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Abstract	Hot springs are unique natural environments that have been used for recreational and/or therapeutic purposes (balneotherapy) since ancient times in various countries, especially, Japan. Japanese hot spring therapy, called "touji", is world-renowned. Multilateral and comprehensive scientific study of hot springs and their benefits could contribute to making Japan a tourism-oriented country. As a first step, microbes living in hot springs and their genes were analyzed to understand the hot spring mircobiome. We detected 27 novel microorganisms and type-specific tRNA degradation. Hot spring water (HSW) consumption is one of the methods of balneotherapy. It has been reported that consumption of hydrogen carbonate- or sulfur-containing water may be prevent and/or improve type 2 diabetes. However, since the molecular mechanisms underlying the effects of balneotherapy have not been well elucidated, the physiological effects of HSW consumption were evaluated using omics-based approaches. In the HSW consumption periods, serum glycoalbumin levels, a glycemic control index, were significantly decreased. Metabolome analysis showed that concentrations of 19 blood metabolites including 4 glycolysis-related metabolites and 3 amino acids were significantly changed in the HSW consumption periods as compared with the tap water consumption periods, suggesting that HSW consumption may induce glycolysis and proteolysis alteration. Additionally, 8 families of gut microbiota were significantly changed, out of which lean-associated bacteria was significantly increased. Moreover, experiment on murine models was also conducted and these models may be useful for screening to evaluate the effectiveness of HSW consumption. The current research provides beneficial information for future studies investigating the molecular basis of balneotherapy. Taken together, our findings provide new insights into the microbial ecosystems in hot springs and the molecular mechanisms underlying the effects of balneotherapy. These findings contribute to the understanding of hot springs and their effect in improving human health.
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Multilateral understanding of hot springs by omics-based approaches

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Abstract

Hot springs are unique natural environments that have been used for recreational and/or therapeutic purposes (balneotherapy) since ancient times in various countries, especially, Japan. Japanese hot spring therapy, called “touji”, is world-renowned. Multilateral and comprehensive scientific study of hot springs and their benefits could contribute to making Japan a tourism-oriented country. As a first step, microbes living in hot springs and their genes were analyzed to understand the hot spring mircobiome. We detected 27 novel microorganisms and type-specific tRNA degradation. Hot spring water (HSW) consumption is one of the methods of balneotherapy. It has been reported that consumption of hydrogen carbonate- or sulfur-containing water may be prevent and/or improve type 2 diabetes. However, since the molecular mechanisms underlying the effects of balneotherapy have not been well elucidated, the physiological effects of HSW consumption were evaluated using omics-based approaches. In the HSW consumption periods, serum glycoalbumin levels, a glycemic control index, were significantly decreased. Metabolome analysis showed that concentrations of 19 blood metabolites including 4 glycolysis-related metabolites and 3 amino acids were significantly changed in the HSW consumption periods as compared with the tap water consumption periods, suggesting that HSW consumption may induce glycolysis and proteolysis alteration. Additionally, 8 families of gut microbiota were significantly changed, out of which lean-associated bacteria was significantly increased. Moreover, experiment on murine models was also conducted and these models may be useful for screening to evaluate the effectiveness of HSW consumption. The current research provides beneficial information for future studies investigating the molecular basis of balneotherapy. Taken together, our findings provide new insights into the microbial ecosystems in hot springs and the molecular mechanisms underlying the effects of balneotherapy. These findings contribute to the understanding of hot springs and their effect in improving human health.

Keywords: Hot spring, Environmental microbes, Balneotherapy, Type 2 diabetes, Gut microbiota

Chapter 1: Introduction

1.1 What is hot springs?

Hot springs are a unique natural environment that are present widely all over the world. Hot springs are commonly used all over the world since over 2,000 years ago. In particular, Japan has the highest number of hot springs in the world [1]. There are over 27,000 active hot springs located over 3,000 hot spring areas, and hot spring bathing and/or hot spring therapy are still familiar to Japanese people. Additionally, Japanese hot spring culture is highly appreciated all over the world. A statistical survey conducted by the Japan Ministry of Land, Infrastructure, Transport and Tourism (http://www.mlit.go.jp/kankocho/siryou/archive/20110325.html) reported that 45% of foreign tourists were looking forward to visiting hot springs in their visit to Japan; this percentage was the third highest, following Japanese food (64%) and shopping (52%). Therefore, Japanese hot springs have high potential as sightseeing spots. Scientific research in this area will help improve the understanding of hot springs and enhance its importance all over the world especially in Japan.

Recently, the distribution of archaea and bacteria in various environments has been represented using microbiome analysis [2, 3]. The studied environments include hot springs [4-7], hydrothermal vents [8], human lacrimal fluids [9], and human gut [10]. In Japan, unique archaea and bacteria have been isolated from hot spring environments: *Thermus thermophilus* was isolated from the Mine hot spring (Shizuoka, Japan) [11], *Sulfolobus* sp. NOB8H2 was isolated from the Noboribetsu hot spring (Hokkaido, Japan) [12], *Vulcanisaeta distributa* was isolated from the Owakudani hot spring (Kanagawa, Japan) [13], and *Sulfolobus tokodaii* was isolated from the Beppu hot spring (Oita, Japan) [14]. Thus, many novel archaea and bacteria have been isolated from hot springs. Additionally, one of the theories proposed that life originated in submarine hydrothermal vents [15]; therefore, microbes inhabiting hydrothermal environments such as hot springs might maintain some features of the ancient, original life forms. Taken together, these reports suggest that the hot spring environments, especially in Japan, are rich sources of unique and interesting microbes and their genes.

Since ancient times, hot springs have been traditionally used as natural baths in various countries such as Greece, Rome, and Japan [1, 16, 17]. From the time of Hippocrates, it was considered that hot springs can exert beneficial effects over various diseases [16]. In Japan, hot spring therapy, called “touji”, was developed in the 14-15th century [1]. At present, the use of hot springs for medicinal purposes is defined as Balneotherapy [18]. However, the essential mineral components and the ideal concentration of each mineral required for the beneficial effects remain unclear [18]. It has been previously reported that balneotherapy has beneficial effects for various diseases such as type 2 diabetes (T2D) [19-21], rheumatism [22], low back pain [23], fibromyalgia syndrome [24], atopic dermatitis [25], and cardiovascular disease [26, 27]. In particular, consumption of hydrogen carbonate- or sulfur-containing water has been reported to prevent or improve T2D [19-21]. Complementary and alternative medicine (CAM) is a group of diverse medical and health care systems, practices, and products that are not considered part of any current Western health care system [28, 29]. Balneotherapy, which is expected to have beneficial effects on various diseases, is also considered CAM [30], and the molecular mechanisms underlying the effects of balneotherapy have not been elucidated. Therefore, further investigations are necessary to determine the effectiveness, safety, standard procedures, and potential side effects of the various CAM methods [29].

