Doctoral Dissertation

Protein-Energy Nutritional Status and Gut Microbiota

March 2022

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Contents

ABBREVIATIONS

AA	Amino acid
ADL	Activity daily living
BCAA	Brain-chained amino acid
BCFA	Brain-chained fatty acid
BMI	Body mass index
BMR	Basal metabolic rate
BSS	Bristol stool scale
CAS	Constipation assessment scale
dL	Deciliter
DLW	Doubly labeled water
DNA	Deoxyribonucleic acid
F/B	Firmicutes/Bacteroides
FFQ	Food frequency questionnaire
g	Gram
GLM	General linear model
HPLC	High-performance liquid chromatography
IDR	Indonesian Dollar Rupiah
kcal	Kilocalories
LPS	Lipopolysaccharide
MCV	Mean corpuscular volume
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
NT-proBNP	N-terminal prohormone of brain natriuretic peptide
OADR	Old-age dependency ratio
OTU	Operational taxonomic unit
P	Probability
PCR	Polymerase chain reaction
PCoA	Principal coordinated analysis
PEM	Protein-energy malnutrition
PNG	Papua New Guinea
PSA	Polysaccharide A
QoL	Quality of life
rRNA	ribosomal ribonucleic acid
RS-3	Resistant starch-3
SCFA	Short-chain fatty acid
SD	Standard deviation
SPSS	Statistical Package for the Social Sciences
TEI	Total energy intake
USA	United States of America
WBC	White Blood Cell
WHO	World Health Organization

Chapter I

Introduction

The number of older populations around the world were increasing over years. There were 40% of the older population (65 years or over) was in Eastern and South-Eastern Asia (260 million from 703 million in 2019). It was the largest and followed by Europe and Northern America. Japan has the highest old-age dependency ratio (OADR) in the world in 2019 and will remain the highest in projection in 2050. The old-age dependency ratio (OADR) is defined as the number of old-age dependents (persons aged 65 years or over) per 100 persons of working age (aged 20 to 64 years) [1]. The demographic showed that Japan has a low birth rate and longevity has been prominent. Japan was classified as a super-aging society in 2007 because the ratio of the aged population reached 21.5%. The extension of the healthy life expectancy was important to improve the quality of life (QoL) for the elderly and reduce the cost of medical care. The issue of nutrition in a super-aging society was to attain an increase in healthy life expectancy, as well as the nursing care and prevention viewpoint. The elderly citizens (75 years old or older) are at risk of overnutrition as well as undernutrition. The elderly can easily fall into a state of protein and energy malnutrition (PEM), due to a variety of causes such as loss of teeth, and a decrease in digestive and physical function. The ratio of elderly citizens with malnutrition is high [2]. Malnutrition, with hypoalbuminemia as an indicator, among the aged population is an important health care problem, as this problem is prevalent across the world, especially among the elderly aged 75 years or older. Malnutrition was not confined to institutionalized or hospitalized elderly individuals but was also seen in community-dwelling genarians. The result of a

5-year and 7-year longitudinal study in Japan showed that decreasing albumin level was associated significantly with aging among community-dwelling older adults aged 65 and over [3,4].

Nutritional assessment and treatment of malnutrition are imperative in order to attempt to minimize the risk of illness or complications associated with old age. Albumin is the most abundant plasmatic protein with one of the main roles assigned to albumin is as an indicator of malnutrition. Studies showed that a progressive reduction of albumin serum concentration between 0.08 and 0.17 g/L per year associated with aging, with greater reductions in men than in women. Meanwhile, albumin values in healthy elderly people in the community stay above 38 g/L until after the age of 90. Therefore, in a situation of clinical stability in the community, in elderly people in the community albumin can be a good marker of nutritional status. Weight loss, reductions in body mass index (BMI), and a decrease in muscle mass are indirect markers of malnutrition. Studies demonstrated that there was an association between hypoalbuminemia and loss of appendicular skeletal muscle mass. Albumin serum concentration is highly sensitive in the diagnosis of malnutrition in the hospitalized elderly, but it has low specificity. The association between autonomy in the activities of daily living (ADL) and albumin levels is more evident. Transversal and longitudinal studies showed that progressive reduction of albumin is associated with the development of greater inability than in those community elderly people who maintain a stable level of albumin. In hospitalized elderly, there was a relationship between higher albumin levels and a shorter mean stay in the hospital, which could mean that

patients with higher levels of albumin achieve the same improvement of functional status in a shorter period of rehabilitation. In the elderly living in the community, there is an association between albumin levels and long-term mortality (between 3 and 12 years). However, in the elderly living in care homes, albumin was associated with short-term mortality (1 year) but not with long-term mortality. Low levels of albumin are associated with higher mortality during a hospital stay for the elderly [5].

Protein-energy homeostasis is a major determinant of healthy aging. In old people, energy expenditure and insulin sensitivity can be altered and will give the risk of protein-energy wasting. A simple way to assess whether energy intake is correct in clinical practice is that the elderly person has a body mass index of more than 21 and maintains a stable weight. During the process of aging, the synthesis of the whole body, as well as specific proteins in muscle, is globally lower than in young subjects during the postabsorptive period [6]. Sarcopenia is a geriatric syndrome defined as the age-related loss of skeletal mass and function. Protein-energy malnutrition was one major risk for the development of sarcopenia. Dietary protein and resistance exercise have a synergistic effect on preserving skeletal muscle. Skeletal muscle mass is regulated by the process of muscle protein synthesis and breakdown (MPS and MPB). Older adults have shown evidence of ‘anabolic resistance’, whereby a higher dose of protein is required to achieve the same MPS response as a younger person. Thus, the recommended daily amount of protein is greater for older people. The aetiologies and mechanisms of anabolic resistance are complexes involving aging physiology,

accumulation of chronic disease, and changes in physical inactivity. The gut microbiome may influence anabolic resistance, either directly or indirectly. Direct effects of the gut microbiome on influencing anabolic resistance through lipopolysaccharide (LPS) modulation, short-chain fatty acid (SCFA) production, and barrier function. Research also implies a role of the gut microbiome in skeletal muscle function and potential mechanism to overcome anabolic resistance in older people [7].

Increased permeability of the intestinal barrier with age has been described across animal species, including worms, flies, mice, and rats. The age-associated remodeling of the gut microbiome in mice was shown to result in increased production of pro-inflammatory cytokines and intestinal barrier failure. In gut dysbiosis (such as aging), declined intestinal barrier integrity (barrier dysfunction) results in the translocation of microbes and microbial particles through the intestinal epithelial cell lining. Reduced microbiota diversity leads to overgrowth of distinct microbes and metabolism instability. Aberrant levels of microbiota-derived metabolites instigate abnormal immune responses resulting in chronic inflammation [8]. There is growing speculation that the gut microbiota may contribute to sarcopenia as aging is also associated with (1) dysbiosis, whereby the gut microbiota becomes less diverse, lacking in healthy butyrate-producing microorganisms and higher pathogenic bacteria, and (2) loss of epithelial tight junction integrity in the lining of the gut, leading to increased gut permeability and higher metabolic endotoxemia. Animal data suggest that both elements may impact muscle physiology, but human data corroborating the

