Openventio PUBLISHERS

ISSN 2377-8350

ADVANCES IN FOOD TECHNOLOGY AND NUTRITIONAL SCIENCES

Open Journal

| November 2015 | Volume 1 | Issue 5 |

ADVANCES IN FOOD TECHNOLOGY AND NUTRITIONAL SCIENCES

Openventio PUBLISHERS

ISSN 2377-8350

Open Journal

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Openventio PUBLISHERS

ISSN 2377-8350 Open Journal http://dx.doi.org/10.17140/AFTNSOJ-1-e005

Editorial

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Volume 1 : Issue 5
Article Ref. #: 1000AFTNSOJ1e005

Article History
Received: October 13th, 2015
Accepted: October 13th, 2015
Published: October 13th, 2015

Citation
Wang B. Application of risk assessment in nutrition. *Adv Food Technol Nutr Sci Open J*. 2015; 1(5): e14-e15. doi: 10.17140/AFTNSOJ-1-e005

Application of Risk Assessment in Nutrition

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In 1995, an agreement was made at the Food and Agricultural Organization of the United Nation/World Health Organization (FAO/WHO) Expert Consultation that risk assessment should be carried out for biological and chemical agents for food standards issues.[1] Since then, risk assessment has been considerably evolved in the last two decades as a science-based activity to inform the regulatory public health policy decision making in agri-food field.[2,3] Food safety related risk assessment provides a systematic framework to evaluate the probability of occurrence of adverse health outcomes due to an excess exposure to a hazardous agent or agents from food consumption.[2] Currently, there has been important improvement in methodologies of data collection, evidence synthesis, and computational techniques for risk assessments that focus on the microbial and chemical hazards along food supply chains. However, risk assessment applications in nutrition (called nutritional risk assessment here) is beyond the areas that have originally been considered as the public health risk assessment realm.

Although, not advanced as microbial and chemical risk assessment in international and/or national/regional levels, nutritional risk assessment has never ceased and becomes more and more critical to public health protection. The increasing use of functional foods, fortified foods, dietary supplements, formulated foods in recent years has increased the intake of nutrient substances among populations around the world. This results in a growing interest in determining the dietary standard of nutrients to prevent chronic adverse disease outcomes due to their excess intake. For example, nutritional risk assessment has been widely accepted as a standard tool to identify the evidence- and science-based Tolerable Upper Intake Levels (ULs) of nutrient substances.[4]

In general, nutritional risk assessment shares the same principles of risk assessment which is applied to chemicals in foods other than nutrients, which is divided into four basic components, hazard identification, hazard characterization, exposure assessment, and risk characterization.[2] As the first step of 'hazard identification', the evidence is collected, evaluated, and synthesized to identify the known and/or potential adverse effects related to the nutrient substances of interest. During 'hazard characterization', the association between the nature and extent of the adverse effects and the specific nutrient exposure is described. Especially, on a quantitative basis, the dose-response relationship is usually established that can translate the level of nutrient intake through food consumption to the probability of having different adverse effects. 'Exposure assessment' characterizes the intake distribution of the specific nutrient among members of the general population or subpopulations of interest. Finally, outputs from the previous three steps are integrated in 'risk characterization' to generate risk estimates.

The major application of nutritional risk assessment is to inform the guidance about diet and supplement intake, such as setting up ULs to avoid the excess intake of nutrients and therefore prevent chronic diseases due to the toxic effect of over-exposed nutrients. In the United States, the Food and Nutrition Board of the Institute of Medicine is the major group working in this field to establish the Dietary Reference Intakes (DRI).[4] In addition, a very important unique aspect that distinguishes nutrient and other non-nutrient hazards is that both high and low nutrient intakes are associated with risks.[5] Therefore, a risk-risk tradeoff analysis or risk-benefit analysis is critical to describe the whole picture by taking into consideration both

ISSN 2377-8350 Open Journal http://dx.doi.org/10.17140/AFTNSOJ-1-e005

the beneficial effects of preventing the adverse effects due to deficient intakes and hazardous effects of promoting the adverse effects due to excess intakes. A risk-benefit analysis mirrors the risk assessment approach, but is a unique method to quantify the balance between health benefits and risks imposed by specific food strategies on population of interest. Hoekstra and colleagues[6] illustrated the risk-benefit analysis method with mandatory fortification of folic acid in bread in Netherland as an example.

The current approaches of nutritional risk assessment has their limitations. Since developed from traditional chemical risk assessment, most existing nutritional risk assessments address the effect of a single or relative specific nutrient substances,[7] which leaves out the possibility to deal with very complex questions. However, the nature of nutritional risks is complex due to many end-points, impact factors, and interactions. In addition, risk assessment cannot compensate for a lake of data and knowledge. However, *ad hoc* data are often utilized for the parameterization of nutritional risk assessment, which indicates the lack of data that were purposely designed for risk assessment practices. This could result in, for example, developing dose-response model based on findings from observational studies due to the lack of randomized controlled trials, which can subsequently lead to misleading interpretations of estimated risks. Therefore, it is crucial for the risk analysts to carefully evaluate uncertainty introduced by lack of high quality data, lack of solid method for dose-response relationship establishment.

In conclusion, although the application of risk assessment in finding answers to the nutrient-related public health questions is still in the early stage, the work of Dietary Reference Intakes (DRI) establishment and risk-benefit analysis studies has shown the benefits to use risk assessment process for nutrition related food standard and food safety issues, and indicated the potential to make further improvement in terms of data collection and methodological research of nutritional risk assessment.

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ADVANCES IN FOOD TECHNOLOGY AND NUTRITIONAL SCIENCES

Openventio PUBLISHERS

ISSN 2377-8350 Open Journal http://dx.doi.org/10.17140/AFTNSOJ-1-e006

Editorial

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Volume 1 : Issue 5
Article Ref. #: 1000AFTNSOJ1e006

Article History
Received: November 14th, 2015
Accepted: November 16th, 2015
Published: November 17th, 2015

Citation
Herrera-Cuenca M. From epigenetics to public policy: a new perspective for early action. *Adv Food Technol Nutr Sci Open J*. 2015; 1(5): e16-e18. doi: 10.17140/AFTNSOJ-1-e006

From Epigenetics to Public Policy: A New Perspective for Early Action

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Epigenetic has been a trendy topic for scientific community in the last two decades at least, yet many people around the world including policy makers and stake holders still think this field has nothing to do with policies or politics. Epigenetics is known as the key regulation of genes expression without changes in deoxirebonucleic acid (DNA) sequence, and the classic epigenetic mechanisms such as DNA methylation or post-translational modifications of histones are the ones most commonly discussed and have been joined by recent biomolecular research by which genes expression might vary according to the ribonucleic acid (RNA) function. Existing literature supports that epigenetics changes are responses to environmental stimulus and exposures; mainly maternal exposures including inadequate nutrition.[1] In addition, it is now suggested the existence of epigenetic windows of susceptibility to environmental insults during sperm development, giving the chance for inclusion of both factors; maternal and paternal in research to promote better understanding of these effects on the health of future generations; and explore the relevant applications on the public policies related for taking early action on the prevention of many diseases.[2]

The last paragraph definitely might sound complicate to a lawyer or a political science specialist or to an economist, still, chances are these professionals are the ones who hold positions of making decisions to take public actions in the path of prevention of chronic diseases. Interaction, understanding and integration between the epigenetic terminology such as DNA methylation and policy analysis terminology such as end type problem, policy problems, problem structuring, preferred policies, observed policy outcomes and expected policy outcomes[3] should be approached as an everyday issue when considering the relevance of the problems itself.

Obesity, type 2 diabetes and other chronic diseases are major health concerns in today's world, so are social inequities that take the global population to harmful exposures in toxic or dangerous environments.[4] Smoking or second hand smoking, inadequate housing, poor water conditions, lack of access to health care services, lack of physical activity due to insecure neighborhoods, inadequate or excessive nutrients intake, excessive weight gain during pregnancies, lack of sleep are some of life conditions to which a large amount of population is exposed.[5] All these conditions when influencing the first thousand days of life might induce alterations in the genes expression without changing the DNA sequence, but definitely deteriorating future health of those exposed.[6]

Having said the above thoughts, we can now see how are the conditions of let's say, an adolescent mother in Caracas, Venezuela, where according to the Venezuelan Health Ministry and the United Nations division for population, 35 of 100 non planned pregnancies occurred in adolescents less than 18 years old in 2010. Living in poor conditions, without access to health services, unemployed and with interrupted education, she can give birth a child who starts life in poor conditions.[7] With no income for buying adequate foods, living in an insecure environment that does not allow her to walk what she needs to in order to achieve the 150 minutes recommended by the World Health Organization (WHO),[8] she might well become overweight or obese or undernourished and have inadequate weight gain during her pregnancy, therefore

establishing a metabolic scenario that will increase the odds for a future development of cardiovascular diseases, type 2 diabetes or obesity in her child.[5] The elevated economic, familiar and ultimately emotional cost of the poor start to life are showing at the moment, that we shall take action for prevention[9] thus making urgent that a space for considering epigenetics into public action should be given, more over when we know epigenetic changes are reversible.[1]

Human development starts with individuals ameliorating their wellbeing. As Amartya Sen refers, the ones who make progress and development are individuals then transferring their wellbeing into societies.[10] A proposal made between academic sectors in Venezuela in 2002 included four policies starting with a family policy that would reinforce the role of the family in its primary care for health, food and nutrition and education, since parents are the first ones involved in feeding their children, ensure that pregnant women food intake is adequate and healthy, introduce children into the education system and should be aware of health control consultation. A structured and healthy family should be the base for human development.[11] Then, three policies: employment, housing and education for sharing and tolerance are needed. Parents need good work plans so they can have an employment that gives the resources for living a life with dignity in a house that is safe and has access to basic services such as adequate water and energy supply and is clean, then education for living into the societal environment expressing the freedoms and respecting limits so everyone can enjoy their space.[11]

Epigenetics and public policy? Yes. Are employment policies related to epigenetic? Yes. When people: fathers, mothers and home leaders have wages that allow them to buy healthy foods, policy makers and stake holders are promoting adequate nutrition on the mothers to be and also in children under their care in the years that are key to growth and development. Are housing policies related to epigenetic? Yes, a safe environment at home allows children to play, to have the adequate stimulus for their development, a house that gets safe water will reduce infectious diseases in children and the rest of the family and non-polluted space will diminish the risk for certain toxic diseases. Finally, education for sharing and tolerance will ensure the knowledge necessary, so the population will be aware of what is important to do for themselves and their children, they will learn how to cooperate with others and understand the role of the family in the improvement of wellbeing. At the end what is interesting is that with the improvement of education, family employment and infrastructure, we are ensuring less methylation of DNA or post-translational modification of histones.