1.2 Objectives

The aim of this study was to multilaterally understand hot springs based on omics-based approaches. As described above, hot springs have not only gained the attention of biological researches, but also of tourists. Scientific research on hot springs could promote development of tourism to these sights as well as advancing our understanding of their health benefits. For this purpose, firstly, microbes living in the hot spring water (HSW) and their genes were analyzed in Chapter 2 to further understanding of what kinds of microorganisms were present in the hot spring environments. Additionally, the physiological effects of HSW consumption were evaluated in Chapter 3 to understand the molecular basis of balneotherapy.

Chapter 2: Metatranscriptome analysis of microbes in an oceanfront deep subsurface hot spring reveals novel small RNAs and type-specific tRNA degradation

This chapter was omitted from the digest due to space reason. Details are available in my doctoral dissertation and article that was published in the Applied and Environmental Microbiology [31].

Chapter 3: The evaluation of the effects of hot spring water consumption on glycemic control based on metabolome and microbiome approaches

3.1 Introduction

Self-medication is an important approach to maintain and promote human health. There are many strategies of self-medication such as improving one's lifestyle and/or dietary habits, consumption of functional foods/beverages and getting adequate exercise [32, 33]. HSW is traditionally used in public baths and balneotherapy in many countries. Balneotherapy is the use of thermal and/or mineral water for treatment of human health by employing various methods such as bathing, drinking, mud therapy, and inhalation [23]. As described in the Chapter 1, it has been reported that balneotherapy has beneficial effects for various diseases [19-23, 26]. Especially, consumption of hydrogen carbonate- or sulfur-containing water has been reported to prevent or improve T2D [19-21]. However, as the reported benefits of the HSW were determined epidemiologically and clinically, the molecular mechanisms of the beneficial effects behind HSW remain unclear.

A recent study has reported that plasma metabolome profiles were altered between healthy people and T2D patients [34]. Moreover, recent studies reported that gut microbiota is involved in various types of diseases such as diabetes [35], obese [36], inflammatory bowel disease [37] and autistic spectrum [38]. The several studies have been indicated that gut microbial composition and function in T2D patients are different from that of healthy subjects [35, 36, 39]. Therefore, preventive and/or therapeutic effects for T2D derived from HSW consumption are expected via influencing blood metabolites concentrations and gut microbial compositions.

For this reason, we conducted clinical trial and animal experiment to elucidate the molecular basis of HSW consumption. In the clinical trial, blood metabolome analysis and gut microbiome analysis were conducted to elucidate the molecular basis of hydrogen carbonate-containing HSW (HSW1) consumption on human glycemic control. In the animal experiment, physiological effects on glycemic control derived from 8 types of HSW (HSW1-8) were evaluated and compared (HSW1 was used both clinical trial and animal experiment). Since previous studies have reported that consumption of hydrogen carbonate- or sulfur-containing water has potential to prevent or improve T2D [19-21], several hydrogen carbonate- and/or sulfur-containing

HSW were used in the animal experiment. Additionally, several other types of HSW such as carbon dioxide- or chloride-containing HSW were also used in the animal experiment to compare the effects derived from consumption of the HSW.

3.2 Methods summary

Methods were briefly described in this digest due to space reason. Full methods are available in my doctoral dissertation.

3.2.1 Tap water and HSW used in this study

In the current study, a total 2 types of tap water (TW and TWAF) and 8 types of HSW (HSW1-8) were used (see details in the doctoral dissertation). Tap water used in the clinical trial was purchased from Nishikawa water treatment plant (Yamagata, Japan), and this commercially available tap water is designated as "TW" in this dissertation. Tap water used in the animal experiment was obtained from the water tap in the animal facility of National Institute of Technology, Tsuruoka College, and this water is designated as "TWAF" in this dissertation. 8 types of HSW (HSW1-8) were obtained from 3 different hot spring areas but their origins of hot springs are different. In the clinical trial, HSW1 was used because it has been orally reported by a local physician that the HSW have beneficial effects for glycemic control. In the animal experiment, all of 8 HSW including HSW1 were used. HSW1 and 2 were obtained from Nagayu hot spring area (Taketa-city, Oita, Japan), HSW3 and 4 were obtained from Hijiori hot spring area (Okura-village, Yamagata, Japan), and HSW5-8 were obtained from Shiobara hot spring area (Nasushiobara-city, Tochigi, Japan).

3.2.2 Clinical trial

This clinical trial was approved by the ethical committees of Japan Health and Research Institute and Keio University Shonan Fujisawa Campus. All subjects were informed of the purpose of this study, and written consent was obtained from all subjects. In this study, HSW (HSW1) consumption test was conducted amongst 19 healthy subjects (7 men and 12 women, ages from 26 to 59, 47 years old on the average). During the test, volunteers drank the 500 ml of TW or HSW1 divided thrice daily (30-60 minutes before breakfast, lunch, and dinner). TW consumption periods and HSW1 consumption periods lasted for a week each and this cycle was repeated twice. Blood and fecal samples were collected on the first day of the test and last days of every week.

3.2.3 Animal experiment

This animal experiment was performed using protocols approved by Animal Studies Committees of National Institute of Technology, Tsuruoka College. 5-weeks-old male C57BL/6JJcl mice were purchased from CLEA Japan Inc. (Tokyo, Japan) and housed in the animal facility of National Institute of Technology, Tsuruoka College under a 12-hour light-dark cycle. Initially, mice were randomly grouped and fed a control diet (CE-2, CLEA Japan Inc.) and TWAF for 1 week to acclimatize to a new environment. After acclimatization, diet was replaced with high fat diet (HFD32, CLEA Japan Inc.). In addition, drinking water was also replaced with each corresponding HSW in HSW1-8 consumption groups. All mice had ad libitum access to food and water. During week 15-17, oral glucose tolerance test (OGTT), intraperitoneal glucose tolerance test (IPGTT), and intraperitoneal insulin tolerance test (IPITT) were performed as described previously with some modifications [40]. Sample size was determined based on published studies [40] using similar assays as well as the previous experience.

