis (Wang et al., 2019). For instance, our result in cecum metabolite profile using CE-TOFMS measurement showed that succinate was increased in ASP supplementation group. Also in phenotype, administration of HFD only group causes deterioration of insulin resistance and impaired glucose tolerance. On the other hands, supplementation of ASP suppressed HFD-induced insulin resistance and glucose intolerance. The part of mechanism is indirect effect by increasing specific bacterial species and its metabolite. This mechanism could be explained by *Parabacteroides*-succinate pathway as previously reported. In addition, *Akkermansia*, the presence of this bacteria is associated with healthy individuals (Dao et al., 2016; Depommier et al., 2019), adheres to enterocytes and strengthens the integrity of the epithelial cell layer (Singh et al., 2019). In our study, the presence of *Akkermansia* have confirmed only in ASP treated group. And epithelial environment including tight junction and anti-peptide and mucin expression is upregulated in ASP treated mice. In addition, ASP reduced the marker of intestinal permeability, lipopolysaccharide (LPS) inflow into the plasma. These results suggest that the presence of *Akkermansia* prevented intestinal permeability and improved intestinal environment induced by the HFD. In adipocyte, white adipose tissue (WAT) which stores fat was significantly miniaturized in ASP supplemented group. This is because, ASP improved adipocyte lipid metabolism including β-oxidation in WAT. In brown adipose tissue (BAT) which induce energy expenditure (Cypess and Kahn, 2010), ASP induced energy expenditure in gene expression level. The mechanism of activated BAT by ASP was interesting discovery. And this investigation remains new claims that why ASP induce the changing BAT function. However, the metabolite which is produced by the gut microbiota succinate level could be support the mechanism in adipocyte in our study. Accumulation of succinate controls activation of adipose tissue thermogenesis (Mills et al., 2018).

In summary, our study showed the importance of microbiota and host crosstalk, we could not reveal how ASP changes microbiota such as anti-bacterial effect, or prebiotics effect. In future study, it could be interesting whether ASP have prebiotics effect or not. In addition, fecal transplantation would make our gut microbiota mediated hypothesis stronger.

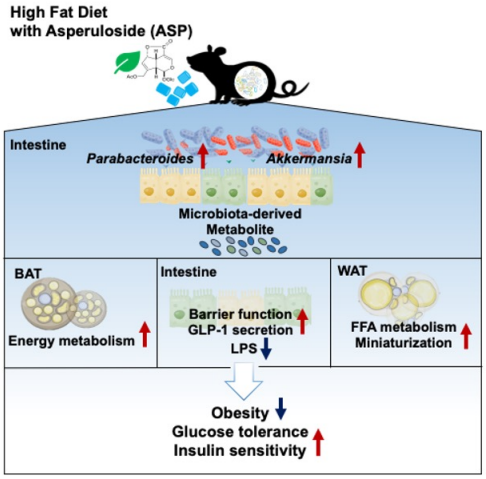

Figure 2 Proposed mechanism of anti-obesity and anti-type 2 diabetic effect by asperuloside

Asperuloside improves the intestinal epithelium environment by changing microbiota composition and its metabolites. Succinate produced by intestinal bacteria may change the metabolic signals in adipocytes and prevent obesity and type 2 diabetes.

Chapter 3
Asperuloside prevents non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) onset

In Chapter 3, we examined the preventive effect of ASP for non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) using high-fat diet (HFD) induced NAFLD model mice and high-cholesterol and high-fat diet (HCD) induced NASH model mice.

NAFLD is the liver phenotype of obesity patients and the hallmark is excessive lipid accumulation in the liver. It is a major health problem because of its high prevalence and the associated risk of progression to liver cirrhosis and liver cancer (Bhurosy and Jeewon, 2014). NASH is a progressive disease of NAFLD and the characteristic is fibrosis and inflammation (Younossi et al., 2018). NASH is becoming the most frequent factor of liver cirrhosis and liver cancer. There are no FDA-approved drugs for NASH (Sanyal et al., 2015). We have already shown ASP attenuates obesity and type 2 diabetes symptoms in Chapter 2. We focused on the effectiveness of the liver phenotype by ASP.