causality of the association between the gut microbiota and muscle mass and strength are lacking. A healthy gut microbiota composition (high concentration of beneficial microbes and diversity) may release higher amounts of SCFA. It will be able to control the translocation of harmful substances from the lumen into circulation. SCFA can improve gut permeability and may positively modulate muscle biology. Reduced translocation of proinflammatory molecules is linked to lower systemic inflammation and may positively influence insulin sensitivity in the muscle. Contrary, unhealthy gut microbiota is associated with a leaky gut, which is less able to regulate the harmful translocation into the bloodstream of microbes (e.g., Bacteroides sp.) and their components (e.g., LPS), as well as proinflammatory cytokines. This leads to low-grade chronic systemic inflammation that may contribute to insulin and anabolic resistance in the muscle. Therefore, healthy gut microbiota may improve protein digestion and absorption by increasing peptide cleavage, and, in addition, promote SCFA production and reduce protein fermentation and the “leaky gut”. As a result, more amino acid (AA) and SCFA, and less gut-derived harmful molecules enter systemic circulation, increasing the postprandial delivery of AA to the muscle and reducing systemic and local inflammation, both in favor of MPS [9]. For these reasons, we explored the intestinal profile in community-dwelling elderly in Japan after receiving rice-koji amazake (act as a prebiotic) for 6 weeks. In Chapter IV, rice-koji amazake intervention in community-dwelling elderly was discussed.

The prevalence of protein inadequacy in developing countries such as sub-Saharan Africa and South Asia was more than 5% [10]. Most of their diets

were dominated by starchy staple foods, and nutrient-dense animal-source foods, fruits, and vegetables often being unavailable or unaffordable [11]. Sago together with taro and yam was claimed to be one of the oldest crops and former staple foods in large areas of Southeast Asia and Oceania before rice largely replaced these crops. Papua Province in Indonesia has a huge sago forest of more than 1 million hectares in total. Coastal communities and the lowlanders in the Papua province consumed papeda (sticky dough, which is considered as cooked rice) and sago lempeng (roasted sago) as their food since ancient times and they still currently do so [12 -15]. Limited production and a higher price of sago compared to imported food products, subsidized particularly imported rice, resulting in a declining consumption of sago [12,14]. Rice policy was integrated into the national transmigration program in 1954/1955 and its implementation continued between the 1970s and the 1990s. This policy was continued by the central government through the "rice for poor" policy since the 2000s [16]. Transmigration started in Papua in 1984 in the form of military transmigrants. Rice policy as part of the transmigration program also began and proceeded with the rice for poor policy since 2002 [17,18]. The history of coastal communities with the consumption of sago as the main staple food shows that they are healthy, physically strong people, and reliable seafarers [13]. Therefore, we aimed to analyze the protein-energy nutritional status of two adults populations in Mimika (low-lying district) with similar hereditary and environmental conditions, one, who consumed sago with a moderately low protein intake compared the other,

who consumed rice with sufficient protein intake. This study was presented in chapter II.

Diverse forms of malnutrition through the life-course are associated with dysbiosis such as lower diversity of gut microbiota in low birth weight, stunting, severe acute malnutrition (6-24 months), sarcopenia, anorexia nervosa (adults), and obesity (adult) [19]. Gut microbiota is implicated in the regulation of energy metabolism through the digestion of indigestible polysaccharides for the host which fermentation by the microbiota leads to the production of SCFA such as propionate, butyrate, and acetate. SCFA represents 10% of the daily energy supply in humans. Thus, an energetic imbalance is linked to gut microbiota alteration [20]. The study on the gut microbiota profile of moderately low protein intake-sago diet compared to sufficient protein intake-rice diet in lowlanders, Papua, Indonesia was presented in chapter III.

Chapter II

Protein-energy nutritional status of moderately low protein intake-sago diet compared to sufficient protein intake-rice diet in well-nourished lowlanders in Papua, Indonesia.

Sago is one of the oldest crops and former staple foods in Southeast Asia and Oceania before being replaced by rice. Indonesia has the largest area of sago palms in Indonesia, with the highest area being in Papua Island (estimated 95%). The sago palms in Papua and West Papua grow in sago forests that have not been managed or cultivated, which can decrease production [12].

The farming behavior of indigenous Papuan farmers is similar to "home-vegetable gardening". They cultivated plants that are resistant to disease and have a minimum risk of failures, such as taroes, sweet potatoes, cassavas, and bananas. Those commodities are also limited in quantities only for their consumption. For sago-eating people, wild sago was obtained in the forest and stored for one week's consumption. Meanwhile, rice-eating people were not cultivating rice, as an alternate, they were provided rice from the government through "rice for the poor policy". These behaviors were influenced by two perceptions that related to local wisdom in preserving nature. First, the potential of natural resources available in the ecological environment is a source to meet the food needs of the local population. Second, the potential of foods crops in nature is perceived as "a blessing" that should not be overused. This phenomenon affects the performance of Papuan farmers such as not cultivating agricultural land regularly, not developing market-oriented agricultural commodities, and tending to grow commodities that can be consumed in limited quantities [21].

The use of sago starch and sago-based food products spread in 21 of the 33 provinces of Indonesia as staple foods or snacks, especially in the coastal or lowland areas. Sago as local food has great potential to be developed to support

food diversification because it contains high carbohydrate productivity. However, other nutrients, including proteins, are very low in sago. As a staple food, sago must be consumed together with a protein food source to fulfill the requirements of protein intake [13,15].

The history of coastal communities with the consumption of sago as the main staple food shows that they are healthy, physically strong people, and reliable seafarers [13]. Mimika is a low-lying district, and people consume sago as their staple food with less protein. Mimika is also classified as a district with a higher risk of food insecurity [22]. However, to date, no report has implied protein deficiency symptoms in adults in this area, contrary to the strong muscular bodies of these people. Rice contains a certain level of protein and is consumed by local people. There is no data available on the health issues involving low protein intake among people who eat sago. This study aimed to analyze the protein-energy nutritional status of two adult populations with similar hereditary and environmental conditions, one who consumed sago with a moderately low protein intake, and the other, who consumed sago with sufficient protein intake.

MATERIALS AND METHODS

Study Population and Ethics Approval

This cross-sectional-analytic study was a part of the main study on analysis of gut microbiota of the lowlanders in Mimika Regency, Papua, Indonesia, conducted in September 2019. The sample size of this study was based on the main study (50 participants with 25 in each group). The inclusion criteria were men and women lived in the lowland of Mimika Regency, Papua with ages ≥ 20 years, body mass indexes between 18,5—24,9 kg/m2, those who consumed traditional carbohydrate-based food (sago) with low protein intake (< 25 g/d) or who consumed modernized carbohydrate-based food with sufficient protein intake (≥ 25 g/d). Participants who had taken antibiotics within the preceding six months, who had taken laxatives, gastric motility medications, prebiotics, or probiotics containing foods or supplements within the preceding month, had a medical history of clinically significant diseases such as cancer, gastrointestinal disorders (irritable bowel syndrome, inflammatory bowel diseases, celiac diseases, constipation, diarrhea, excessive bloating), autoimmune disorders, diabetes, heart diseases, renal failure or previous gastrointestinal surgery, infectious diseases, smokers or those with high alcohol consumption, were excluded from the study. The study was approved by Komite Etik Penelitian Kesehatan (Health Research Ethical Committee), of the Faculty of Medicine, Hasanuddin University, Makassar, Indonesia (Approval Recommendation Number: 554/UN4.6.4.5.31/PP36/2019 and protocol code: UH19070416). This study was

also approved by the Ethics Committee of Okayama Prefectural University (Protocol Code Number: 19-55).