3.2.4 Clinical blood tests

For clinical trial, clinical blood tests were performed at each sampling point, including measurement of fasting plasma glucose, serum glucose, glycoalbumin, insulin, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglyceride, urate, sodium, chloride, calcium, magnesium, and cortisol. The measurement of the concentrations of these parameters was outsourced to RINTEC Co., Ltd (Fukuoka, Japan). Plasma glucose measurement was performed using morning fasting blood samples obtained from only 8 volunteers. For these samples, the homeostasis model assessment ratio (HOMA-R) was calculated from plasma glucose and insulin levels.

3.2.5 Metabolome analysis

Metabolome analysis of blood and fecal samples obtained from clinical trial were conducted as described previously with some modifications [41].

3.2.6 Microbiome analysis

DNA isolation using fecal samples collected from clinical trial and cecal samples collected from animal experiment were performed as described previously with some modifications [42]. 16S rRNA genes in the fecal and cecal DNA samples were analyzed using the MiSeq sequencer (Illumina). The V1-V2 region of the 16S rRNA genes were amplified using bacterial universal primer set 27Fmod (5'-AGRGTTTGATYMTGGCTCAG-3') and 338R (5'-TGCTGCCTCCCGTAGGAGT-3') [43]. MiSeq sequencing was performed according to the manufacturer's instructions. In this study, 2×300 bp paired-end sequencing was employed. Obtained 16S rRNA gene sequences were analyzed using quantitative insights into microbial ecology (QIIME) (v1.8.0) pipeline [44]. Sequences were clustered into operational taxonomic units (OTUs) using 97% sequence similarity, and OTUs were assigned to taxonomy using RDP classifier.

3.2.7 Statistical analysis

For clinical trial, to analyze the intra-individual alterations, statistical evaluation between two groups was performed by Wilcoxon signed-rank test (non-parametric paired test) using the R package exactRankTests or XLSTAT (v2014.6.04) (Addinsoft). For animal experiment, statistical evaluation between TWAF consumption group and each HSW consumption group were performed by Dunnett test using the R package multcomp. Kendall rank correlation coefficients were calculated by using the R software. All statements indicating significant differences show at least a 5% level of probability.

3.2.8 Nucleotide sequence accession number

The microbiome analysis data obtained from clinical trial have been deposited at the DDBJ Sequence Read Archive (http://trace.ddbj.nig.ac.jp/dra/) under accession number DRA004008.

3.3 Results

3.3.1 Mineral components of the water used in this study

For clinical trial and mice experiment to evaluate the molecular level effects derived from HSW consumption, 8 different types of HSW were collected and used. Mineral contents and pH of the tap water and HSW used in this study were measured (Table 3.1). In the almost of all HSW, concentrations of hydrogen carbonate, chloride, sulfate, sodium, magnesium, calcium, and potassium ions were higher as compared with tap water. For undissociated molecules, metasilicic acid, metaboric acid, and carbon dioxide were also observed in high concentrations in HSW. There are no notable differences between tap water used in clinical trial (TW) and animal experiment (TWAF). Only HSW8 contains sulfur (hydrogen sulfide ion and hydrogen sulfide). According to the results of metabolome analysis of HSW, no metabolites were detected.

Table 3.1 Mineral contents and pH of the water used in this study

		Concentrations (mg/kg)									
				HSW							
Minerals	Formula	TW	TWAF	1	2	3	4	5	6	7	8
Hydrogen carbonate ion	HCO_3^-	28.0	27.5	2485	1232	20.7	3340	449	386	458	1084
Chloride ion	Cl^-	11.0	11.4	182	78.3	6.3	679	478	2239	343	906
Sulfate ion	SO_4^{2-}	6.9	6.9	355	185	11.6	0.1	28.7	54.3	46.1	65.3
Carbonate ion	CO_3^{2-}	-	-	1.2	-	-	0.9	-	-	-	0.3
Nitrate ion	NO_3^-	0.7	0.7	1.2	-	1.6	-	-	-	-	-
Fluoride ion	F^-	-	-	0.3	0.2	-	0.7	0.2	0.4	0.5	0.5
Hydrogen sulfide ion	HS^-	-	-	-	-	-	-	-	-	-	22.1
Iodide ion	I^-	-	-	-	0.3	-	0.3	0.6	2.8	0.8	0.8
Bromide ion	Br^-	-	-	-	-	-	1.7	0.7	1.0	0.5	2.7
Hydrogenphosphate ion	HPO_4^{2-}	-	-	-	-	-	0.6	-	-	-	-
Dihydrogenphosphate ion	$H_2PO_4^-$	-	-	-	-	-	-	-	-	-	3.2
Sodium ion	Na^+	10.0	10.3	412	214	7.4	1657	327	1204	286	860
Magnesium ion	Mg^{2+}	1.9	1.9	291	136	3.0	4.8	27.4	37.6	17.0	19.7
Calcium ion	Ca^{2+}	6.1	6.1	177	102	2.8	28	88.1	264	78.7	92.7
Potassium ion	K^+	-	-	80.0	35.3	1.3	8.9	44.9	107	29.1	61.4
Aluminum ion	Al^{3+}	0.2	0.2	0.6	0.2	1.0	0.4	0.6	1.0	0.6	-
Manganese ion	Mn^{2+}	-	-	0.4	0.6	0.1	0.3	0.2	1.5	0.3	0.2
Ferrous ion	Fe^{2+}	-	-	2.3	3.1	-	0.6	0.9	3.7	0.4	-
Ammonium ion	NH_4^+	-	-	1.9	1.0	0.1	2.9	0.1	0.4	0.6	0.5
Metasilicic acid	H_2SiO_3	10.0	10.1	207	164	32.2	24.6	235	92.7	181	177
Metaboric acid	HBO_2	0.8	0.8	6.2	3.5	1.2	35.5	34.5	126	28.0	106
Carbon dioxide	CO_2	0.9	0.9	161	411	241	284	46.6	102	31.2	178
Hydrogen sulfide	H_2S	-	-	-	-	-	-	-	-	-	65.7
Metaarsenious acid	$HAsO_2$	-	-	-	-	-	-	0.1	-	0.1	-
pH		7.58	7.58	7.07	6.28	4.72	6.82	6.63	6.18	6.78	6.58

"-" indicates the value was under detection limit.