In NAFLD model, ASP prevented hepatic liver accumulation and inflammation. We revealed that the suppressed liver lipid accumulation by ASP is explained by the regulating lipogenesis pathway including with the liver X receptor (LXR).

In NASH study, we have showed the preventive effect by ASP on NASH, which is more serious progression model than NAFLD. Our study showed that ASP prevented dietary induced NASH. ASP reduced hepatic lipid accumulation, inflammation and fibrosis which are common phenotype in NASH model. And, ASP prevented liver dysfunction induced by HCD via reducing oxidative stress in the liver.

Our study showed the possibility of effectiveness for NAFLD/NASH by ASP.

Chapter 4
Melon GliSODin prevents NAFLD/NASH onset

In Chapter 4, we have revealed that SOD-like substance, Melon GliSODin prevents NASH onset by improving liver function in diet induced NASH model (Figure 3). Oxidative stress is important role of NASH pathogen (Masarone et al., 2018) and cause the progression of inflammatory condition by upregulating redox signaling pathways, altered gene expression of inflammatory markers creating a vicious cycle. Superoxide Dismutase (SOD) down ameliorates oxidative stress by catalyzing dismutation of the superoxide ($O2^{-}$) radical the enzyme (Sakiyama et al., 2016). Melon GliSODin is a product which makes it possible to deliver SOD to the intestinal tract by avoiding decomposition in the stomach. We hypothesized that increasing of SOD substrate level by administrating Melon GliSODin, may prevent NASH onset via increasing oxidative stress.

As a result, Melon GliSODin prevented NASH onset in dietary induced NASH model mice and the underlying mechanism was multiple effect. Firstly, Melon GliSODin induces antioxidant enzymes and suppresses reactive oxygen species (ROS) marker in the liver. This could be explained by the already known function that Melon GliSODin induces antioxidant enzymes (Vouldoukis et al., 2004). In addition, in adipose tissue, Melon GliSODin reduces the size of miniaturized fat cells and reduces the influx of free fatty acid (FFA) into the liver. NASH has multiple mechanism from several organs. Enlarged adipocytes also promote liver damage via releasing of cytokines and fatty acids (Parekh and Anania, 2007). In Melon GliSODin supplemented mice, the

reduction of FFA in the liver suppressed β-oxidation, which potentially reduces ROS production. Furthermore, fatty acid synthesis is potentially suppressed by Melon GliSODin and thus prevents fat accumulation and inflammation in the liver.

It was clarified that Melon GliSODin regulates adipocyte hypertrophy, suppresses FFA influx into the liver, and prevents over triglyceride (TG) accumulation in the liver and inflammation. However, the mechanism why the Melon GliSODin causes adipocyte miniaturization remains unclear. Furthermore, this finding for adipocyte has not been reported in previous Melon GliSODin study. Our result showed the new insight of Melon GliSODin function for adipocyte. Especially, for obesity and insulin resistance, it is closely linking to adipocyte (Mendez-Sanchez et al., 2018). Further investigation about the effect on the high-fat diet induced metabolic syndrome model mice and molecular mechanism using Melon GliSODin. This experiment would show the new potential of Melon GliSODin for preventing metabolic syndrome.

Together with the results of our study and previous study would make it possible for clinical application to promote active intake of supplements in their habit for the purpose of maintaining liver function and preventing NAFLD and NASH.