Measures

Socio-demographics

A questionnaire was used to collect information on the characteristics of the participants, such as socioeconomic- demographics (age, ethnicity, education, occupation, and income), medical histories (cancer, gastrointestinal disorders, autoimmune disorders, diabetes, heart diseases, renal failure, previous gastrointestinal surgeries, infectious diseases), consumption of medications or supplements (laxatives, gastric motility medications, and prebiotic or probiotic-containing foods or supplements), and lifestyles (smoking and alcohol consumption).

Anthropometric Assessment

Participant height was measured using a mobile stadiometer (SECA 213). Bodyweight, body fat %, bone mass, total body water %, muscle mass, basal metabolic rate (BMR), and visceral fat were measured using a body composition monitor (TANITA BC 730). The measuring platform was placed on a hard, flat surface with minimal vibrations to ensure a safe and accurate measurement. Measurements were taken with minimal clothing and on an empty stomach (no meal before). The socks were removed, and the soles of the feet were cleaned before the participant stepped onto the measuring platform. Before measuring, personal data such as birth dates, gender, and height were input. The participant

was asked to step onto the scale after the scale turned on, stand unassisted in the center of the platform and look straight ahead while standing relaxed but still. The participant was allowed minimal clothing, but a mobile phone, a wallet, and anything heavier were removed. After the measurements were taken, the readings were displayed automatically, including body weight, bone mass, total body water percentages, muscle mass, BMR, visceral fat, and body fat percentages. The assessment was performed twice, and the average was used for the analysis. One of the most common methods for measuring muscle strength is the isometric grip strength test. We measured the isometric grip strength using a handgrip dynamometer. The participant was asked to squeeze the dynamometer as hard as possible with each hand while in a standing position. Before beginning the grip strength testing procedures, the participant was asked a series of questions to obtain information about muscle conditions (any visible limitations for either hand, any surgery of his/her hand, any pain, aching or stiffness, right-handed, left-handed, or equally). The participant remained seated during the preparation and warm-up periods.

Dietary Assessment

The intake of energy, macronutrients (carbohydrate, protein, fat), micronutrients, and fiber were measured using basically 3-day non-consecutive days of a 24-hour food recall (only one person had 2-day non-consecutive days). This assessment was performed on one or two weekdays and on one weekend day to obtain their usual dietary intakes. Face-to-face interviews were conducted to recall their food intake in the last 24-hour. On estimating portion sizes of the food,

plastic food models were used with information on portion sizes, for example, food model of standardized rice with portion sizes information or food model of standardized fish with portion sizes information. Common household measures such as household cups, bowls, spoons, rice spoons, and food photographs were also used to assist the individuals in estimating their portion of the food consumed. Observations on the fish seller were conducted to have accurate data on the type and the portion size of fish. Cooking methods of their local staple food have also been observed to have a precise recipe. At the beginning of the study, a meeting was held together with the head of the public health center to explain the purpose, the benefits, the measurements, and the team member of the study to establish communication with our study population. On collecting food intake data, we were accompanied by staff from the public health center, who is familiar with our study population. The dietary intake was analyzed using the Nutrisurvey 2007 application (www.nutrisurvey.de).

Blood Test

The complete blood counts and plasma albumin levels were measured by Prodia Laboratory, Jayapura, Papua, Indonesia.

Statistical Analysis

All the data were expressed as means ± SDs and medians (minimum, maximum). The data normality distribution was determined using the Shapiro-Wilk test. The differences between groups were determined by the independent t-test (normally distributed data) or the Mann-Whitney U test

(non-normally distributed data). The Pearson test was used to evaluate the correlation between albumin levels and other parameters because albumin was normally distributed. Parameters that correlated with albumin ($P < 0.25$) were further included in multivariate linear regression analyses. Multivariate regression tests were performed on both the rice and sago groups. The results of the regression models are shown as B, standard error, β, t, and *P* values. The Pearson's test was also used to evaluate the correlation of the laboratory profiles, anthropometry profiles, and intake profiles of both the rice and sago groups. All the statistical analyses were performed using the Statistical Package for the Social Sciences version 27 (SPSS Inc, Chicago, IL, USA). Statistical significance was established at a *P*-value < 0.05.

R E S U L T S

Socio-characteristics and Nutrient Intake of the Study Participants

Fifty participants were recruited with 25 each with 12 male and 13 female in the rice and 11 male and 14 female in sago groups. Participants in the sago group were significantly older than those in the rice group (52.91 vs. 43.28-year, $P < 0.05$). Most of the participants in both groups were of Kamoro ethnicity (Table 1). The educational background of both groups was predominantly 6—9 years of schooling. However, > 9 years of schooling was the second most common (24%) educational background in the rice group. Farming with a home-vegetable garden was the most common occupation in both groups; however, temporary employment was the second most common occupation in the rice group. Most of the participants in both groups had incomes < 1.000.000 IDR per month; however, in the rice group, 8% had an income > 2.000.000 IDR per month.

Table 1. Socio-demographic characteristics of the study population.

	Rice (n = 25)	**Sago (n = 25)**
Age (year)	43.28 ± 13.72	52.91 (25.00, 64.00) *
Gender (%)		
Men	48	44
Women	52	56
Ethnic (%)		
Kamoro	92	100
Others	8	0
Marital status (%)		
Married	72	88
Divorced	20	12
Not married	8	0

Variables are presented as means ± SDs, medians (minimum, maximum), and percentages. Abbreviations: *SD* standard deviation; *%* percentage; *IDR* Indonesia Dollar Rupiah; *P* probability. *Significantly different ($P < 0.05$) by Mann-Whitney U test with the rice group.

The intake of most of the nutrients between the rice and sago groups was significantly different. Protein intake in the sago group was significantly lower than in the rice group (19.9 g/d vs. 36.7 g/d, $P < 0.001$). However, the carbohydrate and fiber intake were higher in the sago group than in the rice group (245.5g/d vs. 171.7 g/d, $P < 0.001$; 5.0 g/d vs 3.3 g/d, $P = 0.001$, respectively). The micronutrient intake profile of the rice group was significantly higher than that of the sago group (Table 2).