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ISSN 2377-8350 Open Journal http://dx.doi.org/10.17140/AFTNSOJ-1-e006

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ADVANCES IN FOOD TECHNOLOGY AND NUTRITIONAL SCIENCES

Openventio PUBLISHERS

ISSN 2377-8350 Open Journal http://dx.doi.org/10.17140/AFTNSOJ-1-118

Letter to the Editor

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Volume 1 : Issue 5
Article Ref. #: 1000AFTNSOJ1118

Article History
Received: September 11th, 2015
Accepted: September 29th, 2015
Published: September 30th, 2015

Citation
Piekarski A, Bottje W, Dridi S. Heat stress: a global concern. *Adv Food Technol Nutr Sci Open J.* 2015; 1(5): 102-103. doi: 10.17140/AFTNSOJ-1-118

Heat Stress: A Global Concern

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The agriculture industry provides consumers with food (meat, milk, eggs, and grains) and contributes significantly to the global economy. Due to reliance on specific climate conditions, heat stress (caused by a combination of environmental factors such as: temperature, relative humidity, solar radiation, air movement, and precipitation) can have a very detrimental effect on this industry for both producers and consumers alike. With heat wave frequency and intensity projected to rise during the next century, climate change could make it more difficult to grow crops, raise animals, and harvest fish in the same manner and locations as used in the past.[1] Effects of heat stress can clearly be seen in poultry and livestock, and because animal agriculture is facing substantial challenges, including a steep projected increase in demand and the need to adapt to changing environmental conditions, these issues must be addressed. The United Nations FAO predicted an increase in world population to 9-10 billion, and estimated that by 2050 there will be a 73% increase in meat and egg consumption and a 58% increase in dairy consumption over 2011 levels.[2]

Agriculture is an important sector of the U.S. economy, contributing at least $200 billion each year with Americans consuming more than 37 million tons of meat annually.[3] Heat waves which, as stated earlier, are projected to increase under climate change, could directly threaten livestock. In the U.S., a number of states reported losses of more than 5,000 animals due to a single heat wave alone.[4] To help combat this, producers must provide shade, improved ventilation, and a sufficient quantity of water with temperature further reduced by spraying cool water across the roofs of buildings where animals are housed. Although most poultry are raised in houses, factors such as overcrowding, ambient heat from floors and roofs due to an increase in environmental temperature, can all affect heat stress in these animals. A method, in addition to those aforementioned, is ventilation, which can be provided for air movement by fans and windows.[5] Over time, heat stress can increase vulnerability to disease by increasing gut leakage, parasite infestation, reducing milk production, reducing fertility, and lowering birth weights with greater embryonic mortality in livestock.[6] Heat stress also reduces productivity in poultry by increasing numbers of smaller eggs with thinner eggshells that break during handling and processing, and increasing mortality in embryos and neonates as well as lowering growth and productivity.[7] Because of this, finding ways to help poultry and livestock better adapt to climate change is of uppermost importance.

Modern poultry, especially meat producing poultry (bred for high growth rate and body weight), are particularly sensitive to heat stress due in part to higher normal body temperatures compared to mammals. Because heat production is a natural occurrence and because these animals have highly metabolically active tissue, poultry suffer from heat stress easily with some succumbing to spiraling hyperthermia due to the inability to regulate body temperature in extreme high temperatures.[8] Therefore, much research has gone into finding a way to alleviate or combat heat stress in these animals. Some groups have used "thermal conditioning" which exposes embryos (pre-hatch) or neonates (during the first 4 d post hatch) to high heat conditions, with results showing greater resistance to heat stress and reduced body temperatures.[9] Recently, autophagy, a "self-eating" cell survival pathway, has been applied to this condition. Zhou and colleagues studied heat stress in tomato plants and found that heat stress activates autophagy genes with accumulation of autophagosomes.[10] Preliminary data from our laboratory (data not shown) in poultry suggests a similar mechanism, suggesting a new research avenue that uses the animals own cellular machinery for combating climate change in the agricultural

ISSN 2377-8350 Open Journal http://dx.doi.org/10.17140/AFTNSOJ-1-118

industry.

Due to the complex nature of agricultural systems, there are many factors that need to be considered when assessing how climate change will affect global food production.[11] Therefore, it becomes clear that heat stress is a very real issue and impacts a multi-billion dollar industry that could have significant impacts on global economies in the coming years. With new research aimed at preventing and alleviating issues of climate change, the agriculture sector is set for a surge of new ideas and methods to aid in a potential, eventual elimination of the issue of heat stress.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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ADVANCES IN FOOD TECHNOLOGY AND NUTRITIONAL SCIENCES

Openventio PUBLISHERS

ISSN 2377-8350 Open Journal http://dx.doi.org/10.17140/AFTNSOJ-1-119

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Volume 1 : Issue 5
Article Ref. #: 1000AFTNSOJ1119

Article History
Received: October 15th, 2015
Accepted: October 21st, 2015
Published: October 27th, 2015

Citation
Arambel HR, Donoghue AN, Arsi K, et al. Chitosan supplementation reduces enteric colonization of *Campylobacter jejuni* in broiler chickens and down-regulates expression of colonization genes. *Adv Food Technol Nutr Sci Open J*. 2015; 1(5): 104-111. doi: 10.17140/AFTNSOJ-1-119

Chitosan Supplementation Reduces Enteric Colonization of *Campylobacter jejuni* in Broiler Chickens and Down-Regulates Expression of Colonization Genes

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ABSTRACT

Campylobacter is one of the leading causes of foodborne bacterial gastroenteritis worldwide, and poultry is considered as the most common source of human infections. *Campylobacter* is prevalent in most poultry flocks and a reduction of *Campylobacter* in poultry would greatly reduce the risk of campylobacteriosis in humans. Unfortunately, efforts to reduce *Campylobacter* in pre-harvest poultry have been met with limited success. Preliminary studies with the natural compound, chitosan, demonstrate its ability to kill *Campylobacter*, *in vitro*. The purpose of this study was to determine the ability of feed supplemented chitosan to reduce enteric *Campylobacter* colonization in broiler chickens. Additionally, the effect of chitosan on expression of *Campylobacter's* chicken colonization genes was investigated using real-time quantitative PCR (RT-qPCR). Because chitosan's antimicrobial properties may vary depending upon its molecular weight, selected doses of three molecular weight chitosans were supplemented in the feed and evaluated for efficacy to reduce *Campylobacter* in chickens. Three replicate trials were conducted, and in each trial, birds were divided into 10 treatments (n=10 birds/treatment) and were fed 0% (controls), 0.25%, 0.5% or 1% (wt./wt.) of a low, medium or high molecular weight chitosan. Birds were fed treated feed for the duration of the study and orally challenged with a four-strain mixture of wild type *C. jejuni* on day 6. On day 15, the ceca samples were collected for enumeration of *Campylobacter*. In all three trials, the 0.5% dose of the medium molecular weight chitosan reduced cecal *Campylobacter* counts ($P<0.05$). RT-qPCR analysis revealed that chitosan down-regulated the expression of chicken colonization genes as compared to control ($P<0.05$). These results suggest that supplementation of chitosan in feed is a potential strategy to reduce the enteric colonization of *Campylobacter* in pre-harvest chickens.

KEYWORDS: *Campylobacter jejuni*; Chitosan; Broiler chickens; Pre-harvest; Colonization gene; Real-time quantitative PCR.

ABBREVIATIONS: RT-qPCR: Real-time quantitative PCR; IBS: Irritable Bowel Syndrome; ReA: Reactive arthritis; IBD: Inflammatory Bowel Disease; BPD: Butterfield's Phosphate Diluent; NCBI: National Center for Biotechnology Information; LMW: Low Molecular Weight; MMW: Medium Molecular Weight; HMW: High Molecular Weight; ECDC: European Centre for Disease Prevention and Control; SIC: Sub-Inhibitory Concentration.

ADVANCES IN FOOD TECHNOLOGY AND NUTRITIONAL SCIENCES

Openventio PUBLISHERS

ISSN 2377-8350 Open Journal http://dx.doi.org/10.17140/AFTNSOJ-1-119

INTRODUCTION

Campylobacter is one of the most frequently reported food-borne pathogens and causes an estimated 1.3 million infections in the United States annually.[1] While the majority of *Campylobacter* cases result in acute gastroenteritis, infection has also been associated with more severe diseases, including Guillain-Barré syndrome, Reactive arthritis (ReA), Irritable Bowel Syndrome (IBS), and Inflammatory Bowel Disease (IBD).[2] Epidemiological evidence indicates that the most common source for *Campylobacter* infections in humans is due to consumption of poultry products.[3] This is typically caused by the consumption of improperly cooked chicken or cross-contamination from handling raw chicken.[1,4] *Campylobacter* colonization in poultry is common; as many as 90% of US broiler flocks are contaminated with this food-borne pathogen.[5] Therefore, a reduction or elimination of *Campylobacter* in poultry is a research priority to reduce the risk of infection in humans. Many pre-harvest strategies have been evaluated for reducing *Campylobacter* in poultry, such as bio-security, probiotics, competitive exclusion, bacteriocins, bacteriophages, vaccines, and natural compounds, often with limited success.[6] Recently, the natural product chitosan has shown potential to reduce colonization of another food-borne pathogen, *Salmonella* Typhimurium, in pre-harvest poultry[7] and may have application against *Campylobacter*. Chitosan has also shown efficacy against other Gram-negative species, including *Escherichia coli* and *Pseudomonas fluorescens*.[8,9] Chitosan, a natural by-product derived from the deacetylation of chitin, is obtained from crab and shrimp shell waste.[10,11] Chitosan is a potential natural food preservative with broad antimicrobial benefits.[8,12,13] Although the exact mode of action of chitosan is not completely understood, researchers have previously determined that chitosan is capable of interacting with the outer cell membrane of bacterial pathogens, altering its permeability, disrupting cellular physiology and causing cell death.[9,14] To our knowledge, the ability of chitosan to reduce *Campylobacter* colonization in poultry has not been evaluated. The purpose of this study was to determine the efficacy of in-feed supplementation of chitosan on *Campylobacter* colonization in broiler chicks. Young chickens were used in this study because previous results from our laboratory demonstrated that young birds can be used as a reliable model to study *Campylobacter* colonization in market age birds.[15,16]

MATERIALS AND METHODS

Chitosan Materials

Chitosan of molecular weight 50-190 kDa and 190-310 kDa was obtained from Sigma-Aldrich (St. Louis, MO, USA), and 400-600 kDa chitosan was purchased from Spectrum Chemicals (New Brunswick, NJ, USA).