3.3.2 Comparisons of clinical parameters between TW and HSW1 consumption periods in the clinical trial

In the clinical trial, intra-individual alterations of clinical parameters were firstly analyzed. Serum glycoalbumin level, a glycemic control index, was slightly but significantly decreased during HSW1 consumption periods as compared with TW consumption periods (Fig. 3.1A). Serum glucose level was not significantly decreased, but tended to be lowered by HSW1 consumption (P value = 0.092). Other parameters related to glycemic controls like plasma glucose levels, insulin concentrations, and HOMA-R, were not different between TW and HSW1 consumption periods (see details in the doctoral dissertation). These results suggest that HSW1 consumption may have the possible benefit in glycemic control. However, the amounts of changes of glycoalbumin levels were slight and improvement of glycemic control could not completely supported by the alterations of blood glucose levels. Since the current study targeted healthy subjects, alterations of glycoalbumin and glucose levels might be slight. Therefore, it is important to perform additional studies among diabetes patients to further evaluate the beneficial effects of HSW1 consumption for glycemic control and/or T2D.

3.3.3 HSW1 consumption-related changes of physiological metabolism

To evaluate the effect of HSW1 consumption on glycoalbumin reduction, blood metabolome analysis was performed using CE-TOFMS. A total of 152 metabolites were detected from blood samples at least from 1 subject and 1 time point, and concentrations of these metabolites were compared within subject (see details in the doctoral dissertation). Over 85% of metabolites were not significantly changed after HSW1 consumption periods, and it was expected that the concentrations of most metabolites remained consistent due to physiological homeostasis. However, the concentrations of 19 metabolites were significantly different between TW and HSW1 consumption periods (see details in the doctoral dissertation).

Metabolites that may be related to glycemic control such as glycolysis-related metabolites (3-phosphoglycerate, pyruvate, ATP, and ADP), amino acids (tyrosine, methionine, and glycine), and UDP-N-acetylglucosamine were included in significantly changed metabolites. In addition, 3 amino acids were significantly decreased but almost of all amino acids were also lowered after HSW1 consumption periods (Fig. 3.1B). Since the 4 glycolysis-related metabolites were significantly changed in the HSW1 consumption periods, relative concentrations of the metabolites corresponding to glycolysis and citric acid cycle were also represented (Fig. 3.1C-D).

3.3.4 HSW1 consumption-related changes of intestinal environment

To investigate the compositions and changing of gut microbiota during the test, microbiome analysis was conducted using fecal samples that were collected weekly during the study. A total of 7,075 OTUs were constructed from 16S rRNA gene sequences derived from 19 subjects. To investigate the intra-individual changes of gut microbiota during the test, relative abundances of each microbial taxon were compared between TW and HSW1 consumption periods within subjects. From the results, it was observed that over 85% of families were not altered similarly to blood metabolites, but relative proportions of 8 families especially Christensenellaceae and Porphyromonadaceae were significantly different between TW and HSW1 consumption periods (Fig. 3.1E). As species level analysis, relative abundances of each OTU were compared between TW and HSW1 consumption periods, 23 OTUs including *Clostridium* cluster III, IV, and XIVa were significantly altered after HSW1 consumption periods (see details in the doctoral dissertation).

Recently, gut microbiota-derived metabolites such as short-chain fatty acids (SCFA) and bile acids have received a lot of attention. Therefore relative concentrations of several short chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs), and bile acids were compared between TW and HSW1 consumption periods. As a result, butyrate, pentanoate, decanoate, dodecanoate, and deoxycholate were significantly increased after consumption of HSW1 (Fig. 3.1F).

3.3.5 Evaluation of the physiological effects derived from HSW consumption in animal experiment

According to the clinical trial, HSW1 consumption has the possible potential to prevent and/or improve T2D, therefore it was consequently investigated in high-fat diet-induced obese mice. In addition, the physiological effects derived from consumption of various types of HSW were also investigated. Firstly, body weights of all groups were not different during the test. Although the body weights were not changed, food intakes of HSW4, 7, and 8 consumption groups, and water intakes of HSW1, 2, 4, 7, and 8 groups were significantly increased as compared with TWAF consumption group (see details in the doctoral dissertation). However, fasting plasma glucose levels (measured before glucose administration in OGTT) of HSW 3 and 8 consumption groups were significantly decreased. Additionally, plasma glucose level of HSW1, 3, 7, and 8 consumption groups at 15 min after glucose administration were significantly decreased as compared with TWAF consumption group (Fig. 3.2A). Therefore these 4 types of HSW were defined as “effective HSW”. As a result of OGTT, plasma glucose levels were significantly decreased at only 15 min after glucose administration in the effective HSW consumption groups. We suspected that this phenomenon might have occurred due to increment of insulin level via increased incretin secretion such as GLP-1. However, plasma GLP-1 (active form) and insulin levels were not improved in the effective HSW consumption groups (see details in the doctoral dissertation). Instead, insulin levels at 15 and 60 minutes after glucose administration in HSW8 consumption group were significantly decreased. To evaluate the insulin resistance, IPGTT and IPITT were also performed but significant improvements were not observed between TWAF and each effective HSW consumption group. In fact, plasma glucose levels were significantly increased in HSW3 and 7 consumption groups in IPGTT and IPITT, respectively (see details in the doctoral dissertation).

Finally, alterations of gut microbiota compositions derived from HSW consumption were investigated. For this purpose, microbiome analysis was conducted using the cecal contents that were collected from the mice of each HSW consumption group. Some OTUs were significantly changed between TWAF and each HSW consumption group (see details in the doctoral dissertation). Subsequently, the correlation between mineral concentrations in the drinking water and relative abundances of each OTU were analyzed. As a result, there are 71 OTUs that have more than 0.7 or less than -0.7 of Kendall rank correlation coefficients (Fig. 3.2B). Almost of all the OTUs were corresponded to Bacteroidales or Clostridiales, but the correlation patterns were not discriminated at order level taxonomy.