Table 2. Nutrient intake of the study population

	Rice (n = 25)	**Sago (n = 25)**	***P* value**
Energy intake (kcal)	1029 ± 373	1233 ± 381	0.062
Protein intake (g)	36.7± 16.0	19.9 ± 8.0	< 0.001[a]
Fat intake (g)	20.0 (4.6; 67.7)	16.2 ± 10.0	0.295
Carbohydrate intake (g)	171.7 (64.9; 319.8)	245.5 ± 79.5	< 0.001[b]
Fiber intake (g)	3.3 ± 1.2	5.0 ± 1.9	0.001[a]
Vitamin A intake (µg)	262 (48; 854)	162 ± 108	0.008[b]
Vitamin B1 intake (mg)	0.33 (0.17; 0.60)	0.23 ± 0.14	0.006[b]
Vitamin B2 intake (mg)	0.30 (0.13; 0.87)	0.13 (0.03; 0.43)	< 0.001[b]
Vitamin B6 intake (mg)	0.7 ± 0.2	0.3 (0.1; 0.9)	< 0.001[b]
Vitamin C intake (mg)	20 ± 12	13 ± 10	0.031[a]
Sodium intake (mg)	74 (18; 618)	78 (35; 629)	0.600
Potassium intake (mg)	791.8 ± 313.3	555.6 ± 253.5	0.005[a]
Calcium intake (mg)	122 (42; 440)	69 (26.; 262)	0.008[b]
Magnesium intake (mg)	122 (56; 205)	75 ± 29	< 0.001[b]
Phosphorus intake (mg)	515 ± 210	272 (149.; 749)	0.002[b]
Iron intake (mg)	3.2 (1.6; 9.5)	3.2 ± 1.3	0.393
Zinc intake (mg)	3.27 (1.13; 11.53)	1.43 (0.57; 4.17)	< 0.001[b]

Variables are presented as means ± SDs and medians (minimum, maximum). Abbreviations: SD standard deviation; kcal kilocalories; g gram; µg microgram; mg milligram; P probability; [a] Significant difference between the rice and sago groups with the independent t-test; [b] Significant difference between the rice and sago groups with Mann-Whitney U test.

Our food groups showed that the rice group gained the largest energy from the rice (607 kcal/day), while the sago group did it from sago (810 kcal/day). Rice (11.0 g/day) and fish (13.1 g/day) were the sources of protein in the rice-eating

group, while in the sago group, fish (12.2 g/day) was the major source of the protein (Table 3 and 4).

Table 3. Food groups consumed per day of the rice group

	The amount (g/day)	**Energy (kcal)**	**Protein (g)**	**Fat (g)**	**Carbohydrate (g)**
Rice	467.0 (167.0; 533.0)	607 (217; 813)	11.0 (4.0; 14.0)	1.0 (0.0; 18.0)	129.0 (48.0; 153.0)
Sago	0.0 (0.0; 121.0)	0 (0; 461)	0.0 (0.0; 0.0)	0.0 (0.0; 3.0)	0.0 (0.0; 110)
Sweet potato	0.0 (0.0; 67.0)	0 (0; 75)	0.0 (0.0; 2.0)	0.0 (0.0; 0.0)	0.0 (0.0; 18.0)
Noodle	0.0 (0.0; 33.0)	0 (0; 94)	0.0 (0.0; 3.0)	0.0 (0.0; 0.0)	0.0 (0.0; 19.0)
Fish	76.2 ± 45.5	80 (10; 288)	13.1 ± 8.0	5.0 (0.0; 21.0)	0.0 (0.0; 0.0)
Meat	17.0 (0.0; 80.0)	47 (0; 221)	4.0 (0.0; 18.0)	3.0 (0.0; 16.0)	0.0 (0.0; 2.0)
Egg	0.0 (0.0; 67.0)	0 (0; 125)	0.0 (0.0; 8.0)	0.0 (0.0; 10.0)	0.0 (0.0; 1.0)
Tempeh/tofu	0.0 (0.0; 67.0)	0 (0; 186)	0.0 (0.0; 10.0)	0.0 (0.0; 14.0)	0.0 (0.0; 8.0)
Green leafy vegetables	71.6 ± 40.7	23 (2; 83)	1.0 (0.0; 3.0)	2.0 (0.0; 7.0)	2.0 (0.0; 5.0)
Other vegetables	0.0 (0.0; 42.0)	0 (0; 14)	0.0 (0.0; 1.0)	0.0 (0.0; 1.0)	0.0 (0.0; 3.0)
Sugar	13.0 (0.0; 47.0)	48 (0; 181)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	13.0 (0.0; 47.0)
Tea/coffee	1.0 (0.0; 16.0)	1 (0; 21)	0.0 (0.0; 1.0)	0.0 (0.0; 0.0)	0.0 (0.0. 4.0)
Snack (cake, bread, fried banana, doughnut)	17.0 (0.0; 117.0)	45 (0; 384)	0.0 (0.0; 8.0)	1.0 (0.0; 16.0)	5.0 (0.0; 54.0)
Fruit	0.0 (0.0; 67.0)	0 (0; 54)	0.0 (0.0; 1.0)	0.0 (0.0; 0.0)	0.0 (0.0; 14.0)
Milk	0.0 (0.0; 35.0)	0 (0; 159)	0.0 (0.0; 6.0)	0.0 (0.0; 5.0)	0.0 (0.0; 21.0)

Variables are presented as means ± SDs and medians (minimum, maximum). Abbreviations: SD standard deviation; g gram, kcal kilocalories.

Table 4. Food groups consumed per day of the sago group

	The amount (g/day)	**Energy (kcal)**	**Protein (g)**	**Fat (g)**	**Carbohydrate (g)**
Rice	67.0 (0.0; 233.0)	87 (0; 303)	2.0 (0.0; 6.0)	0.0 (0.0; 6.0)	19.0 (0.0; 67.0)
Sago	219.4 ± 78.6	810 ± 304	1.0 (0.0; 2.0)	0.0 (0.0; 17.0)	189.0 ± 73.6
Sweet potato	0.0 (0.0; 110)	0 (0; 233)	0.0 (0.0; 1.0)	0.0 (0.0; 5.0)	0.0 (0.0; 46.0)
Noodle	0.0 (0.0; 60.0)	0 (0; 85)	0.0 (0.0; 3.0)	0.0 (0.0; 0.0)	0.0 (0.0; 17.0)
Fish	69.4 ± 34.6	95 ± 58	12.2 ± 5.9	2.0 (0.0; 18.0)	0.0 (0.0; 0.0)
Meat	0.0 (0.0; 33.0)	0 (0; 111)	0.0 (0.0; 9.0)	0.0 (0.0; 8.0)	0.0 (0.0; 1.0)
Egg	0.0 (0.0; 17.0)	0 (0; 32)	0.0 (0.0; 2.0)	0.0 (0.0; 3.0)	0.0 (0.0; 0.0)
Tempeh/tofu	0.0 (0.0; 40.0)	0 (0; 82)	0.0 (0.0; 3.0)	0.0 (0.0; 8.0)	0.0 (0.0; 1.0)
Green leafy vegetables	48.6 ± 43.0	10 (0; 64)	1.0 (0.0; 2.0)	0.0 (0.0; 5.0)	1.0 (0.0; 4.0)
Other vegetables	0.0 (0.0; 42.0)	0 (0; 14)	0.0 (0.0; 1.0)	0.0 (0.0; 0.0)	0.0 (0.0; 3.0)
Sugar	13.0 (0.0; 57.0)	52 (0; 219)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	13.0 (0.0; 57.0)
Tea/coffee	0.0 (0.0; 8.0)	0 (0; 9)	0.0 (0.0; 1.0)	0.0 (0.0; 0.0)	0.0 (0.0; 2.0)
Snack (cake, bread, fried banana, doughnut)	8.0 (0.0; 117.0)	13 (0; 372)	0.0 (0.0; 8.0)	1.0 (0.0; 17.0)	1.0 (0.0; 52.0)
Fruit	0.0 (0.0; 67.0)	0 (0; 61)	0.0 (0.0; 1.0)	0.0 (0.0; 0.0)	0.0 (0.0; 16.0)
Milk	0.0 (0.0; 20.0)	0 (0; 93)	0.0 (0.0; 4.0)	0.0 (0.0; 4.0)	0.0 (0.0; 10.0)

Variables are presented as means ± SDs and medians (minimum, maximum). Abbreviations: SD standard deviation; g gram, kcal kilocalories.