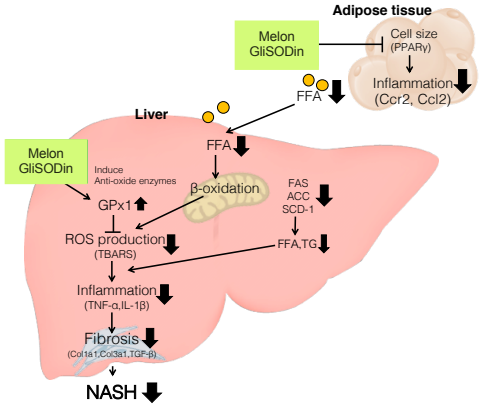

Figure 3 Melon GliSODin attenuates hepatic lipid accumulation and fibrosis

Melon GliSODin reduced hepatic lipid accumulation, inflammation and fibrosis in the liver. In white adipocyte, miniaturized adipocyte cell size and influx of FFA to the liver.

Chapter 5
Agaricus brasiliensis KA21 attenuated NAFLD/NASH progression

In Chapter 5, we have revealed that *Agaricus brasiliensis KA21* have preventive property for NASH onset. *Agaricus brasiliensis KA21* is a mushroom which native to Brazil, and widely cultivated in Japan for its medicinal uses. It is considered as one of the edible use mushrooms to cure several diseases. In previous study, *Agaricus brasiliensis KA21* has anti-inflammation property and prevent oxidative stress under the chronic disease state (Gonçalves et al., 2012; Saiki et al., 2017). We examined whether *Agaricus brasiliensis KA21* have functions for preventing effect on NASH, which is a disease based on inflammation and accumulation of oxidative stress.

As a result, we revealed that *Agaricus brasiliensis KA21* exerted preventive effect in NASH model mice. *Agaricus brasiliensis KA21* prevented liver lipid accumulation and plasma lipid accumulation. Also, fibrosis and inflammation were prevented in *Agaricus brasiliensis KA21* supplementation mice. Thiobarbituric Acid Reactive Substances (TBARS) as marker of oxidative stress was decreased in *Agaricus brasiliensis KA21* supplemented mice liver. We also investigated why the oxidative stress was decreased in *Agaricus brasiliensis KA21* supplementation. The mechanism of reducing oxidative stress could be explained by reducing NADPH oxidase (NOX) activity pathway which generating ROS production. Our study showed that *Agaricus brasiliensis KA21* exerts not only anti-inflammatory effect but also an effect of preventing lipid accumulation and oxidative stress accumulation under the NASH condition (Figure 4). The administration of *Agaricus brasiliensis KA21* activated anti-inflammatory and antioxidant pathways in the liver, which has not been reported previously.

In our study, we used whole dried powder of *Agaricus brasiliensis KA21*. However, the characteristic of *Agaricus brasiliensis KA21* is rich in containing β-glucan and polyphenols. Our study remains the issue what is the main active compound for NASH prevention by *Agaricus brasiliensis KA21*. In the future study, some purified compound focusing β-glucan and

polyphenols which extracted from *Agaricus brasiliensis KA21* could solve this claim. Adding such purified components to the primary hepatocyte and examining anti-inflammatory and antioxidant effects, would approaches more detailed mechanisms. In addition, to investigate the property of *Agaricus brasiliensis KA21* the trial comparing chow diet and chow diet with *Agaricus brasiliensis KA21* is interesting to know its new potential for health benefit of this product.

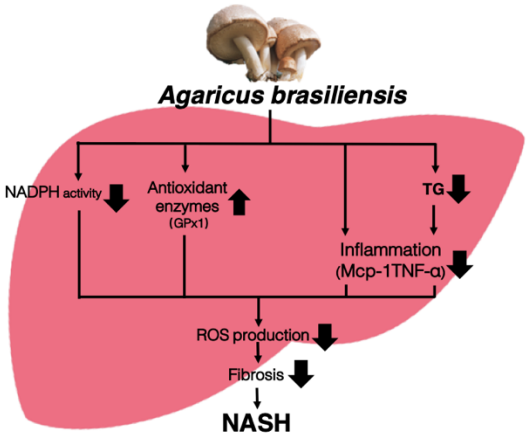

Figure 4 *Agaricus brasiliensis KA21* attenuates liver inflammation and fibrosis

Agaricus brasiliensis KA21 prevented lipid accumulation and inflammation in the liver. *Agaricus brasiliensis KA21* prevented NADPH activity and generating oxidative stress level in the liver.