In vitro Susceptibility of *C. jejuni* to Chitosan

Antimicrobial activity of each molecular weight chitosan, low (50-190 kDa), medium (190-310 kDa) and high (400-600 kDa), in a 0.5% (wt./vol.) solution was determined by inoculating each solution with a four-strain mixture of wild-type *C. jejuni*. Preparation of the *Campylobacter* inoculum was done as described previously by Farnell and others.[17] In brief, working stock cultures of the four wild-type strains of *C. jejuni* were obtained by individually inoculating each strain into fresh *Campylobacter* Enrichment Broth (CEB, Acumedia, Neogen Corporation, Lansing, MI, USA) from frozen glycerol stock and successively sub-culturing twice at 42 ºC for 48 h under microaerophilic conditions. Strain mixtures were then combined centrifuged at 3000 * g for 10 minutes and the cell pellet re-suspended in 10 mL Butterfield's Phosphate Diluent (BPD). A 1% stock solution (wt./vol.) of each molecular weight of chitosan was prepared in 50 mM acetic acid as described by Ganan and others.[18] For the experiment, the stock concentration of each of the chitosan solutions and the acetic acid control was diluted 1:1 with an inoculum containing 10^8 CFU/mL of *C. jejuni*, resulting in a final concentration of 0.5% for each chitosan. Sample time points included 0, 2, 4 and 8 h post inoculation. At each time point, an aliquot from the treatments and control was taken and 1:10 serial dilutions were direct plated on Campy Line Agar.[19] The plates were incubated for 48 h at 42 ºC in a microaerophilic atmosphere. Direct enumeration of *Campylobacter* colonies was converted to CFU/mL for each treatment. Each susceptibility assay was repeated in duplicate.

In vivo Susceptibility of *C. jejuni* to Chitosan

Day of hatch Cobb broiler chicks (Siloam Springs, AR, USA) from a local commercial hatchery were utilized for the animal experiments. In each of three replicate trials, 100 chicks per trial were randomly divided into 10 treatments, which consisted of three concentrations (0.25%, 0.5%, or 1% wt./wt.) of each molecular weight chitosan, which was added to the feed and a positive control (0% chitosan). Birds were placed in floor pens and provided feed and water *ad libitum*; treated feed was provided throughout the entire trial.

The *Campylobacter* challenge was prepared as mentioned above. Birds were challenged by oral gavage with 0.25 mL of a four-strain mixture of wild-type *C. jejuni* on day 6, at a concentration of 10^7-10^8 CFU/mL. On day 15, birds were euthanized and the ceca were excised for *Campylobacter* enumeration. Cecal contents were serially diluted 10-fold with BPD and plated on CLA for direct enumeration. Plates were incubated at 42 ºC under microaerophilic conditions for 48 h and enumerated for *Campylobacter* colonies as previously described by our laboratory.[20] All the experiments conducted in this study were approved by the Institutional Animal Care and Use Committee of the University of Arkansas.

Chitosan Solution Preparation and Determination of Sub-Inhibitory Concentration (SIC)

The chitosan solution was prepared as mentioned pre-

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viously.[18] The SIC of chitosan was determined using previously published protocol.[21] Briefly, 24 well polystyrene plates (Costar, Corning, NY, USA) containing CEB (2 mL/well) supplemented with two-fold dilutions of chitosan (0, 0.2, 0.1, 0.05, 0.025, 0.0125 and 0.00625%), were inoculated with ~5.0 log CFU of *C. jejuni* wild strain, followed by incubation at 42 ºC for 24 h. Bacterial growth was determined by culturing on CLA agar plates. The highest concentration of chitosan that did not inhibit *C. jejuni* growth during mid-log (8 h), and stationary phase (24 h) were selected as the SIC for the compound.

RNA Isolation, cDNA Synthesis and Real-time Quantitative PCR

The effect of SIC of chitosan on the expression of *Campylobacter* genes critical for colonization in chicken was investigated using real-time quantitative PCR (RT-qPCR), as described previously.[21] The wild type *C. jejuni* strain was randomly selected from the four strains used in the *in vivo* trials for gene expression analysis. The strain was cultured with or without SIC of chitosan at 42 ºC in CEB to mid-log phase (8 h) and total RNA was extracted using the RN easy Mini kit (Qiagen, Valencia, CA, USA), followed by complementary DNA synthesis (iScript cDNA synthesis kit, Bio-Rad). The cDNA synthesized was used as the template for RT-qPCR. The amplification product was detected using SYBR Green reagent (iQ SYBR Green Supermix, Bio-Rad). The primers for each gene (Table 1) were designed from published GeneBank *C. jejuni* sequences using Primer 3 software National Center for Biotechnology Information (NCBI) and synthesized from IDT DNA. The relative expression of candidate genes was determined using the comparative critical threshold (ΔΔCt) method on a Quant Studio 3 real-time PCR system (Applied Biosytems, Thermo Fisher Scientific, Carlsbad, CA, USA). Data were normalized to the endogenous control (16S rRNA), and the level of expression of target genes between treated and untreated samples were analyzed to study effect of chitosan on expression of each gene. Duplicate samples were used and the assay was repeated three times.

Statistical Analysis

Cecal *Campylobacter* counts were logarithmically transformed before analysis to achieve homogeneity of variance.[22] Analysis of the data was done using the PROC GLM procedure of SAS.[23] Treatment means were partitioned by LSMEANS analysis and probability of $p<0.05$ was required for statistical significance. Data comparisons for the gene expression study were performed using multiple t-test with GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California, USA, www.graphpad.com).

RESULTS

Chitosan *in vitro*

Campylobacter counts were reduced by approximately 1 log at 2 and 4 h when co-incubated with 0.5% for all three molecular weights of chitosan as compared with controls (Table 2). At 8 h, all three chitosan preparations produced a 4.5 to 5 log reduction in counts when compared with controls.

Chitosan *in vivo*

In trial 1, *Campylobacter* counts were reduced in six of the chitosan treatments: 0.25% and 0.5% Low Molecular Weight (LMW), 0.25% and 0.5% Medium Molecular Weight (MMW), 0.25% and 1% High Molecular Weight (HMW), in comparison to the positive control (Table 3). Trial 2 showed a significant reduction of *Campylobacter* by four of the chitosan treatments: 0.5% LMW, 1% LMW, 0.25% MMW, and 0.5% MMW (Table 3). Results from Trial 3 showed a significant reduction of *Campylobacter* by one of the chitosan treatments: 0.5% MMW (Table 3).

Gene with Accession no.	Primer	Sequence (5'- 3')	Gene description
16S-rRNA (NC_002163.1)	Forward Reverse	5'-TGAGGGAGAGGCAGATGGAA-3' 5'-TCGCCTTCGCAATGGGTATT-3'	Ribosomal RNA (housekeeping gene)
cadF (NC_002163.1)	Forward Reverse	5'-CGCGGGTGTAAAATTCCGTC-3' 5'-TCCTTTTTGCCACCAAAACCA-3'	Outer membrane fibronectin-binding protein
jlpA (NC_002163.1)	Forward Reverse	5'-AGCACACAGGGAATCGACAG-3' 5'-TAACGCTTCTGTGGCGTCTT-3'	Surface exposed lipoprotein
ciaB (NC_002163.1)	Forward Reverse	5'-TCTCAGCTCAAGTCGTTCCA-3' 5'-GCCCGCCTTAGAACTTACAA-3'	Invasion antigen protein
fliA (NC_002163.1)	Forward Reverse	5'-AGCTTTCACGCCGTTACGAT-3' 5'-TCTTGCAAAACCCCAGAAGT-3'	Flagella biosynthesis RNA polymerase sigma factor
motA (NC_002163.1)	Forward Reverse	5'-AGCGGGTATTTCAGGTGCTT-3' 5'-CCCCAAGGAGCAAAAAGTGC-3'	Flagellar motor protein
motB (NC_002163.1)	Forward Reverse	5'-AATGCCCAGAATGTCCAGCA-3' 5'-AGTCTGCATAAGGCACAGCC-3'	Flagellar motor protein

Table 1: Primers used for real time quantitative PCR (RT-qPCR) analysis.

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Treatment	Time in hours			
	0	2	4	8
Positive controls	$6.35*10^7$	$8.15*10^7$	$5.45*10^7$	$3.5*10^7$
Low Molecular Weight	$3.42*10^7$	$6.8*10^6$	$1.24*10^6$	$3.0*10^2$
Medium Molecular Weight	$8.55*10^7$	$2.55*10^6$	$1.82*10^6$	$5.5*10^2$
High Molecular Weight	$7.45*10^7$	$2.59*10^6$	$2.00*10^6$	$6.5*10^2$

[1]0.5% concentration of: low molecular weight chitosan is 50-190 kDa; medium molecular weight chitosan is 190-310 kDa; or high molecular weight chitosan is 400-600 kDa, in 50 mM acetic acid.
[2]*Campylobacter* inoculum was added to each chitosan treatment and sampled at 0, 2, 4, and 8 h; samples were plated and enumerated after 48 h incubation.
[3]Values represent average campylobacteriosis counts of two separate replicate trials.

Table 2: The effect of different molecular weight chitosans on growth of *Campylobacter jejuni in vitro*[1,2,3] *Campylobacter* counts, *in vitro*.