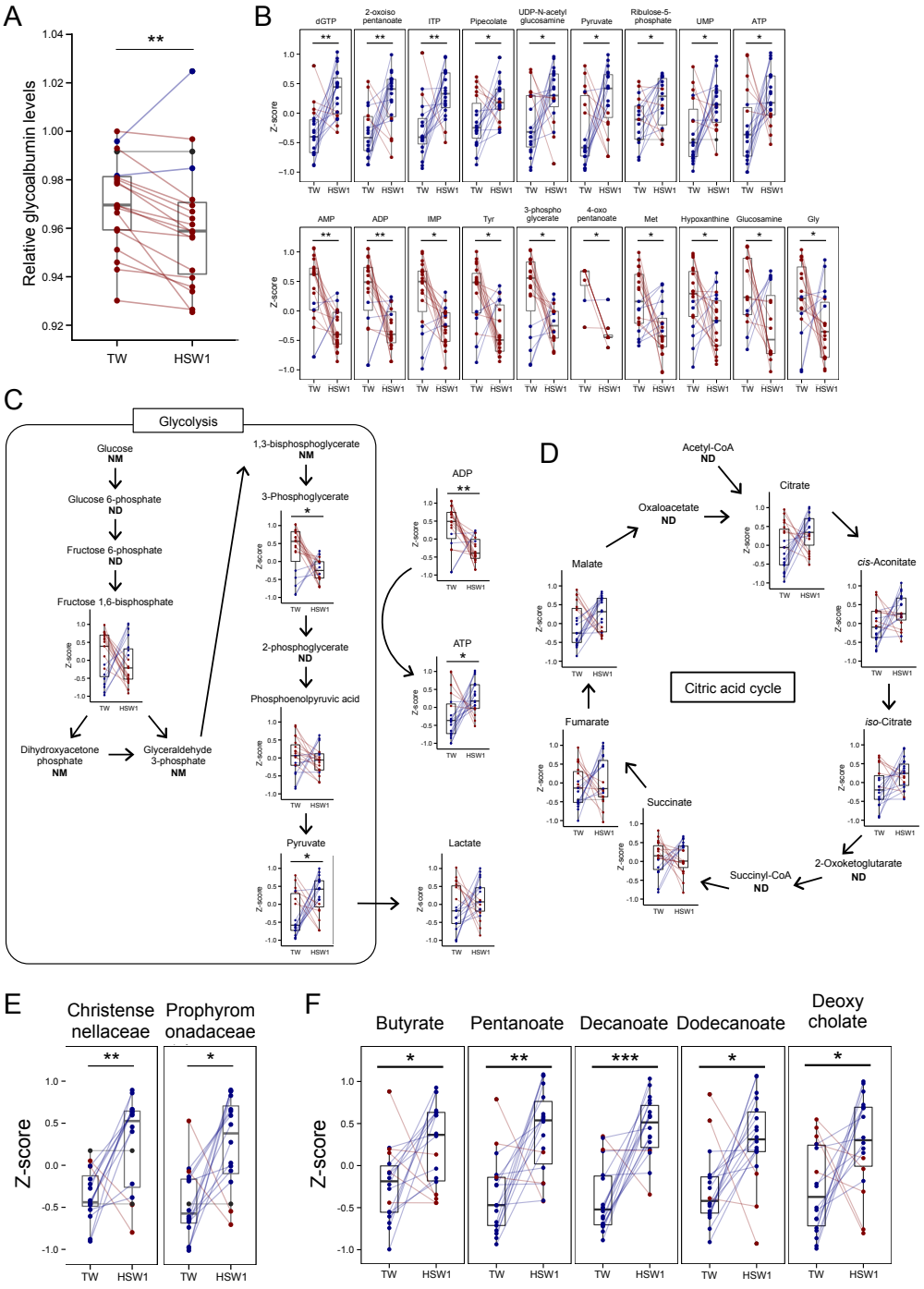

Figure 3.1 Alterations of serum glycoalbumin level, blood metabolites, fecal microbiota and fecal metabolites.

Individual data of mean relative serum glycoalbumin level (A), blood amino acids (B), blood metabolites related to glycolysis (C) and TCA cycle (D), fecal microbiota (E), and fecal metabolites (F) of week 1 and 3 (TW); and week 2 and 4 (HSW1) were shown in dot plots overlaid on box plots. Plots corresponding to same individuals were connected with red, blue or gray lines when the values were decreased, increased or not changed in HSW1 consumption periods as compared with TW consumption periods, respectively. Plots were also colored in the same color as their lines. **P* < 0.05; ***P* < 0.005; ****P* < 0.0005.