Chapter 6
General Conclusion

In chapter 6, we have summarized Chapter 2 to Chapter 5. We have shown that three natural compounds are effective for prevention of lifestyle-related diseases. In particularly, ASP is effective for obesity and type 2 diabetes, NAFLD and NASH, Melon GliSODin and *Agaricus barasiliensis KA21* have preventive effect for NASH. These three compounds were already reported as anti-inflammation and anti-oxidative stress property. We strengthened the evidence that substances which have been reported to have antioxidant and anti-inflammatory effect have possibility to preventing NASH. And the importance of oxidative stress and inflammation condition under the metabolic disorders.

In addition, we discussed the future work of this study. Our study has investigated the mechanism using the mice model after the pathological condition has been completed. However, in order to approach the more detailed mechanism, the study which set multiple time points until the pathological condition is completed and its omics analysis using transcriptome, metabolome and microbiome analysis would make it clear how the compounds work. In addition, the effect under the normal diet condition could be interesting to deepen the characteristics of the compounds and new insight of its function.

In NASH study, we have not compared with positive control because there are no approved drugs in NASH disease. However, Vitamin E and thiazolidinedione drugs are recognized as potentially effective compound to NASH (Al-Busafi et al., 2012; Bansal et al., 2020). Trials comparing these drugs, ASP, Melon GliSODin and *Agaricus brasiliensis KA21* about the preventive effects of NASH are important for showing the efficacy of three compounds and would be a very interesting study.

As a limitation of this study, although this study aims to the human application finally, there is a difference in eating habits and living environment between mice and humans, so this result always has not the same effect in humans. In addition, it should be noted there is no method which effective for all subjects because the differences of their genetic and environmental background. Finally, we hoped that by disseminating these findings to society, many people will be able to incorporate such products into their daily dietary habits as preventive medication.

References

Al-Busafi, S.A., Bhat, M., Wong, P., Ghali, P., and Deschenes, M. (2012). Antioxidant therapy in nonalcoholic steatohepatitis. Hepat. Res. Treat. 2012, 947575.

Bansal, G., Thanikachalam, P.V., Maurya, R.K., Chawla, P., and Ramamurthy, S. (2020). An overview on medicinal perspective of thiazolidine-2,4-dione: A remarkable scaffold in the treatment of type 2 diabetes. J. Adv. Res. 23, 163–205.

Bhurosy, T., and Jeewon, R. (2014). Overweight and obesity epidemic in developing countries: A problem with diet, physical activity, or

socioeconomic status? Sci. World J. 2014, 964236.

Cani, P.D., and Knauf, C. (2016). How gut microbes talk to organs: The role of endocrine and nervous routes. Mol. Metab. 5, 743–752.

Cypess, A.M., and Kahn, C.R. (2010). Brown fat as a therapy for obesity and diabetes. Curr. Opin. Endocrinol. Diabetes Obes. 17, 143–149.

Dao, M.C., Everard, A., Aron-Wisnewsky, J., Sokolovska, N., Prifti, E., Verger, E.O., Kayser, B.D., Levenez, F., Chilloux, J., Hoyles, L., et al. (2016). Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: Relationship with gut microbiome richness and ecology. Gut 65, 426–436.

Depommier, C., Everard, A., Druart, C., Plovier, H., Van Hul, M., Vieira-Silva, S., Falony, G., Raes, J., Maiter, D., Delzenne, N.M., et al. (2019). Supplementation with Akkermansia muciniphila in overweight and obese human volunteers: a proof-of-concept exploratory study. Nat. Med. 25, 1096–1103.

Fock, K.M., and Khoo, J. (2013). Diet and exercise in management of obesity and overweight. J. Gastroenterol. Hepatol. 28, 59–63.