	Chitosan dose	Trial 1	Trial 2	Trial 3
Positive controls Control	0%	$8.77\pm.17^{a}$	$7.05\pm.69^{a}$	$8.36\pm.24^{a}$
Low Molecular Weight	0.25%	$7.06\pm.58^{cde}$	$7.1\pm.29^{ab}$	$8.59\pm.20^{a}$
	0.5%	$7.68\pm.27^{bcd}$	3.96 ± 1.02^{c}	$7.88\pm.38^{ab}$
	1.0%	$7.96\pm.15^{abc}$	ND^{d}	$7.76\pm.40^{ab}$
Medium Molecular Weight	0.25%	$6.76\pm.34^{de}$	4.83 ± 1.08^{bc}	$8.47\pm.21^{a}$
	0.5%	$7.4\pm.38^{bcd}$	$3.25\pm.94^{c}$	$7.28\pm.70^{b}$
	1.0%	$8.03\pm.14^{abc}$	$7.45\pm.34^{a}$	$8.57\pm.17^{a}$
High Molecular Weight	0.25%	$7.45\pm.19^{bcd}$	$7.49\pm.31^{a}$	$8.16\pm.29^{ab}$
	0.5%	$8.43\pm.18^{ab}$	$7.8\pm.35^{a}$	$8.34\pm.26^{a}$
	1.0%	$6.3\pm.74^{e}$	$7.31\pm.30^{a}$	$8.51\pm.19^{a}$

[1]Low molecular weight chitosan is 50-190 kDa; medium molecular weight chitosan is 190-310 kDa; high molecular weight chitosan is 400-600 kDa.
[2]ND: non-detectible.
[3]Day-of-hatch birds were fed chick starter treatments of 0.25%, 0.5% or 1% of either low molecular weight, medium molecular weight or high molecular weight chitosan, respectively, for the entire 15-day study; bird were inoculated with *Campylobacter jejuni* mixture on Day 6 and cecal contents were collected on Day 15 for campylobacteriosis enumeration.
[4]Means within columns with no common superscript differ significantly ($p<0.05$).

Table 3: The effect of different concentrations and molecular weight chitosans on cecal *Campylobacter jejuni* counts (means±SEM) in 15-day old broiler chicks during three separate trials[1,2,3,4] *Campylobacter* counts, *in vivo*.

SIC and Gene Expression Analysis

Since MMW chitosan was found to consistently reduce *Campylobacter* counts *in vivo*; we selected MMW chitosan for the gene expression analysis. One of the *C. jejuni* wild strain used in the *in vivo* study was randomly selected for the mechanistic study. Based on growth curve results (data not shown), the SIC of MMW chitosan that did not inhibit *C. jejuni* strain growth as compared to control was 0.0125%. This concentration of MMW chitosan was used for subsequent gene expression analysis. RT-qPCR results (Figure 1) revealed that MMW chitosan significantly reduced the transcription of genes coding for *Campylobacter* motility; namely, *fliA, motA, motB* and adherence (*cadF, jlpA, ciaB*) as compared to control ($P<0.05$). The expression of chicken colonization genes was not significantly affected by acetic acid ($P>0.05$).

DISCUSSION

Preliminary *in vitro* results utilizing a 0.5% dose demonstrate that the three molecular weight chitosan treatments reduce *Campylobacter* counts in comparison to the untreated controls (Table 2). To evaluate the ability of chitosan to reduce enteric *Campylobacter* colonization in chickens, the 0.5% concentration of all three molecular weight chitosans, plus a lower (0.25%) and higher dose (1%) were also evaluated. In the first trial, cecal *Campylobacter* counts were reduced in 6 out of 8 of the treatments (Table 3). When conducted in a second trial, 4 of the 8 treatments were effective; whereas in the third replicate trial, the 0.5% MMW reduced enteric *Campylobacter* counts

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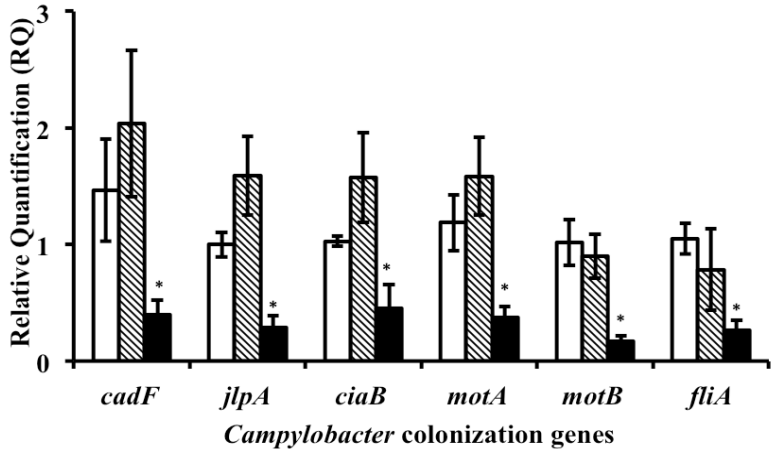

[1]RNA from wild type strain cultured either in the presence or absence (control) of 0.0125% chitosan to mid-log (8 hour) was used for cDNA synthesis and gene-expression analysis. 16S-rRNA was used as endogenous control.
*Means that differ significantly from the control (P<0.05).

Figure 1: Effect of 0.0125% MMW chitosan on the expression of chicken colonization genes (means ±SEM) in *Campylobacter jejuni*.[1]

when compared with controls (Table 3). Although there is variability between replicate trials, the 0.5% MMW chitosan dose consistently reduced *Campylobacter* in all three trials.

To determine the potential mechanism of action of chitosan, we investigated the effect of SICs of MMW chitosan on the expression of critical chicken colonization genes of *Campylobacter*. SICs of antimicrobials, including antibiotics are known to alter pathophysiology of microbes by modulating gene transcription.[21,24-26] In *C. jejuni,* the flagellar biosynthesis gene, *fliA* regulates a large number of genes involved in motility, protein synthesis and colonization.[27] A mutation in *fliA* has been shown to reduce motility and colonization potential in chicken cecum.[28] Similarly, *motA, motB* are critical for flagella motor function and facilitate motility and colonization.[27] *CadF* is another important virulence gene that encodes a 37 kDA outer membrane protein, that along with CiaB and JlpA, promotes adherence to intestinal cells and colonization in the avian intestinal tract.[27,29] We observed that SIC of MMW chitosan significantly decreased the expression of motility genes as well as adherence genes as compared to control (Figure 1), indicating that the anti-colonization effect of chitosan could be potentially mediated through reduced transcription of critical genes.

The importance of replicating results demonstrating a significant reduction in enteric *Campylobacter* counts in pre-harvest poultry cannot be underestimated. Previous research conducted by our laboratory[15,20,30] and others[31-33] have highlighted the variability between trials when evaluating pre-harvest treatments against enteric *Campylobacter*. Because of this inherent variability associated with *Campylobacter* colonization studies, results from a single pre-harvest study may not fully evaluate the consistency or effectiveness of a *Campylobacter* intervention strategy.[34-37]

Feed application of chitosan is a viable application for reducing *Campylobacter* colonization in chickens; however, water application is also a possible option. Unfortunately, chitosan is insoluble in water within the normal pH range.[38,39] This problem can be resolved by mildly acidifying the water, as accomplished in our *in vitro* studies. It is possible this will enhance the efficacy of chitosan as proposed by Qin and co-workers.[39] Acidifying water lines is already being performed in some poultry operations, which can reduce another foodborne pathogen, *Salmonella;*[22] thereby, this aids in the reduction of *Campylobacter* as well in the water lines and during feed withdrawal prior to processing, without altering the gut epithelium.[40,41] Thus, acidifying water in poultry houses could have a number of positive effects on bird health and reduce the potential zoonotic transfer of pathogens to humans. This possibility is currently under investigation.

The use of pre-harvest intervention strategies to reduce *Campylobacter* colonization (e.g., chitosan) can be part of a multifaceted approach to reduce the incidence of this foodborne pathogen. It has been proposed that a 2-log reduction in *Campylobacter* on the chicken carcass could reduce the risk of human campylobacteriosis by up to 30-fold.[42] Perceivably "small" reductions of Campylobacter in chickens could result in large reductions of campylobacteriosis incidences in humans. Olson and colleagues compiled data relevant to the consistent rise of campylobacteriosis incidences from the 1980's through 2006 as occurred in many countries, including Denmark, England, Wales, Norway, Sweden, New Zealand, and Australia, many of which are currently monitored by the European Centre for Disease Prevention and Control (ECDC).[43] In the 2000's, New Zealand focused on poultry as the primary source of *Campylobacter,* and by applying required regulatory implementations, along with the assistance of voluntary interventions, New Zealand saw a 54%

decline in campylobacteriosis incidences in 2008, compared to the period 2002-2006.[44] This decline was associated with a reduction in *Campylobacter* counts in chicken meat.[45] New Zealand's well-documented reduction of campylobacteriosis cases sets precedence for global reduction of *Campylobacter* by focusing intervention strategies on the poultry industry.

In conclusion, enteric *Campylobacter* counts were consistently reduced by in-feed supplementation of 0.5% MMW chitosan in three replicate trials. The use of this chitosan in pre-harvest poultry may be incorporated into a multifaceted strategy to reduce *Campylobacter* counts in chickens. Also, chitosan can be used to further reduce or inhibit *Campylobacters* surviving on the chicken carcass or meat. Further studies are warranted to explore the potential use of chitosan for reducing *Campylobacter* contamination in pre- and post-harvest poultry and the potential mechanism of action through whole transcriptome analysis.

ACKNOWLEDGEMENTS

Funded in part by the USDA-NIFA-OREI 2011-01955.

CONFLICTS OF INTEREST

Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that are suitable.

The authors declare that they have no conflicts of interest.