Anthropometric and Laboratory of the Study Participants

Body composition (muscle mass, fat mass, visceral fat, and basal metabolic rate) showed no significant differences between the rice and sago groups. The hand-grip strength did not differ between the groups (Table 5). Muscle strength of both groups showed within in normal range. In the rice group, men had a median

of 37.3 kg and women had a mean of 22.3 kg with the reference value in men 35.5—55.3 kg and women 18.9—32.7 kg for 40—44-year-old. While in the sago group men had a median of 36.7 kg and women had a median of 22.5 kg with the reference value in men 34.7—54.5 kg for 45—49-year-old and women 17.7—31.5 kg for 55—59-year-old.

Table 5. Body composition of the study population.

	Rice (n = 25)	**Sago (n = 25)**	***P* value**
Height (cm)	158.5 ± 6.8	160.5 ± 4.1	0.208
Body weight (kg)	55.3 ± 6.8	56.9 ± 6.0	0.375
BMI (kg/m2)	22.0 ± 1.8	22.1 ± 1.9	0.852
Muscle mass (kg)	37.4 (29.6; 54.4)	36.4 (17.8; 52.1)	0.823
Muscle mass (%)	71.4 ± 7.0	68.2 (30.9; 86.5)	0.327
Muscle strength (kg)	25.7 (15.8; 78.0)	27.7 (16.7; 41.0)	0.786
Fat mass (%)	25.0 ± 7.3	25.7 ± 7.3	0.756
Basal metabolic rate (kcal)	1196 ± 194	1201 (986; 1518)	0.938
Bone mass (kg)	2.2 ± 0.4	2.2 (1.9, 2.9)	0.632
Visceral fat (rating)	6.0 (1.5; 16.0)	7.2 ± 3.4	0.397
Total body water (%)	52.3 ± 4.5	51.2 ± 4.5	0.395

Variables presented as means ± SDs and medians (minimum, maximum). Abbreviations: SD standard deviation; cm centimeter; kg kilogram; % percentage; kcal kilocalories; P probability.

Laboratory profiles showed a significant difference between the rice and sago groups in the hemoglobin and hematocrit levels of the males (13.7 g/dL vs 12.6 g/dL, $P = 0.044$; 43% vs 41% $P = 0.027$, respectively). Men in the rice group had normal hemoglobin levels, while those in the sago group had hemoglobin levels lower than the reference values. Meanwhile, the hematocrit levels of the men in both groups were within the reference values. The hemoglobin levels in the

women in both groups were lower than the reference values but did not differ between the groups. A lower MCV value than the reference value was found for both rice and sago groups. Iron intake did not differ between groups of men and women but was lower than the reference value. Serum albumin levels did not differ between the rice and sago groups and were within the reference values (Table 6).

Table 6. Laboratory profile of the study population.

	Rice (n = 25)	**Sago (n = 25)**	***P* value**	**Normal Value**
Hemoglobin (g/dL)	13.7 (12.3; 19.6)	12.6 ± 1.4	0.045*	13.2—17.3 (man)
	10.4 ± 1.0	10.9 ± 1.3	0.265	11.7—5.5 (woman)
Hematocrit (%)	43 (38; 60)	41 (11; 43)	0.027*	40—52 (man)
	32 (12; 38)	36 (15; 41)	0.058	35—47 (woman)
Red blood cell (10^6/µL)	5.6 ± 0.9	5.3 (1.4; 6.0)	0.452	4.4—5.9 (man)
	4.7 (1.2, 5.4)	4.7 (1.8; 5.8)	0.458	3.8—5.2 (woman)
MCV (fl)	75 (57; 104)	75 ± 5	0.861	80—100
MCH (pg)	25 (17; 86)	24 (19; 79)	0.861	26—34
MCHC (g/dL)	32 (29; 88)	32 (29; 94)	0.786	32—36
RDW-CV (%)	15.8 ± 2.5	15.1 (12.9; 25.0)	0.977	11.5—14.5
Thrombocyte (10^3/µL)	269 ± 95	233 ± 95	0.188	150—400
White blood cell (10^3/µL)	7.8 ± 2.5	7.0± 1.4	0.358	3.8—10.6 (man)
	8.9 ± 2.4	6.8 (5.4, 18.8)	0.055	3.6—11 (woman)
Albumin (g/dL)	4.1 ± 0.3	4.0 ± 0.4	0.455	3.4—4.8

Variables are presented as means ± SDs and medians (minimum, maximum). Abbreviations: SD standard deviation; g gram; dL deciliter; µL microliter; fl femtoliter; pg picogram; % percentages;

MCV mean corpuscular volume; MCH mean corpuscular hemoglobin; MCHC mean corpuscular hemoglobin count; RDW-CV red blood cell distribution width; p probability. * Significantly different between the rice and sago groups with Mann- WhitneyU test.

Multivariate Analysis Results

A multivariate analysis of albumin for both groups was conducted to analyze the correlation of body composition and laboratory profile with albumin. Multivariate linear regression analysis showed that a better prediction of albumin in the rice group was featured by hemoglobin (β = 0.354, *P* = 0.089), and the white blood cell counts (WBC) (β = 0.396, *P* = 0.059). This model explained 12.8% of the albumin variability (Table 7). We accepted this model even though *P*-value > 0.05 (the software was set to maintain variable at least *P*-value = 0.1) because hemoglobin and albumin were found to be correlated in anemic patients. While albumin and white blood cell were reported to be correlated in diabetic patients.

Table 7. Multivariate analysis of factors related to albumin of the rice group.

	Model	**B**	**S.E.**	**β**	**T**	***P* value**
1	Constant	3.206	0.391		8.188	< 0.001
	Hemoglobin (g/dL)	0.042	0.023	0.354	1.780	0.089
	White blood cell (10^3/μL)	0.046	0.023	0.396	1.989	0.059

Analysis with multivariate linear regression backward method found that Albumin = 3.206 + 0.042*hemoglobin + 0.046*WBC (R^2=12.8%) in the rice group. All linear regression assumptions (linearity, normality, zero residue, no outlier residue, independent, constant, and homoscedasticity) were fulfilled. Abbreviations: WBC white blood cell; kcal kilocalories; kg kilogram; g gram; dL deciliter; μL microliter; d day; B unstandardized beta; SE standard error; β standardized beta; t the t-test statistic; P probability.

On the one hand, in the sago group, the better model for the multivariate association in determining albumin was characterized by the MCV (β= -0.524, P

= 0.007). This model explained 24.3% of albumin variability in the sago group (Table 8).

Table 8. Multivariate analysis of factors related to albumin of the sago group.