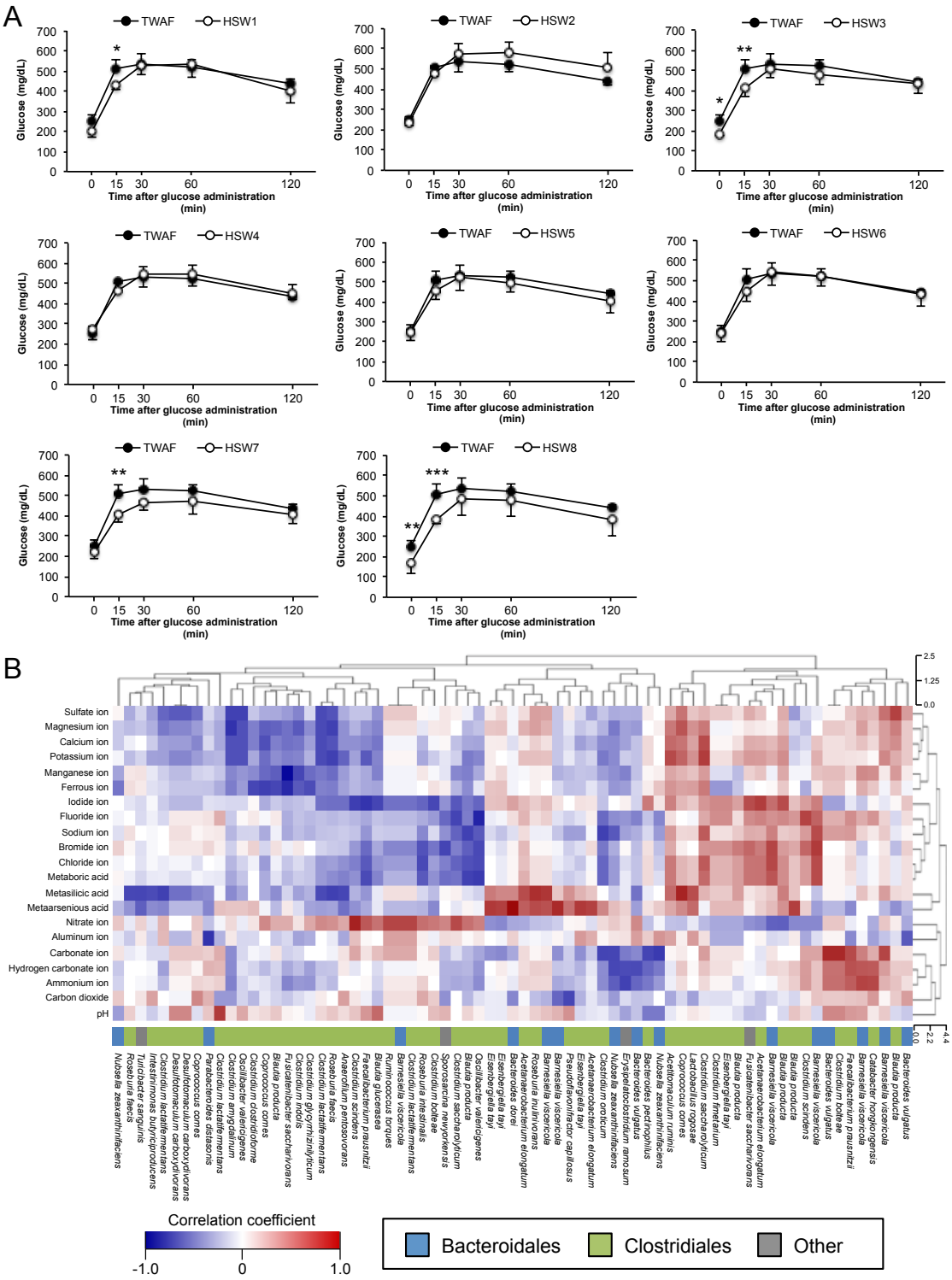

Figure 3.2 OGTT and correlation between mineral contents and cecal microbiota

(A) OGTT was performed on the TWAF or HSW1-8 consumption mice (n = 4-5). *P < 0.05; **P < 0.005; ***P < 0.0005.

(B) Kendall rank correlation coefficients between mineral concentrations of each mineral water and mean relative abundances of each OTU were shown as heat map using blue-white-red scheme.

3.4 Discussion

In the present study, physiological effects especially on glycemic control derived from HSW consumption were investigated. Firstly, it was shown that the relative serum glycoalbumin level was slightly but significantly decreased after HSW1 consumption as compared with TW consumption in the clinical trial. The amounts of reductions of glycoalbumin levels in the HSW1 consumption periods were only 0.93% of initial value as compared with the TW consumption periods in average. In addition, coefficient of variation (CV) of reproducibility of glycoalbumin assay kit used in this study has been reported as 2.8-3.2%. Therefore, reduction of glycoalbumin levels was statistically significant but it is not clear whether there are actually biological and/or medical impacts such as prevention and/or improvement of T2D. Besides, blood glucose levels were not decreased significantly, but tended to be lowered by HSW1 consumption, although glycoalbumin level has been known to reflect blood glucose levels during short-term (at least the last 14 days) [45]. This might be attributed that blood glucose levels are influenced by external factors such as food intake and exercise [46]. Since the current study targeted healthy subjects, alterations of glycoalbumin and glucose levels were expected to be minimal. It is important to perform further studies among borderline diabetes patients to further evaluate the preventive and/or therapeutic effects for T2D and/or glycemic control derived from HSW1 consumption.

According to the metabolome analysis of blood samples, ATP and pyruvate were significantly increased whereas 3-phosphoglycerate and ADP was significantly decreased. Additionally, other members of glycolysis, fructose 1,6-bisphosphate and phosphoenolpyruvic acid, were tended to decrease, whereas lactate was slightly increased. These results suggest that glycolysis may be upregulated after the HSW1 consumption periods. In the citric acid cycle, citrate, *cis*-aconitate, *iso*-citrate, and malate were tended to increase, but there were no significant differences. Therefore HSW1 consumption may induce enhancement of glycolysis, but not citric acid cycle. Since blood glucose levels were not decreased significantly, but tended to be lowered as observed in our results, it may be attributed to glycolysis enhancement.

On the other hand, concentrations of 3 amino acids (tyrosine, methionine, and glycine) were significantly lowered in the HSW1 consumption periods. A previous study reported that high concentrations of various amino acids especially tyrosine in blood is one of the risk factor of T2D [47]. Additionally, it has been also reported plasma concentrations of several amino acids including tyrosine and methionine were significantly high in hyperinsulinemia (it is often observed in early stage of T2D) patients as compared to healthy subjects [48]. Our results demonstrated that concentrations of 3 amino acids including tyrosine and methionine in blood were significantly decreased and that of other standard amino acids were tended to be lowered after in the HSW1 consumption periods. As such, it can be suggested that the HSW1 consumption may have the possible potential to avoid risk factors of T2D through the alterations of metabolism in the body.

Since recent studies reported the relationships between gut microbiota and T2D and/or obesity [49-51], we hypothesized that beneficial effect for glycemic control derived from HSW1 consumption might involve gut microbiota. As expected, alterations of gut microbiota compositions derived from HSW1 consumption were observed. In this study, family Christensenellaceae was the most significantly increased taxon. Previous study reported that Christensenellaceae was enriched in lean group (BMI < 25) as compared with obese group (BMI > 30) [52]. Additionally, it was also reported that transplantation of *Christensenella minuta* to germ-free mice reduced weight gain. Additionally, the family Porphyromonadaceae was also significantly increased after HSW1 consumption, it has been reported that the taxon was negatively correlated with type 1 diabetes [53],

and were decreased in high-fat/high-sucrose diet fed mice [54]. Therefore, our results suggested that consumption of HSW1 may have the possibly potential to prevent getting obese and/or diabetes via alteration of the gut microbiota compositions.

Recently, many studies reported the importance and positive or negative effects of gut microbiota-derived metabolites such as SCFAs and bile acids [40, 42, 55-57]. Therefore, relative changes of short- and medium-chain fatty acids and bile acids between TW and HSW1 consumption periods were analyzed in this study. 2 SCFAs (butyrate and pentanoate) were significantly increased after HSW1 consumption. Previous studies reported that SCFAs enhance incretin secretion [57] and insulin sensitivity in the muscle and liver [40]. Therefore, beneficial effects for glycemic control derived from HSW1 consumption may be mediated by increasing of intestinal SCFA concentrations. Additionally, it has been reported that butyrate induces the differentiation of regulatory T cells in the colon and suppresses colonic inflammation [42], therefore HSW1 consumption may have the possible potential to prevent and/or improve not only T2D but also inflammatory bowel disease and allergic diseases. Whereas, deoxycholate is one of the secondary bile acids produced by gut microbiota especially *Clostridium* cluster XI and XIVa [56, 58], and it has been reported that deoxycholate is the key molecule to develop obesity-associated hepatocellular carcinoma. In the present study, deoxycholate was increased after HSW1 consumption, it was attributed to increase relative abundances of 3 *Clostridium* cluster XIVa species (*Clostridium aminophilum*, *Clostridium lavalense*, and *Ruminococcus obeum*). After HSW1 consumption, SCFAs and deoxycholate were increased, and it has been reported that SCFAs have mainly positive effects whereas deoxycholate has mainly negative effects, therefore HSW1 consumption may have both positive and negative effects.