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ISSN 2377-8350 Open Journal http://dx.doi.org/10.17140/AFTNSOJ-1-120

Case Report

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Volume 1 : Issue 5
Article Ref. #: 1000AFTNSOJ1120

Article History
Received: October 30th, 2015
Accepted: November 20th, 2015
Published: November 23rd, 2015

Citation
Hirakawa A, Watanabe S, Tanaka S. Koda's fasting therapy: energy balance and intestinal bacterial flora. *Adv Food Technol Nutr Sci Open J*. 2015; 1(5): 112-123. doi: 10.17140/AFTNSOJ-1-120

Koda's Fasting Therapy: Energy Balance and Intestinal Bacterial Flora

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ABSTRACT

Since ancient times, fasting has been performed for religious reasons or as a cure of illnesses. Mitsuo Koda developed fasting dietary therapy and confirmed beneficial effects for many patients with intractable diseases. About 900-1000 kcal/day by unpolished brown rice, green vegetable paste and *tofu* constitute the basic regimen of Koda's therapy. This case report deals with women patient (M) who had suffered spino-cerebellar degeneration at a young age and recovered by using Koda's method. She had been living on only one glass of fresh vegetable juice per day for 19 years since her acute episode at age 20. Four other persons who were experimenting Koda's method were asked to join the study for comparison. In our case series, the Basic Metabolic Rate (BMR) was 1000-1200 kcal, which was equivalent with the intake, but in case of M the nutritional intake was almost one tenth of others. M's ketone bodies, especially β-Hydoxy Butyrate (BHB) in the blood, were more than 3 mM, so the main energy should come from ketone bodies. Biochemical changes of M coincided with the metabolic adaptation to yield BHB, as shown by elevated Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Creatine Kinase (CK). High aspartate was a reflection of above metabolic change. Increased BHB was observed in two participants, M and H, and both of them had *Bifidobacteriacae* in fecal bacteria. Energy balance and biochemical changes were discussed in these five subjects with calorie restriction.

KEYWORDS: Fasting therapy; Energy balance; Ketone bodies; β-hydroxy butyrate; *Bifidobacteriacae.*

INTRODUCTION

Since ancient times, fasting has been performed for religious reasons or as a cure of illnesses. Dr. Takahira[1] was at the vanguard of fasting therapy in 1910-1920. He studied precisely the metabolic changes caused by fasting, and reviewed the efficacy of fasting therapy in various disease states. Cahill[2] studied metabolic changes during 40 days of starvation and found that β-hydroxy butyrate (BHB) replaces glucose as a source of energy.

Koda's method is the combination of a low-energy dietary therapy, vegetarian diet, fasting, and physical exercise to stimulate the self-healing capacity.[3,4] Using this regimen, Mitsuo Koda had recovered himself from chronic hepatic failure and gastro-intestinal tract weakness, and he could also confirm beneficial effects for many patients with intractable diseases.[4] Unpolished brown rice and green vegetable paste constitute the basic regimen of Koda's therapy, as it improves the intestinal environment by resolving constipation. Koda's method could maintain the balance of the autonomic nervous system, so it might not only be effective for gastro-intestinal conditions, but also for some neurodegenerative diseases.[4]

This case study basically deals with a women patient (M) who had suffered spino-cerebellar degeneration at a young age and recovered by using Koda's method. She had been

ISSN 2377-8350 Open Journal http://dx.doi.org/10.17140/AFTNSOJ-1-120

living on only one glass of fresh vegetable juice per day for 19 years since her acute episode at age 20. Four other persons who were experimenting Koda's method were asked to join the study for comparison. These five participants were studied with a special focus on their energy balance, metabolic state, and intestinal microflora.

The study protocol was approved by the ethical committee of the Life Science Promoting Association (No. H24-1).

MATERIALS AND METHODS

Subjects Past History and Dietary Analysis

Past history, daily lifestyle, and dietary data were obtained by interviews at the Mori Clinic. Three-days dietary records were confirmed by a registered dietitian, and the intake of nutrients was calculated by using the Functional Food Factors (FFF) database.[5]

M (case 1) was a vivid woman. All five participants looked well, but B (case 2) and S (case were lean, owing to their breast cancer and gastric cancer, respectively. Confined to a wheelchair, W (Case 3) was alert, and displayed well-developed upper extremities. H (case 5) was a vivid, Muscular Professor. Summary case reports are described below for each patient.

Case 1: M

This 50 years-old, vivid and healthy-looking woman was born in December 25, 1962. She suffered spino-cerebellar degeneration at age twenty.[6] She could not keep her posture, so she could not walk but instead crawled on the ground. After diagnosis, her doctor predicted that she would probably not survive more than 5-years. She tried many treatments in despair. Finally she visited Koda's clinic, where fasting therapy was prescribed. She twice tried one-month fasting courses, and her neurodegenerative symptoms miraculously disappeared. Put on regular Koda's diet composed of brown rice and vegetables, she saw her disease relapse. She thus finally decided to live on one glass of fresh green-yellow vegetable smoothy paste per day. For her last 7 years of life, she was only taking filtered vegetable juice, adding 10 tablets each of *Ebios* (fermented beer remnant)[7] and *Spiren* (chlorella supplement)[8] and 1 g vitamin C tablet. Her vegetable juice was a mixture of grinded and filtered green vegetable soup, composed of 30 g kale, 20 g radish leaves, 20 g *komatsuna* (saltgreen), 20 g *chingen-sai,* 20 g carrot leaves, 20 g sunny lettuce, 20 g *kikuna* (*garland chrysanthemum*), and 20 g *yukina* (winter saltgreen). In addition, she drank 500 ml of water and up to 4-5 cups of persimmon-leave tea *ad libitum* when feeling thirsty.[9]

She usually went to bed at 0:00 and got up 3:00 am. She used to work hard at her acupuncture clinic during daytime. She was often asked to lecture outside, and travelled to many places. She had no other complaint about her health. Her height was 154.4 cm and body weight 57.7 kg.

Case 2: B

This 60 years-old lean female patient was born in July 23, 1963. Her height was 152.6 cm and her body weight was 38.2 kg. She suffered allergy and gastro-intestinal tract problems in childhood. At age 19, she received a tonsillectomy. At age 33, an ovarian cyst was removed surgically. At age 35 she was hospitalized in Koda's clinic for her gastro-intestinal tract weakness, and fasting therapy was prescribed with clear soup for 2 weeks. At that time her body weight was 48 kg, but she preferred to eat sweet foods, so after discharge her body weight rebounded to 55 kg. At age 58, after a health check-up, a left breast cancer was detected and resected. In November 2011, she started again Koda's raw vegetable diet. She has been taking daily *sui-mag* (di-hydroxy magnesium solution 100 mg/dl) at 6.30 am, Koda's diet (red vegetable soup with 5 g salt) at 12:00, 100 g brown rice powder with 30 g honey, 200 ml green vegetable soup, 300 g soy bean curd with 1ml soy sauce, and 10 ml of *kabosu* (citrus) juice at 19:00. Her body temperature was slightly low at 35.8, but her physical examination was otherwise normal.

Case 3: W

This 36 years-old male patient was born on September 26, 1976. He appeared on a wheelchair. At age 26, after a motorcycle accident, he had a fractured thoracic vertebra with paraplegia below the chest. His lower extremities showed marked muscular atrophy and his body weight was 37.8 kg, despite a height of 170.5 cm. Believing that Koda's therapy might cure the paralysis, he practiced Koda's vegetarian diet for 10 years in addition to rehabilitation. He needed manual removal of 100-150 g feces twice a day, and when bowel movements were absent, he drunk a cup of *sui-mag*. He took two glasses of red and green vegetable juice daily, 10 tablets each of *ebios* and *spiren* at 12:00, red and green vegetable juice, 300 g *tofu* with 1 ml *shoyu*, 120-150 g brown rice powder with 30 g honey, and 10 g salt occasionally.

Case 4: S

This 55 years-old male patient was born on April 3, 1958. He was a short-track athlete at high school. His optimal weight was 60 kg, but it increased to 80 kg at age 45 during work at a computer company, where he gradually developed hypertension. At age 54, a stage-3c carcinoma of the gastric cardia was detected by endoscopy, and a Billroth-II gastrectomy was performed. One month after the operation he started Koda's raw vegetable diet. Two months later he had edema due to low proteinemia, so fish was added to the diet. Three months ago, a metastasis in Virchow's lymph node was found, but he refused chemotherapy and continued acupuncture and dietary therapy. He took daily two glasses of red and green vegetable juice, 10 tablets of each *ebios* and *spiren* at 12:00, and 300 g *tofu* with 1 ml *shoyu* and 120-150 g brown rice powder with 30 g honey

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were added to the above two juice at 18:00 p.m. Two small cups of *Sui-mag* was added at 20:00 p.m.

Case 5: H

This 71-years old muscular man was born on August 31, 1942. He had been a long-distance runner at university. After graduation he started body building and became a champion. He used to eat a lot of meat to develop his muscles, but he became easily fatigued. He started Koda's raw vegetable diet after hearing Koda's lecture, and participated in the 1985-1986 clinical study on the effects of complete raw vegetable diet. He felt that the diet changed his body and his physical performance increased. He suffered retinal detachment in his left eye in 2000, so he returned to Koda's raw vegetable diet. He was fine to the point of being able to go for skiing with students. He lived on Koda's vegetarian diet at least twice a day, but he liked fruits at breakfast, with a large bowel of raw vegetable salad and two cooked dishes for lunch and dinner. He took occasional sweets as a snack with black coffee. He preferred Japanese burdock noodle occasionally. He regularly passed 200-300 g stools daily.

Energy Expenditure

Measurements of basal metabolic rate (BMR) were conducted as previously described.[10] The subjects came to the clinic in the early morning and were asked to minimize walking prior to the BMR measurement. BMR was measured in the post-absorptive stage at least 12 hours after the last meal. Measurements were performed in a room at a constant temperature of approximately 25 °C. After entering the clinic, the subjects rested in the supine position wearing a face mask for at least 30 minutes. The samples of expired air were collected in Douglas bags over two 10-minutes periods, and the mean of the two values was used for the analyses.

The expired O_2 and CO_2 concentrations were measured using a gas analyzer (Arco System, AR-1, Kashiwa, Japan) with a galvanic O_2 sensor and an infrared CO_2 sensor. Prior to each of the consecutive measurements, the gas analyzer was calibrated using atmospheric air. The volume of expired air was determined using a dry gas volume meter (Shinagawa, DC-5, Tokyo, Japan) and then converted to the volume under conditions of standard temperature, pressure, and dry gas. BMR (kcal/day) was calculated using Weir's equation.[11]

Laboratory Tests

Venous blood samples were collected after a fast of at least 12 hours for measurement of blood biochemistry. The following blood parameters were analyzed in the Serum Research Laboratories, Tokyo.[12]

Whole blood was used for Hemoglobin (Hb), Red Blood Cell (RBC) count, White Blood Cell (WBC) count, Hematocrit (HT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), platelet count, and blood morphology, such as STAB, SEG, EOSIN, BASO, MONO, LYMPH (T/B).