Gonçalves, J.L., Roma, E.H., Gomes-Santos, A.C., Aguilar, E.C., Cisalpino, D., Fernandes, L.R., Vieira, A.T., Oliveira, D.R., Cardoso, V.N., Teixeira, M.M., et al. (2012). Pro-inflammatory effects of the mushroom Agaricus blazei and its consequences on atherosclerosis development. Eur. J. Nutr. 51, 927–937.

Masarone, M., Rosato, V., Dallio, M., Gravina, A.G., Aglitti, A., Loguercio, C., Federico, A., and Persico, M. (2018). Role of Oxidative Stress in Pathophysiology of Nonalcoholic Fatty Liver Disease. Oxid. Med. Cell. Longev. 2018, 9547613.

Mendez-Sanchez, N., Cruz-Ramon, V.C., Ramirez-Perez, O.L., Hwang, J.P., Barranco-Fragoso, B., and Cordova-Gallardo, J. (2018). New Aspects of Lipotoxicity in Nonalcoholic Steatohepatitis. Int. J. Mol. Sci. 19.

Mills, E.L., Pierce, K.A., Jedrychowski, M.P., Garrity, R., Winther, S., Vidoni, S., Yoneshiro, T., Spinelli, J.B., Lu, G.Z., Kazak, L., et al. (2018). Accumulation of succinate controls activation of adipose tissue thermogenesis. Nature 560, 102–106.

Parekh, S., and Anania, F.A. (2007). Abnormal lipid and glucose metabolism in obesity: implications for nonalcoholic fatty liver disease. Gastroenterology 132, 2191–2207.

Sackner-Bernstein, J., Kanter, D., and Kaul, S. (2015). Dietary intervention for overweight and obese adults: comparison of low- carbohydrate and low-fat diets. a meta- analysis. PLoS ONE 10, e0139817.

Saiki, P., Kawano, Y., Van Griensven, L.J.L.D., and Miyazaki, K. (2017). The anti-inflammatory effect of Agaricus brasiliensis is partly due to its linoleic acid content. Food Funct. 8, 4150–4158.

Sakiyama, H., Fujiwara, N., Yoneoka, Y., Yoshihara, D., Eguchi, H., and Suzuki, K. (2016). Cu,Zn-SOD deficiency induces the accumulation of hepatic collagen. Free Radic. Res. 50, 666–677.

Sanyal, A.J., Friedman, S.L., Mccullough, A.J., and Dimick-Santos, L. (2015). Challenges and opportunities in drug and biomarker development for nonalcoholic steatohepatitis: Findings and recommendations from an American Association for the Study of Liver Diseases-U.S. Food and Drug Administration Joint Workshop. Hepatology 61, 1392–1405.

Singh, R., Chandrashekharappa, S., Bodduluri, S.R., Baby, B. V, Hegde, B., Kotla, N.G., Hiwale, A.A., Saiyed, T., Patel, P., Vijay-Kumar, M., et al. (2019). Enhancement of the gut barrier integrity by a microbial metabolite through the Nrf2 pathway. Nat. Commun. 10, 89.

Vouldoukis, I., Lacan, D., Kamate, C., Coste, P., Calenda, A., Mazier, D., Conti, M., and Dugas, B. (2004). Antioxidant and anti-inflammatory properties of a Cucumis melo LC. extract rich in

superoxide dismutase activity. J. Ethnopharmacol. 94, 67–75.

Wang, K., Liao, M., Zhou, N., Bao, L., Ma, K., Zheng, Z., Wang, Y., Liu, C., Wang, W., Wang, J., et al. (2019). Parabacteroides distasonis Alleviates Obesity and Metabolic Dysfunctions via Production of Succinate and Secondary Bile Acids. Cell Rep. 26, 222-235, e5.

Younossi, Z., Anstee, Q.M., Marietti, M., Hardy, T., Henry, L., Eslam, M., George, J., and Bugianesi, E. (2018). Global burden of NAFLD and NASH: Trends, predictions, risk factors and prevention. Nat. Rev. Gastroenterol. Hepatol. 15, 11–20.