	Model	B	S.E.	β	t	*P* value
1	Constant	7.192	0.945		7.612	< 0.001
	MCV (fl)	-0.034	0.012	-0.493	-2.832	0.010
	Muscle mass (%)	-0.009	0.006	-0.263	-1.509	1.46
2	Constant	6.733	0.919		7.326	0.000
	MCV (fl)	-0.036	0.012	-0.524	-2.950	0.007

Multivariate linear regression backward analysis revealed albumin = 6.733 + (-0.036) *MCV (R^2=24.3%) in the sago group. All linear regression assumptions (linearity, normality, zero residue, no outlier residue, independent, constant and homoscedasticity) were fulfilled. Abbreviations: MCV mean corpuscular volume; fl femtoliter; g gram; kg kilogram; d day; % percentage; B unstandardized beta; SE. standard error; β standardized beta; t the t-test statistic; P probability.

In the sago group. there were significant correlations with the MCV and visceral fat (r = 0.415, *P* = 0.039), the visceral fat and basal metabolic rates (r = 507, *P* = 0.010).

DISCUSSION

The present study showed that there was no differences in the body composition and serum albumin levels between the sago-moderately low protein and rice-sufficient protein groups. The hemoglobin and hematocrit levels in the males were the only items that were significantly different between the two groups. This study also analyzed the serum albumin level as a marker for protein-energy malnutrition caused by insufficient protein intake. The multivariate analysis revealed that the predictive factors of serum albumin levels were different between the two groups. Hemoglobin and WBC counts were the determinants of the serum albumin level in the rice-sufficient protein group. On the other hand, the MCV was a predictor of the serum albumin levels in the sago-moderately low protein group.

The sago-eating participants were older than the rice-eating participants. A similar finding was reported in a study in Riau Province, Indonesia, where the people who consumed more sago were older (> 50 years old) compared to those who consumed less sago [23]. Sago is considered to be the major food of ancient times in the sago-producing areas of Indonesia. Recently sago consumption has been reduced and replaced by the consumption of rice. The socioeconomic status of the rice consumers in our study showed that they had higher educational backgrounds, occupations, and incomes than the sago consumers. A study in Maluku, Indonesia also showed that better household incomes and education will reduce sago consumption and production, shifting to rice consumption. Rural

people also perceived sago as inferior food, while rice was perceived as superior food [14,16].

Most of the nutrient intakes of sago-eating participants such as protein and micronutrient intake were lower than that those of rice-eating participants in our study. Energy intakes, carbohydrate intakes, and fiber intakes were higher in the sago group than in the rice group. Syartiwidya et al had similar findings that participants consumed more sago (> 140 g/day) compared to participants who consumed less sago (< 140 g/day) and had higher percentages of severe deficits in the adequacy of protein levels (43% vs. 38.3%) [23]. Based on the Indonesian Nutrient Composition Food, while sago per 100 g edible portion had a higher carbohydrate content than white rice cooked (85.6 g vs. 26.0 g) it had a lower protein content (0.6 g vs. 2.4 g) [24].

This is the first study on the dietary intake of lowlanders in Papua, Indonesia with energy and protein intake were 1029 kcal and 36.7 g for the rice group and 1233 kcal and 19.9 g for the sago group. A study by Okuda, T et al in 1981 on Papua New Guinea highlanders showed that the mean daily energy intake was 2390 kcal, and the daily protein intake was 35.2 g. This profile was exceptionally high because there was a yearly festival season in the village when the data were collected. In comparison with them, our result on lowlanders in Papua, Indonesia had lower energy intake. The lowlanders in Papua, Indonesia were having daily foods with small varieties and in contrast, the highlanders in Papua New Guinea were having special food for the yearly festival. The yearly festival season on

highlanders and the wages from coffee plantations can affect these differences [25].

The dietary intake of the two groups showed that the main differences were in the source of carbohydrate and protein intake. Resistant starch type 3 (RS 3) derived from sago contained higher RS (31—38%) than those derived from rice starch (21—26%). This implicated on the production of SCFA mainly butyrate as the preferred energy source for colonocytes [26].

Stunting was a nutritional problem in Indonesia. According to Indonesian National Health and Research Survey in 2018, national data showed there was a decreasing prevalence of severe stunting from 18.0% in 2013 to 11.5% in 2018 but slightly increased of stunting from 19.2% in 2013 to 19.3% in 2018 [27]. Mimika district had better stature compared to other districts in Papua province (Mimika profile is 5.68% of severe stunting, 14.29% of stunting, and 80.03% of normal) [28]. A study by Fikawati, S. et al in 2021 among preschool children in Central Jakarta, Indonesia found that children who lacked energy and protein intakes were at a higher risk of stunting than children who had sufficient intakes. Macronutrient intakes are important and should be consumed in sufficient quantities every day to prevent stunting [29]. Inadequate energy and protein intake in our population study can be the risk of having stunting on their children. Height among our participants showed that their height was comparable to the highlanders of Papua New Guinea. Highlanders of Papua New Guinea had a height of 158.3 cm, and it was similar to our height lowlanders of Papua Indonesia 158.5 cm for the rice group and 160.5 cm for the sago group [25]. The sago-eating

people can get more resistant starch 3 compared to rice-eating people. This implicated to have a higher production of SCFA mainly butyrate, the energy source for colonocytes in the sago-eating people than rice-eating people [26]. Meanwhile, the inadequate protein should be corrected to the level adequate to prevent stunting in children in their family members.

The amount of protein intake in the sago and rice groups was 0.35 g/kg/d and 0.66 g/kg/d, respectively. The minimum intakes for nitrogen equilibrium were the main factors of the lower limits of successful adaptation at which an appropriate body composition can be maintained. The mean adult requirement that is recommended by WHO, 2007 is 0.66 g/kg indicated by the nitrogen balance studies implied an overall efficiency of utilization of dietary proteins of approximately 50% in replacing the obligatory nitrogen loss. The risk of protein deficiency started to increase when the intake decreased to below the range of the true minimum requirements (that is, a value that is currently unknown but likely to be between 0.40 and 0.50 g/kg/d) [30]. A series of studies of highlanders in Papua New Guinea, who depended largely on sweet potatoes and vegetables with low protein intake (4% of total calories) showed negative nitrogen balances with little malnutrition within the population. It was hypothesized that there was a fixation of nitrogen by the intestinal microflora [31]. A study by Huang et al suggested that there was no intestinal nitrogen fixation to occur in sweet potato eaters in slightly below requirement levels of protein intake [32]. Intake of protein also found significant differences at different locations on the highlanders of Papua New Guinea using a delta nitrogen stable isotope (Δ15N), the discriminant

factor between diets and scalp hair. The estimated Δ15N values were correlated negatively with several indicators of animal protein intake [33] . A study developing and validating a semi-quantitative food frequency questionnaire (FFQ) for assessing protein intake found that the highlanders of Papua New Guinea did not meet the biologically required protein intake. Although closer to an urban center, it showed higher protein intake than the more remote communities [34].

Serum albumin levels in the sago group did not differ from those in the rice group and fell within the normal range. Multivariate linear regression analysis showed different predictive factors for albumin levels in these groups. Hemoglobin and white blood cells were factors related to albumin in the rice group, while MCV was a predictor of albumin in the sago group. Based on our working hypotheses, basal metabolic rate influences serum albumin levels in the sago group indirectly. Protein intake in the sago group can be classified as a moderately low protein intake. A moderately low protein intake does not change the basal metabolic rate or energy expenditure. Serotonergic and β-adrenergic systems are believed to be involved in the mechanism of the changes in energy balance. These are only two of the neurotransmitter systems involved in regulating food intake. Moderately low protein-high carbohydrate increases adiposity and fat in the liver. In an animal study, the lowest protein consumption increased the proportion of energy deposited as carcass fat. The higher the fat in the liver, the lower is the iron in the liver tissue. In this study, lower iron levels were marked by a lower level of MCV. This condition induces an adaptation mechanism of protein metabolism to maintain body protein balance (albumin

within normal range). We assumed that there was decreased protein turnover (protein synthesis, amino acid oxidation, protein degradation) with maintained post-absorptive whole-body protein and basal muscle protein synthesis as the mechanism of long-term low proteins intakes in our participants (Figure 1).