As nutritional markers, Total Protein (TP), Albumin (Alb), Triacylglycerol (TG), Total Cholesterol (T-Cho), Free Cholesterol (F-Cho), Low-Density Lipoprotein-cholesterol (LDL-cholesterol), Malondialdehyde-modified low density lipoprotein (MDA-LDL), Oxidized low-density lipoprotein (Ox-LDL), High-Density Lipoprotein-cholesterol (HDL-cholesterol) were measured. In addition, the following vitamins and minerals were quantitatively measured: vitamin A, vitamin B_1, vitamin B_2, vitamin B_6, pyridoxan, pyridoxal, pyridoxin, vitamin B_{12}, folic acid, vitamin C, 25-OH vitamin D, 25-$(OH)_2$ vitamin D, and vitamin E, and Ca, P, Na, K, Cl, Mg, Fe, Unsaturated Iron Binding Capacity (UIBC).

To assess the liver function, Total Bilirubin (T-Bil), bilirubin (direct and indirect), Asparagic acid aminotransferase (AST)), Alanine aminotransferase (ALT), Lactate Dehydrogenase (LD), Alkaline phosphatase (ALP), Leucine Amino Peptidase (LAP), Alanine aminotransferase (γ-GTP), Choline esterase (ChE) were measured.

As metabolic markers of muscle, pancreas and kidney, Amylase (AMY), Creatine Kinase (CK) or Creatine Phosphokinase (CPK), blood Urea Nitrogen (UN), creatinin, Uric Acid (UA) were measured. HbA1c and fasting blood glucose were also ordered, but failed by clotting during transportation to the laboratory.

As a special nutritional reference, ketones in the venous blood, such as acetoacetic acid, 3-hydroxy butyric acid, acetone and total ketone bodies were quantitatively measured. Ordinary and highly-sensitive CRP tests were performed as markers of inflammation. In addition, Leptin, Adiponectin (LA), resistin, and 39 serum amino acids profiles were determined.

Intestinal Bacteria

Fresh feces were collected in separate sterile feces containers (Sarstdt, Germany) containing 2 ml of RNA *later* (Ambion, Inc., USA) and were stored at room temperature. The samples were stored at 4 °C until using for extraction of fecal DNA. DNA was extracted from stool samples by using the bead-bearing method as previously described by Matsuki, et al.[13] The V6-V8 region of the bacterial 16S rRNA gene was PCR amplified using the barcode-tag universal primer sets Q-968F-#(5'-CWSWSWWSHTWACGCGARGAACCTTACC-3') and Q-1390R-#(5'CWSWSWWSHTTGAGGGCGGATGWGTAC-3') (where # indicates a series of 128 barcode sequence tags underlined in the sequence). The PCR and pyrosequencing were performed as described by Nakayama, et al.[14]

All sequences were examined for possible chimeric artifacts by the CHECK CHIMERA program of the Ribosomal

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Database Project (RDP).[15] Sequence data were aligned with the CLUSTAL W package and corrected by manual inspection. The results were summarized at Phylum level (*Firmicutes, Bacteroidetes, Actinibacteria, Proteobacteria, Fusobacteria, Verrucomicrobia, Cyanobacteria, Lentisphaerae, and Tenericutes*) and at family level (*Bifidobacteriaceae, Bateroidaceae, Porphyromonadaceae, Prevotellaceae, Streptococcaceae, Clostridiaceae, Lachnospiraceae, Ruminococcaceae, Violonellaceae, Fusobacteriaceae, Enterobacteriaceae, Verrucomicrobiaceae, and others such as bacteroidales*).

RESULTS

Demographic Data of Subjects and Total Energy Expenditure

Demographic data of participants are summarized in Table 1. Their Body Mass Index (BMI) was low except for M. In the case of W, severe muscular atrophy of the lower extremities contributed to the loss of body weight. Blood pressure and body temperature also tended to be within the lower range.

The basal metabolic rate (BMR) was 1000-1200 kcal/day. In the case of M, the morning measurement was 1092 kcal/day and the evening measurement was 1228 kcal/day. As Total Energy Expenditure (TEE) could be estimated BMR * 1.5, TEE would be around 1500 kcal/day.[10]

Nutrient Intake from Koda's Diet

Koda's diet provides about 1000 kcal energy source per day from brown rice, vegetable juice and *tofu*. There are two different juices: one is pure green yellow vegetable leaves (green juice), and the other contains vegetable roots, such as carrot, radish, lotus and *taro* (red juice).[16]

M took only green juice for nearly 19 years with supportive vitamin C tablets and *ebios* (fermented beer remnant).[7] She took only 68 kcal/day from juice, and it became 122 kcal/day including supplements. H took *soba* noodles and occasionally pancakes with sweet red beans (*dorayaki*) because he liked sweet taste. The total calorie intakes of B, W, S, and H were 1065, 1084, 1084, and 1674, respectively. It was about two third in 3 subjects and in Case of M it was about 1/15 of necessary energy intake. Protein intakes were in the range of 40-50 g/day, fat 18-23 g/day, carbohydrate 170-190 g/day, except for M (Table 2). Vitamin and mineral intakes fulfilled the Japanese Dietary Reference Intakes (DRI) 2010.[17] There was no cholesterol, vitamin B_{12} and vitamin D intake. Dietary fiber intake was more than 20 g. Fat intake was also low, less than 20 g, and in case of M it was almost none. In her case, many nutrients were less than one tenth of average. Dietary fiber intake was only 5.6 g/day, although plant-derived vitamins and minerals were closer to normal.

Blood and Biochemistry

Biochemical data are summarized in Table 3. Total protein, albumin and High-Density Lipoprotein (HDL) cholesterol were normal in all subjects. The total cholesterol level was rather high, except for W. TG was low in all, but remained within normal range.

Liver enzymes concentrations, such as aminotransferases (AST), ALT, LD and ALP were high in all, except H. Creatine Kinase (CK) was extremely high in M, in whom ketone bodies were remarkably high. The β-hydroxy butyrate/aceto-acetate ratio was 4.9. H also showed high concentrations of ketone bodies, with a ratio of 3.0, but liver function tests showed normal values. Both continued Koda's diet for more than 10 years.

UN was within normal range, and creatinine was low in Band W. CRP was only elevated in S with cancer. Leptin, adiponectin and resistin were within the normal range.

Amino-acid profiles showed elevated aspartic acid, especially in M and H who showed hyperketonemia (Table 4). In M, α-aminobutyric acid, Branched-Chain Amino Acids (BCAA) and Fisher ratio were high. By contrast, asparagine was low in all. Essential Amino Acids (EAA) and BCAA were low in B. Other amino-acids did not show notable deviations from normal.

Name	M	B	W	S	H
age yrs.	52	61	38	56	72
sex	female	female	male	male	male
height (cm)	154.4	152.6	170.5	172.8	157.8
body weight (kg)	57.7	38.2	37.8	51.5	50.8
BMI	24.2	16.4	13	17.2	20.4
blood pressure (systolic/diastolic)	95/72	105/65	82/55	95/65	105/70
body temperature	36.5	35.7	36	35.7	36.2
Total Energy Expenditure/day	1092/1228	1287	974	935	1130

Table 1: Demographic data of the participants.

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Name	M		B	W	S	H
Diet	veg_juice	supplument*	KodaB	KodaB	KodaA	KodaB+snack
Duration	19 yrs		3 yrs	10 yrs	1 yr	10 yrs
Energy	44	77.4	1084	1084	1065	1674
Protein	5	14.9	49	49	41	65
Fat	1	2.3	23	23	18	27
Carbohydrate	7.3	0.03	174.1	174.1	189.0	294
Water soluble DF	0.83	na	5.31	5.31	5.14	5.31
Insoluble DF	4.15	na	15.67	15.67	15.14	15.67
All dietary fiber	5.64	7.7	22.3	22.3	21.6	30.2
Ash	2.4	na	21.1	21.1	25.2	21
Na	34	na	4085	4085	6024	4085
K	735	41.3	3145	3145	2991	3499
Ca	320	4	938	938	819	990
Mg	43	na	401	401	360	401
P	84	na	1121	1121	983	1121
Fe	3.9	0.1	12.3	12.3	11.4	15.0
Zn	0.7	na	7.1	7.1	6.4	7.1
Cu	0.1	na	1.4	1.4	1.2	1.4
Mn	0.7	na	5.8	5.8	5.2	5.8
I	0.9	na	28.8	28.8	23.9	28.8
Se	1.8	na	25.7	25.7	21.5	25.7
Cr	1.2	na	9.2	9.2	7.5	9.2
Mo	16.5	na	291.9	291.9	244.5	291.9
cryptoxanthin	12.3	na	13.08	13.08	13.08	13.08
βcarotene_eq	5200	5830	23405	23405	23405	23405.0
αtocopheraol	3.10	na	7.01	7.01	6.69	7.006
βtocopherol	0.02	na	0.58	0.58	0.47	0.58
γtocopherol	0.39	na	12.95	12.95	9.84	12.95
vitamin K	4339	na	8363	8363	8350	8363
vitamin B_1	0.2	3.24	1.3	1.3	1.2	1.5
vitamin B_2	0.3	5.2	0.6	0.6	0.6	0.8
niacin eq.	1.9	na	24.6	24.6	22.2	24.6
vitamin B_6	0.3	0.4	1.6	1.6	1.5	1.6
vitamin B_{12}	0	52	0	0	0	0
Folic acid	201.8	na	439.6	439.6	425.2	439.6
Pantothenic acid	0.6	na	4.5	4.5	4.3	4.5
Biotin	2.5	na	29.6	29.6	25.3	29.6
vitamin C	90.3	na	134.3	134.3	135.2	134.3
sat. fatty acid	0.023	na	4.02	4.02	3.22	4.02
monosat. FA	0.005	na	4.68	4.68	3.76	4.68
polysat. FA	0.067	na	10.06	10.06	7.86	10.06

Sat: saturated, FA:\fatty acid, *na: not applicable, provider's data.