In the animal experiment, it was investigated whether beneficial effects for glycemic control can be observed by consumption of HSW1 and other types of HSW. Results indicated that HSW consumption could not suppress weight gain, therefore there is no potential in preventing obesity in mice. Since the increments of lean-associated bacteria were estimated as one of the triggers to prevent getting obese in the clinical trial, but lean-associated bacteria detected from human volunteers were not present in the mice cecum according to the microbiome analysis of cecal contents. Therefore, weight gain could not be suppressed by HSW consumption even though the potential to prevent getting obese derived from HSW1 consumption was suggested by clinical trial.

Although the body weights were not significantly changed, glucose tolerance was slightly improved in HSW1, 3, 7, and 8 consumption groups but not in other HSW consumption groups. It suggests that HSW1 consumption may have the possible beneficial effect to improve glycemic control in the current clinical trial, therefore murine experiments might be able to use for the screening of effectiveness for glycemic control that were derived from HSW consumption. It has been reported that the consumption of hydrogen carbonate- or sulfur-containing water could prevent or improve T2D [19-21]. Because HSW1 and 7 contain hydrogen carbonate, and HSW8 contains sulfur, their beneficial effects were reasonable. However, HSW2, 4, and 5 also contain hydrogen carbonate but improvement of glucose tolerance was not observed in the mice experiment. Additionally, glucose tolerance was slightly improved in HSW3 consumption group, but the concentrations of both hydrogen carbonate and sulfur of HSW3 were almost same as TW. These results suggest that some types of HSW containing hydrogen carbonate or sulfur actually have the beneficial effects for glycemic control as reported previously, but the concentrations of hydrogen carbonate and sulfur were not sufficient enough to be discriminated of having the potential benefit for glycemic control.

According to the results of OGTT, blood glucose levels were significantly decreased at 15 min after glucose administration in the effective HSW consumption groups. As the previous studies reported that GLP-1 induces the food-mediated early insulin secretion [59, 60], these results were expected to occur by increasing of insulin secretion via increasing of GLP-1. As such, GLP-1 and insulin concentrations in blood, and insulin sensitivity were analyzed to investigate the reason behind the slight improvement of glucose tolerance in effective HSW consumption groups, but there are no significant differences as compared with TWAF consumption group. Instead, insulin concentrations at 15 and 60 minutes after glucose administration were significantly decreased in the HSW8 consumption group, although the glucose levels were significantly decreased at 15 min after glucose administration in this group. Taken together, insulin sensitivity of effective HSW consumption group might not have been improved, thus we hypothesize that the suppression of glucose uptake from gut occurred. Therefore, expression levels of glucose receptors such as GLUT2 [61] should be compared in the future studies. However, the reason why glucose tolerance was improved in HSW1, 3, 7, and 8 consumption groups remains unclear in the current study.

Microbiome analysis in mice experiment showed that relative abundances of microbes were influenced by HSW consumption. Previous study has reported that magnesium and calcium concentrations in culture medium affected the growth of rumen bacteria [62], and as such, mineral consumption is expected to provide various effects for gut microbiota. As expected, there are many positive or negative correlations between mineral contents and compositions of OTUs. These results suggested that various types of minerals have the potential to increase or decrease relative abundances of gut microbiota. Although recent studies have reported that dietary habits are important in shaping the structure of gut microbiome [63, 64] thereby influencing host health status, there are abundant numbers of studies focusing on the effects of gut microbiota derived from dietary fat and/or fiber [65, 66]. Our present study shows the importance of mineral consumption to control the structure of gut microbiome. Therefore, the impact for gut microbiota compositions and their functions derived from consumption of various types of minerals should be analyzed in the further studies.

Chapter 4: Concluding remarks

Hot springs are natural environments that are found globally. They are familiar to people in various countries such as Japan. However, there are a lot of debatable and unresolved questions with regards to hot springs.

In Chapter 3, physiological effects of HSW consumption were evaluated based on the results of metabolome and microbiome analyses [67], since the molecular underlying the effects of balneotherapy are not well understood. In this study, we have shown that serum glycoalbumin levels were significantly decreased after consumption of HSW1, which was collected from the Nagayu hot spring area. In addition, it was also suggested that glycolysis may be enhanced and proteolysis may be suppressed in humans in the HSW1 consumption periods. Lean-associated bacteria and concentrations of several organic acids in the intestine were also significantly increased. Therefore, HSW1 consumption may exert beneficial effects for glycemic control in humans by alteration of metabolic dynamics and intestinal environments. As previous studies of balneotherapy were often limited to epidemiological and clinical evaluations, findings of the current research may become very important as the first step for towards understanding the molecular basis of balneotherapy. However, it should be noted that many points still require to be considered to confirm and clarify the

beneficial effects of HSW consumption on glycemic control, and subsequently, clinical trials among borderline diabetes patients and/or large-scale clinical trials would also be required in the future.

In the experiment on mice, it was possibly useful as screening to evaluate the effectiveness of HSW consumption. Additionally, we also proposed that the potential importance and availability of mineral consumption because it may be able to control the balance of intestinal flora. According to our results, several OTUs mainly corresponding to Bacteroidales and Clostridiales were highly correlated with mineral concentrations in drinking water.

In conclusion, the findings in this dissertation contribute to advancing the understanding of ecosystems in hot spring environments and the molecular basis of balneotheraphy. I hope these findings impact not only the scientific world, but also find applications in the real world.