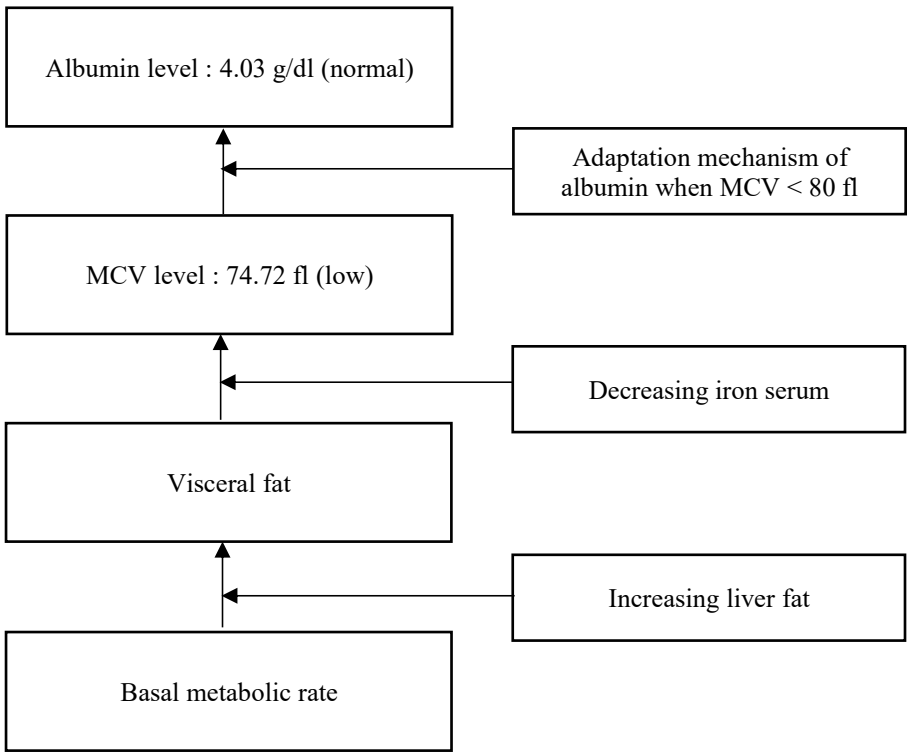


Figure 1. Flow chart of the working hypotheses on how basal metabolic rate influences albumin in the sago group. Abbreviation: MCV mean corpuscular volume, g gram, dL deciliter, fl femtoliter.

The serum albumin levels in the moderately low protein sago diet showed a negative prediction according to the MCVs. Similar findings were obtained in a study by Suk-Hwan Yang on the relationship between liver function tests and MCVs in 157 persons (patients with liver disease and a healthy control group) indicating that albumin was related significantly and negatively with MCVs

(stepwise multiple regression analysis). The MCV levels were higher, but albumin levels were lower in patients with liver disease than in the controls [35]. The adaptation mechanism of albumin in the present study was triggered by a decrease in the MCV levels (iron serum). An *in vitro* study by Higashida et al found that iron deficiency decreases iron-containing protein and reduces protein synthesis in basal and branched-chained amino acid (BCAA)- and insulin-stimulated conditions in muscle cells [36]. Animal and human studies have shown that there is a reduction in the synthetic and catabolic rates of albumin in protein-deficient states. There was an increasing transfer of albumin from the extravascular pool to the intravascular pool to maintain the serum albumin within normal levels [37,38]. Animal studies have also shown a decrease in albumin synthesis (as a percentage of total liver protein synthesis) from 15% to 8% on a protein-free diet (2-9 days) in rats [39]. Reducing the protein intake of humans from the required intake (0.6 g/kg/d) to inadequate levels (0.1 g/kg/d) will decrease leucine flux, body protein synthesis, and protein breakdown with a smaller reduction in leucine oxidation [40].

The rate of albumin synthesis in healthy participants consuming a diet with very low protein (e.g., 10 g/day) in an isocaloric diet, showed a decrease in albumin synthesis by 20%—65%. However, when both protein and calories are restricted (i.e., starvation), albumin synthesis remained close to normal. The catabolism of body proteins to provide energy releases sufficient amino acids to maintain normal albumin synthesis [41]. Albumin synthesis was also modulated

by the proportion of animal and vegetable protein in the diet. Studies on isoenergetic and isonitrogenous diets in healthy men showed that the albumin synthesis rate in the group consuming 63% of vegetable protein was reduced in comparison with the group consuming 74% of animal protein [42]. Albumin is the most abundant antioxidant in whole blood, and oxidative stress has now emerged as a major pathway with pathological relevance for many cardiovascular diseases, such as atherosclerosis, coronary artery diseases, heart failure, atrial fibrillation, strokes, and venous thromboembolism [43]. A study on the very old, centenarian, and supercentenarian in Japan showed that plasma albumin levels were almost associated with all-cause mortality in these populations. Plasma albumin levels were correlated significantly with cholinesterase levels, inflammation, and N terminal- prohormone of brain natriuretic peptide (NT-pro BNP) levels (biomarkers of endogenous cardioprotective molecules) [44].

Muscle mass in the moderately low protein sago diet was not significantly different from that in the sufficient protein rice diet. Healthy humans can maintain lean body mass and body protein balance when protein intake is restricted. Integrated and adaptive metabolic changes in the body occur by decreasing amino acid oxidation and protein degradation with more efficient use of amino acids derived from protein degradation. Maintaining skeletal muscle protein synthesis is an important component of the “adaptation” to low protein intake [45] . A randomized parallel study by Hursel et al, with 15 participants either high (2.4 g/kg/d) or low protein intake (0.4 g/kg/d) showed that low protein intake induces prolonged adaptation of body mass and fat-free mass by lowering body protein

turnover rates (protein synthesis, protein breakdown, and protein oxidation), but maintains post-absorptive whole body net protein balance and maintains basal muscle protein synthesis in 12 weeks [46]. Mosoni et al showed that short-term food deprivation induced the inhibition of muscle protein synthesis and liver protein synthesis after 112—114 hours of food deprivation and 5—7 hours of re-feeding in rats. After re-feeding, liver synthesis was more stimulated than muscle protein synthesis. A coordinated response of liver and muscle protein metabolism allowed sparing of muscle proteins during food deprivation at the expense of liver proteins [47].