Table 2: Dietary intake by Koda's diet.

Name	reference range	unit	unit	M	B	W	S	H
TP	6.7-8.3	g/dL		8.4	8.1	7.3	7	7.2
Alb	3.8-5.2	g/dL		5.1	4.4	4.6	3.8	4.2
TG	50-149	mg/dL		61	80	73	54	79
T-Cho	150-219	mg/dL		183	264	140	199	256
F-Cho	30-60	mg/dL		46	68	36	57	62
LDL-chol	70-139	mg/dL		101	134	65	105	192
MDA-LDL		U/L		115	na	75	97	182
HDL-chol	M 40-86	F 40-96	mg/dL	59	98	54	81	56
T-Bil	0.3-1.2	mg/dL		0.8	0.4	0.4	0.4	0.7
direct bil	<0.4	mg/dL		0.2	0.1	0.1	0.1	0.2
indirect bil	<0.8	mg/dL		0.6	0.3	0.3	0.3	0.5
AST (GOT)	10-40	U/L		48	69	33	117	25
ALT (GPT)	5-40	U/L		23	72	70	93	24
LD (LDH)	115-245	U/L		258	335	138	302	210
ALP	115-359	U/L		270	346	772	367	166
LAP	35-73	U/L		61	77	63	82	52
γ-GTP	M <70	F <30	U/L	25	38	12	76	19
ChE	M 242-495	F 200-459	U/L	327	323	322	189	422
AMY	37-125	U/L		78	71	99	90	102
CK (CPK)	M 62-287	F 45-163	U/L	1212	67	122	68	171
UN (BUN)	8.0-22.0	mg/dL		10.4	12.3	14.8	11.4	15
creatinine	M 0.61-1.04	F 0.47-0.79	mg/dL	0.53	0.38	0.27	0.7	0.8
UA	M 3.7-7.0	F 2.5-7.0	mg/dL	4.6	3.2	3.7	4.1	7.4
Ketone bodies (venous blood)								
acetoacetate	<55	μmol/L		635	7	11	8	69
3-hydroxy butyrate	<85	μmol/L		3136	33	32	19	207
total ketone bodies	<130	μmol/L		3771	40	43	27	276
CRP	<0.30	mg/dL		0.05	0.06	0.02	0.45	0.05
high sensitive CRP		ng/mL		na	na	85	3840	287
leptin		ng/mL		3.7	na	1.5	1.8	4.5
adiponectin (LA)	>4.0	μg/mL		9	na	18.6	14.6	9.1
resistin		ng/mL		7.1	na	3.9	4	9

na: not applicable.

Table 3: Biochemical data of participants.

amino acid/ name	M	B	W	S	H	standard
taurine	60.7	75.3	81.7	82.6	278.6	39.5-93.2
aspartic acid	16.4	2.9	2.6	5.4	8.7	<2.4
hydroxyproline	tr	tr	tr	14.6	tr	<21.6
threonine	88.8	84.7	124.8	80.2	129.8	66.5-188.9
serine	141.6	91	131.4	97.4	105	72.4-164.5
asparagine	44.7	39.5	43.1	43	50	44.7-96.8

ISSN 2377-8350 Open Journal http://dx.doi.org/10.17140/AFTNSOJ-1-120

glutamic acid	43.6	22.5	21.2	25.5	55.2	12.6-62.5
glutamine	493.7	564.3	537.7	522.9	516.2	422.1-703.8
sarcosine	nd	nd	nd	nd	nd	tr
a-aminoadipic acid	tr	nd	nd	nd	nd	nd
proline	114	144.1	89.2	97.5	162.3	77.8-272.7
glycine	278.6	205.5	198	227.8	212.3	151-351
alanine	283.9	345.2	250.3	299.2	284	208.7-522.7
ctrulline	21.2	22.6	19.2	27.2	32.5	17.1-42.6
a-aminobutyric acid	44.4	8	9.5	7.5	23	7.9-26.6
valine	270.3	138.1	142.4	143.8	238.8	147.8-307
cystine	19.4	9.8	11.1	20.8	25.5	13.7-28.3
cystathionine	nd	nd	nd	nd	nd	tr
methione	24.3	14.5	234	17.6	21.7	18.9-40.5
isoleucine	121.5	33.2	40.9	50.3	72.2	43-122.8
leucine	189.1	69.2	79	93.9	129.4	76.6-171.3
tyrosine	53.7	41.7	43	57	57.2	40.4-90.3
phenylalanine	66.5	33	32.6	48	56.5	42.6-75.7
g-amino-b-hydroxybu-tyric acid	nd	nd	nd	nd	nd	nd
b-alanine	3.3	3.6	3	3.4	4.5	tr
b-amino-isobutyric acid	nd	nd	tr	tr	tr	tr
g-aminobytyric acid	nd	nd	nd	nd	nd	nd
monoethanolamine	8.8	tr	tr	tr	6.2	<10.4
homocystine	nd	nd	nd	nd	nd	nd
histidine	62	58.1	68.9	58.2	67.1	59-92
3-methylhistidine	tr	tr	nd	tr	tr	<5
a-methylhistidine	nd	nd	nd	nd	nd	<18.5
carnosine	nd	nd	nd	nd	nd	nd
asserine	nd	nd	nd	nd	nd	nd
tyryptophan	39.6	42.5	41.4	42.3	46.4	37-74.9
hydroxylysine	nd	nd	nd	nd	nd	nd
ornithine	39.3	54.4	53.8	52	69.7	31.3-104.7
lysine	115.8	139.3	151.2	132.7	150.6	108.7-242.2
arginine	88.6	87.3	53.3	88.3	73	53.6-133.6
total AA	2677.3	2318.7	2240.8	2313.6	2842.6	2068.2-3510.3
NEAA	1699.4	1706.1	1535.6	1646.6	1930.1	1381.6-2379.4
EAA	977.9	612.6	705.2	667	912.5	660-1222.3
BCAA	580.9	240.5	262.3	288	440.4	265.8-579.1
EAA/NEAA	0.58	0.36	0.46	0.41	0.47	0.4-0.63
BCAA/Total AA	0.22	0.1	0.12	0.12	0.15	0.11-0.18
Fisher ratio	4.83	3.22	3.47	2.74	3.87	2.43-4.4

Table 4: Plasma free amino acid profile of participants.

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Vitamins and minerals were well provided by the brown rice or brown rice powder, except for vitamin A, 25-OH vitamin D. As for the vitamins, all showed high folic acid concentrations, and in H 25-$(OH)_2$ vitamin D and vitamin E were high. As for mineral intake, K and Mg tended to be high. Ca was low in 2 out of 5. In M iron was low and UIBC was high. (Table 5)

As for the blood features, all subjects were within the normal range, except for S who had low Hb (9.5 g/dl) due to metastatic gastric cancer. The RBC count was more than 0.4 bil/l in all, and the WBC count was elevated to 8400/µl in M, but others were around 4000/µl. Platelets were all nearly 200000/µl. Lymphocyte counts were low in M and S, but T lymphocytes represented more than 80% in all subjects (data not shown)

Fecal Bacteria

The distribution of fecal bacteria by family is shown in Figure 1. High ketonemia was recognized in two participants, M and H. Both continued more than 10 years Koda's diet. *Bifidobacterium* are the dominant bacteria in these two participants. It is a major family of *Actinobacteria*, which is known to decompose non-digestible oligosaccharides.

DISCUSSION

The Koda dietary therapy has proven effective for many intractable diseases in Japan.[3,4] It is composed of brown rice and vegetable smash or soup, intermingled fasting therapy, as an alternative medicine. It seems to stimulate the human nervous and endocrine systems, and to increase self-healing ability.[4] Also, it changes the metabolic state. For example, Osame[18] reported changes in blood sugar and various hormones during his own fasting experiment, and found that the lowering blood glucose was followed by an increase in free fatty acids. Glucagon, glucocorticoids, adrenalin, and growth hormone were simultaneously increased.

In our case series, the daily intake of energy during Koda's diet was only 900-1000 kcal. The total energy expenditure, however, was predicted more than 1500 kcal/d from BMR (1000-1200 kcal and physical activity of more than 1.5 in free-living condition), and this discrepancy was enormous in case of M. Although the nutritional intake was insufficient (one tenth of average), nutritional biomarkers, such as hemoglobin, protein, albumin, TG, and cholesterol were almost within the normal range.

Name	Reference			M	B	W	S	H
vitamin A	97-316	IU/dL		59	131	76	84	182
vitamin B1	24-66	ng/mL		27	32	34	31	46
vitamin B2	66.1-111.4	ng/mL		84.5	54.4	68.3	61.5	78.7
vitamin B6								
pyridoxamin	M<0.6	F<0.6	ng/mL	0.2	0.2	0.2	0.2	0.2
pyridoxal	M 6.0-40.0	F<4.0-19.0	ng/mL	9.4	5.9	9.3	4.7	9.3
pyridoxin	M<3.0	F<3.0	ng/mL	3	3	3	3	3
vitamin B12	180-914	pg/mL		879	205	296	333	407
Folic acid	>4.0	ng/mL		22	21.7	17.8	12.9	19.8
vitamin C	5.5-16.8	µg/mL		15	10.4	8.9	12.9	11.8
25-Oh vitamin D	7-41	ng/mL		5	5	5	5	26
25-(OH)2 vitamin D	20.0-60.0	pg/mL		42.8	26.4	9.8	11.1	66.2
vitamin E	0.75-1.41	mg/dL		0.76	nd	0.53	1.16	1.42
Ca	8.5-10.2	mg/dL		9.1	9.2	7.8	9	8.3
P	2.4-4.3	mg/dL		4.1	3.2	3.6	3.8	3.4
Na	136-147	mEQ/L		136	140	139	141	142
K	3.6-5.0	mEQ/L		5.8	4.8	4	4.9	6.2
Cl	98-109	mEQ/L		105	109	106	108	104
Mg	1.8-2.6	mg/dL		2.6	2.8	2.5	2.9	2.1
Fe	M 54-200	F 48-154	µg/dL	52	86	72	59	54
UIBC	M 104-259	F108-325	µg/dL	436	245	248	231	209

Table 5: Serum vitamins and minerals of the participants.