References

1. Serbulea M & Payyappallimana U (2012) *Health & Place* 18(6):1366-1373.
2. DeLong EF & Pace NR (2001) *Systematic Biology* 50(4):470-478.
3. Smith MI, *et al.* (2014) *F1000prime Reports* 6:51.
4. Barns SM, *et al.* (1994) *Proceedings of the National Academy of Sciences of the United States of America* 91(5):1609-1613.
5. Kvist T, *et al.* (2007) *FEMS Microbiology Ecology* 59(1):71-80.
6. Hall JR, *et al.* (2008) *Applied and Environmental Microbiology* 74(15):4910-4922.
7. Inskeep WP, *et al.* (2010) *PLoS One* 5(3):e9773.
8. Xie W, *et al.* (2011) *The ISME Journal* 5(3):414-426.
9. Murakami S, *et al.* (2015) *Keio SFC Journal* 15(1):382-400.
10. Arumugam M, *et al.* (2011) *Nature* 473(7346):174-180.
11. Oshima T & Imahori K (1974) *International Journal of Systematic Bacteriology* 24(1):102-112.
12. Schleper C, *et al.* (1995) *Journal of Bacteriology* 177(15):4417-4426.
13. Itoh T, *et al.* (2002) *International Journal of Systematic and Evolutionary Microbiology* 52(Pt 4):1097-1104.
14. Suzuki T, *et al.* (2002) *Extremophiles* 6(1):39-44.
15. Baross JA & Hoffman SE (1985) *Origins of Life and Evolution of the Biosphere* 15(4):327-345.
16. Jackson R (1990) *Medical history. Supplement* (10):1-13.
17. van Tubergen A & van der Linden S (2002) *Annals of the Rheumatic Diseases* 61(3):273-275.
18. Nasermoaddeli A & Kagamimori S (2005) *Environmental Health and Preventive Medicine* 10(4):171-179.
19. Gutenbrunner C (1993) *Physikalische Medizin, Rehabilitationsmedizin, Kurortmedizin* 3(04):108-110.
20. Ohtsuka Y, *et al.* (2003) *The Journal of the Japanese Society of Balneology, Climatology and Physical Medicine* 66(4):227-230.
21. Schoppen S, *et al.* (2007) *Nutricion Hospitalaria* 22(5):538-544.
22. Annegret F & Thomas F (2013) *Rheumatology International* 33(11):2839-2850.
23. Karagulle M & Karagulle MZ (2015) *Clinical Rheumatology* 34(2):207-214.
24. Ablin JN, *et al.* (2013) *Evidence-Based Complementary and Alternative Medicine* 2013:638050.
25. Choi YJ, *et al.* (2013) *Annals of Dermatology* 25(4):462-470.
26. Perez-Granados AM, *et al.* (2010) *The Journal of Nutritional Biochemistry* 21(10):948-953.
27. Pagourelias ED, *et al.* (2011) *International Journal of Biometeorology* 55(5):657-663.

28. Tabish SA (2008) *International Journal of Health Sciences* 2(1):V-IX.
29. Esteghamati A, *et al.* (2015) *International Journal of Endocrinology and Metabolism* 13(2):e19678.
30. Wieland LS, *et al.* (2011) *Alternative Therapies in Health and Medicine* 17(2):50-59.
31. Murakami S, *et al.* (2012) *Applied and Environmental Microbiology* 78(4):1015-1022.
32. Salas-Salvado J, *et al.* (2011) *Nutrition, Metabolism, and Cardiovascular Diseases* 2:B32-48.
33. Walker KZ, *et al.* (2010) *Journal of Human Nutrition and Dietetics* 23(4):344-352.
34. Kaur P, *et al.* (2013) *Molecular BioSystems* 9(2):307-317.
35. Larsen N, *et al.* (2010) *PLoS One* 5(2):e9085.
36. Qin J, *et al.* (2012) *Nature* 490(7418):55-60.
37. Norman JM, *et al.* (2015) *Cell* 160(3):447-460.
38. Hsiao EY, *et al.* (2013) *Cell* 155(7):1451-1463.
39. Karlsson FH, *et al.* (2013) *Nature* 498(7452):99-103.
40. Kimura I, *et al.* (2013) *Nature Communications* 4:1829.
41. Mishima E, *et al.* (2015) *Journal of the American Society of Nephrology* 26(8):1787-1794.
42. Furusawa Y, *et al.* (2013) *Nature* 504(7480):446-450.
43. Kim SW, *et al.* (2013) *DNA Research* 20(3):241-253.
44. Caporaso JG, *et al.* (2010) *Nature Methods* 7(5):335-336.
45. Koga M & Kasayama S (2010) *Endocrine Journal* 57(9):751-762.
46. Moebus S, *et al.* (2011) *European Journal of Epidemiology* 26(9):719-728.
47. Wang TJ, *et al.* (2011) *Nature Medicine* 17(4):448-453.
48. Nakamura H, *et al.* (2014) *Nutrition & Diabetes* 4:e133.
49. Turnbaugh PJ, *et al.* (2006) *Nature* 444(7122):1027-1031.
50. Musso G, *et al.* (2010) *Diabetes Care* 33(10):2277-2284.
51. Ridaura VK, *et al.* (2013) *Science* 341(6150):1241214.
52. Goodrich JK, *et al.* (2014) *Cell* 159(4):789-799.
53. Wen L, *et al.* (2008) *Nature* 455(7216):1109-1113.
54. Parks BW, *et al.* (2013) *Cell Metabolism* 17(1):141-152.
55. Fukuda S, *et al.* (2011) *Nature* 469(7331):543-547.
56. Yoshimoto S, *et al.* (2013) *Nature* 499(7456):97-101.
57. Yadav H, *et al.* (2013) *The Journal of Biological Chemistry* 288(35):25088-25097.
58. Ridlon JM, *et al.* (2006) *Journal of Lipid Research* 47(2):241-259.
59. Drucker DJ (1998) *Diabetes* 47(2):159-169.
60. Lugari R, *et al.* (2002) *Hormone and Metabolic Research* 34(3):150-154.
61. Thorens B (2015) *Diabetologia* 58(2):221-232.
62. Morales MS & Dehority BA (2014) *Animal* 8(9):1427-1432.
63. Claesson MJ, *et al.* (2012) *Nature* 488(7410):178-184.
64. Voreades N, *et al.* (2014) *Frontiers in Microbiology* 5:494.
65. Trompette A, *et al.* (2014) *Nature Medicine* 20(2):159-166.
66. Caesar R, *et al.* (2015) *Cell Metabolism* 22(4):658-668.
67. Murakami S, *et al.* (2015) *Evidence-Based Complementary and Alternative Medicine* 2015:824395.