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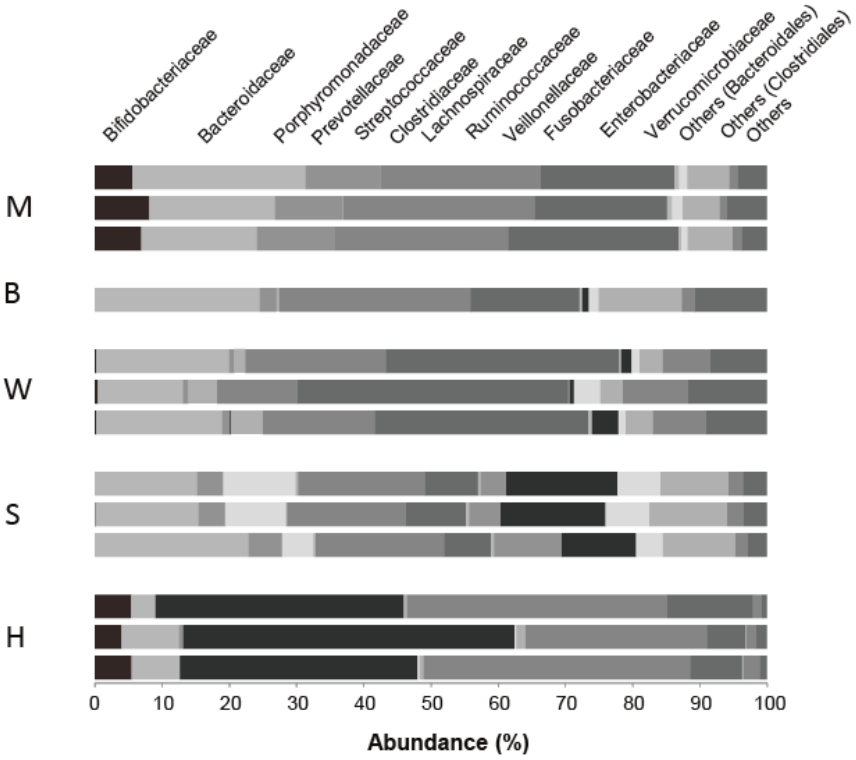

Figure 1: Comparison of microbiota of participants by family level.

M and H cases, who showed elevated beta-hydroxy butyrate showed noticeable population of *bifidobacteriaceae* (left part of column).

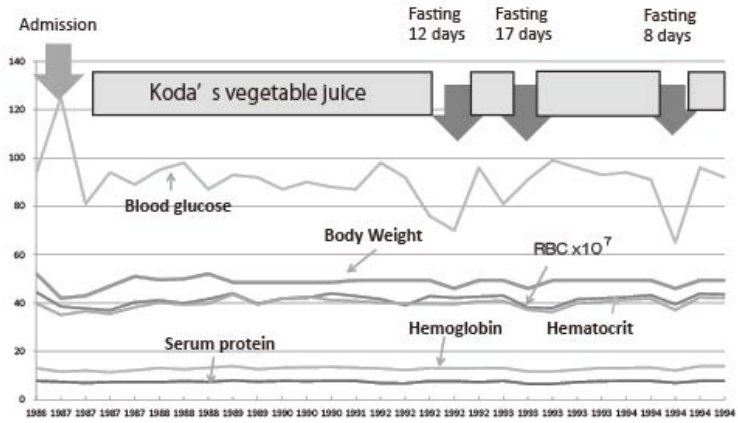

Figure 2: Changes of body weight and laboratory data for 10 years since onset of disease in Case of M.

Blood glucose level became low during fasting, and slight decrease of body weight is recognized, but serum protein, hemoglobin, hematocrit and RBC count remain within the normal value. Body weight first decreased but it recovered 2-3 later.

It has been said that weight decreases by several kilograms in the first 2-3 months after starting Koda's diet, but it becomes stable afterward, and weight gain can happen after several months.[4] Mori's long course showed such a curve (Figure 2). It was called the Koda curve and it is thought that the metabolic efficiency of the whole body improves.

Cahill[2] studied the glucose metabolism of people who let themselves fast for 40 days. A rise of β-hydroxybutyrate (BHB) to approximately 6 mM was characteristic. During starvation, insulin levels are extremely low and facilitate acyl-CoA entry into mitochondria, producing significant amounts of acetyl-CoA that cannot be metabolized in the Krebs cycle and is diverted towards ketone bodies synthesis.[19] Cahill[20,21] reported that in the starving human adult, BHB and aceto-acetate are produced in the liver from long-chain fatty acids and released into the blood.

ISSN 2377-8350 Open Journal http://dx.doi.org/10.17140/AFTNSOJ-1-120

The ratio of acetoacetate to 3-β-hydroxybutyrate depends on the redox status in the liver mitochondria (i.e., the NAD+/NADH ratio). Under normal circumstances, the BHB to aceto-acetate ratio is around 1. However, in diabetic ketoacidosis, this may increase to more than 10. The reference range of BHB is less than 0.4-0.5 mmol/L. In normal individuals BHB and acetoacetate are less than 0.1 mM and acetone essentially not measurable. It has been said that levels of more than 1 mmol/L require further action, whereas levels of more than 3 mmol/L require immediate medical review.[20-27]

Glucose, BHB, acetoacetate should be used for the energy source of the brain of these people. Liver, fat, muscle, and kidney affected the fasting energy production. The liver produced two-fifths, and the kidney three-fifths of the remainder. As for the gluconeogenesis of 80 g that brain requires a day, it was estimated that lactic acid from glycerol made 15-20 g, pyruvate reuse 20 g, 35-40 g from ketone bodies by an alanyl course, and 10-11 g/day by protein. With BHB as substrate, Nicotinamide Adenine Dinucleotide (NADH) increases relative to NAD^+ and CoQ10 increased relative to $CoQH_2$. This provides an increase in energy for Adenosine triphosphate (ATP) synthesis as compared to that in the heart using glucose. BHB increased contractility, and oxygen consumption decreased.

Increased BHB was observed in two participants, M and H. M's ketone bodies in the blood were more than 3 mM, so the main energy should come from ketone bodies. This level is seen in ketoacidosis, but the acidity would be neutralized by magnesium hydroxyde (*sui-magu*). Biochemical changes of M coincided with the metabolic adaptation to yield BHB, as shown by elevated AST, ALT, and CK. High aspartate was a reflection of above metabolic change. H was occasionally omnivorous, although he showed increased BHB. In his case all hepatic enzymes and CK were within normal range. The three other patients also showed elevated AST, ALT, LD and/orγ-GTP, without increase of CK. Any carbohydrate ingested, in even small amounts, decreases the level of BHB.[19] Thus, these 3 patients did not show the increase of ketone bodies.

As only ketone bodies increased without symptoms of ketoacidosis, BHB would be produced by enterobacteria. Intake of dietary fibers seemed not to be enough as the energy source, so the contribution of enterobacteria should be examined. As only two of the five patients produced high ketone bodies, it is plausible that intestinal bacteria would produce ketone bodies or precursors.

We analyzed the intestinal bacteria of participants who continued to take a Koda-type restriction diet for more than one year. The human digestive tract harbors trillions of bacteria, many of which establish lifetime, symbiotic relationships with their hosts. Those bacteria feed us with the products and by-products of their own digestive activities. The gut microbiome had evolved to encode a variety of digestive enzymes that break down hard-to-digest polysaccharides from food plants. In the case of M, vegetable juice was filtered to remove insoluble fibers, so only soluble fibers could contribute to the energy source. Some *Bacteroides* had xylanase or cellulose activities, so these species may play an important role in fiber degradation in a strict vegetarian. *Bifidobacterium* was also known to digest insoluble oligosaccharide.

Hayashi, et al.[28] had previously analyzed the fecal bacteria of Mori 15 years ago. They found that *Clostridium* and *Bacteroides* were the dominant groups, but they did not find *Fusobacterium*. They also found many *Bifidobacterium* by direct culture, but they could not find a relationship with vegetarian diet by Polymerase Chain Reaction (PCR) method.

We found that the high BHB was only recognized in two subjects with *Bifidobacteriacae*. Noack-Loebel, et al.[29] reported that many *Bidifobacterium spp*. were detected in the lacto-ovo-vegetarian diet consumed by children. *Fusobacterium prausnitzii* is one of the most frequent and numerous species detected in the human large bowel using 162 eDNA library method (approximately 4-10% of the total clone population. Finegold, et al.[30] failed to detect this species in a vegetarian. All of our subjects did not show fusobacterium except for the man with gastric cancer. H, who was not a strict vegetarian, showed the dominant *Prevotellaceae* but this finding is difficult to interpret. It was noteworthy that 4 participants showed the dietary intake was less than two third of Transesophageal echo TEE, so at least one third energy should come from intestinal microbiota. Calorie restriction diet may clarify the contribution of microbiota for energy production.

Numerous animal models consistently demonstrated that gut microbiota can modulate host energy homeostasis and adiposity through different mechanisms.[31] Although, extensive experimental data suggested that microbiota manipulation can beneficially affect host glucose metabolism, a causal relationship will needs to be proven in humans.[31-34]

In addition to the dietary therapy, Koda's therapy aimed at autonomic nerve training to improve the nerve-muscular reflex by letting exercise to perform waist twisting, movements to strengthen the capillary circulation, alternate bathing in warm and cool water, naked body therapy to be exposed to cool air.[4] These could maximize the self-healing ability to reach the spiritual level of living. This is a target of study in integrative medicine in near future.[35] We shall start to call volunteers to participate in the cross-sectional and prospective study on Japanese traditional brown rice vegetarians for health. Some of them would practice fasting for their health by themselves.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

CONSENT

As for the Patients consent statement, All participants agreed to publish the manuscript, entitled "Koda's fasting therapy: Energy balance and intestinal bacterial flora" in Advances in Food Technology and Nutritional Sciences - Open Journal, and provided the written informed consent (written in Japanese). The study was conducted at the Mori Acupuncture Clinic, Osaka as a collaborative work with authors.

ACKNOWLEDGEMENT

The authors appreciate Dr. Hirokazu Tsuji, Yakult Central Research Institute, for his support to analyze fecal bacteria. Authors also thank Dr. Philippe Calain for discussion and English revision of this manuscript. The authors appreciate the Life Science Promoting Association for funding this study.

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