Visceral fat and MCV were correlated positively in the moderately low protein sago group. Different findings from an animal study by Visscher et al (2017) showed that the level of fatty acids in the liver had a strong negative correlation with the iron content. The livers of turkeys that died from hepatic lipidosis were analyzed for their fat and iron levels. The higher fat content in the liver, the lower the iron content in the liver tissue [48]. A study by Siddique et al, on nonalcoholic fatty liver disease patients found that iron deficiency was prevalent, and this was associated with the female sex, increased body mass indexes, and non-white race. Interestingly, serum hepcidin levels were low in iron-deficient participants, indicating that serum hepcidin was not a primary cause of iron deficiency, rather it was a physiological response to decreasing levels of iron [49]. Hepatic iron can cause liver injury. Adipose tissue iron can be linked to adipose tissue function, including the dysregulation of adipokines, enhanced adipose tissue lipolysis and adipose tissue inflammation. These might be the

possible mechanisms linking adipose tissue iron to liver injury [50]. Unfortunately, serum iron and other iron profiles were not assessed in our study, but low MCV was related to iron deficiency. Low MCV was found in both groups, and iron intake was below the requirement. We assumed that the iron levels were related to the visceral fat in the sago-moderately low protein group but not in the rice-sufficient protein group.

In our study, sago-eating participants with a moderately low protein diet showed more visceral fat (liver fat) than rice-eating participants. A study by Du et al, on food intake, energy balance, and serum leptin concentrations in rats fed low-protein diets found several low levels of dietary proteins from total calories (2%, 5%, 8%, 10%, 15% and 20% casein) influenced their energy intake and body fat. The lowest protein consumption increased the proportion of energy deposited as carcass fat. The body fat content showed a positive correlation with serum leptin concentrations [51]. Another animal study by Pezeshki et al showed that diets with 10% moderately low protein calories in obesity-prone rats had an increased liver fat % (hepatic lipidosis). While this moderately low protein diet caused hyperphagia (increasing energy intake) without altering energy expenditure, body fat, and lean mass, it promoted hepatic lipidosis [52].

The sago group with moderately low protein intake showed a similar basal metabolic rate compared to the rice group. A study by Pezeskhi et al, on the effects of diets varying in protein concentrations on energy balance in obesity-prone rats found that protein-free (0% protein calories) diets decreased energy intake and increased energy expenditure, very low protein (5% protein)

diets increased energy intake and expenditure, whereas moderately low protein (10% protein) diets increased energy intake without altering expenditure, relative to the control diet (15% protein). Serotonergic and β-adrenergic signaling coupled with the upregulation of key thermogenic markers in brown fat and skeletal muscle was thought to be the mechanism of the change in energy balance [52]. Different findings by Miyatani et al on the basal metabolism of various types of protein diets with the rice diet and sweet potato diet of the Papua New Guinea highlanders found that there were no significant differences on the BMRs among the sweet potato diet with low protein (0.3 g/kg/d), the sweet potato diet with protein (0.5 g/kg/d), rice diet with low protein (0.6 g/kg/d), and rice diet with protein (1.4 g/kg/d), however, the respiratory quotients were different [53].

Our study found that body composition and serum albumin levels were not significantly different between the groups of lowlanders of Mimika, Papua, Indonesia. Multivariate linear regression showed that there were different predictors of albumin between the sago-eating group and the rice-eating group. An adaptation mechanism for the sago-moderately low protein intake may have maintained the albumin levels within the normal range.

This study was a part of the main study on analysis of gut microbiota of rice-eating people with sufficient protein intake and sago-eating people with low protein intake of the lowlanders in Mimika Regency, Papua, Indonesia. We are interested in their habitual low protein intake of sago-eating people, but they appeared to be healthy. Therefore, the measurement of protein intake was carried out carefully. Besides using plastic fish models with the portion size, we also

looked at the fish that they consumed in the fish seller and compare them with our plastic models. On the other hand, on the measurement of carbohydrates, we used plastic rice models with the standard portion size such as “100 grams of rice on a plate”. This standard portion sizes of plastic models may not capture their real carbohydrate intake precisely. They might have eaten more than they reported. Underreporting energy intakes were prevalent in economically deprived populations, and this is one of our limitations in this study. Therefore, we think that underreporting of energy intake is from underreporting of carbohydrate intake. A review article by Maurer, J. et al, 2006, on the psychosocial and behavioral characteristics related to energy misreporting explained that lower carbohydrate intake was one of the commonest dietary patterns of underreporting [54] . Underreporting of energy intakes in our study also can be influenced by their education level and their social-economic status. A study by Olendzki, B.C., et. al in 2008 found that energy intake underreporting was prevalent in the low-income, low-literacy of the Carribean Latino population [55] . Others factors associated with misreporting energy intake were demographics and diet. Women, older adults, and people with less education tend to underreport energy intake [54]. A study by Sawaya, A.L. et. al in 1996 on comparing four different methods of the dietary survey such as a 7 day-weighing method, a 24-hour food recall (in duplicate), and two types of food intake frequency survey among young and older women with doubly labeled water (DLW) found that the total energy intake (TEI) by the 24-hour food recall method was the closest to the total energy consumption calculated by DLW for young women. The TEI/DLW was 86.7% for young

women (mean age 25.2 ± 3.5 years) and for older women (74.0 ± 4.4 years) was 75.2% [56].

Another limitation in our study was the limited number of indicators for assessing the energy-protein nutritional status such as urinary excretion of nitrogen per unit of creatinine to measure nitrogen losses. No data on menopause in relation to iron metabolism (there were eleven women with the age 55 to 64 years old and it was the age of menopause period according to the world health organization (WHO) were obtained. The other limitation of the study was the mismatch in some parameters of the two groups (the sago group was older than the rice group, some participants in the rice group had better socioeconomic status). Further research is needed to examine the interactions between the liver (fatty acid, iron, and albumin profiles) and the muscles (amino acid profile) with the regulatory hormones in participants with prolonged sago-moderately low protein intakes.

Investigating the adaptation mechanisms of protein metabolism to maintain albumin levels within the normal range may have a beneficial impact on gerontology. Malnutrition, with hypoalbuminemia as an indicator, among the aged population is an important health care problem, as this problem is prevalent across the world, especially among the elderly aged 75 years or older. Malnutrition was not confined to institutionalized or hospitalized elderly individuals but was also seen in community-dwelling genarians. The result of a 5-year and 7-year longitudinal study in Japan showed that decreasing albumin level was associated significantly with aging among community-dwelling older adults aged 65 and

over [3,4]. An epidemiological survey found that lower albumin levels, even within the normal range, were related factors of frailty measures, trace elements, and inflammation markers in the general population [57].

Chapter III

Gut microbiota related to protein-energy nutritional status on moderately low protein intake-sago diet compared to sufficient protein intake-rice diet of well-nourished lowlanders in Papua, Indonesia

Diet and other environmental factors can modulate the composition and metabolic activity of the human gut microbiota, which can affect the health of the host. Life stage, lifestyle, and diet had been known to be related to the gut microbiota of the host [58] . Diet, particularly macronutrients, has a major role in shaping the composition and activity of these complex gut microbiota populations. Each type of macronutrient (proteins, dietary fibers, fat) influences the gut microbiota specifically. Specific categories of species will be stimulated depending on the major macronutrient consumed [20].

Western-style diets are associated with gut microbial populations that are typified by a *Bacteroides* enterotype whereas traditional diets rich in plant polysaccharides are associated with a *Prevotella* enterotype [20, 58, 59] . Vegetarian and vegan diets have a high carbohydrate content, so their gut microbiota was dominated by bacteria with high carbohydrate fermenting bacteria such as *Prevotella*, *Clostridium clostridioforme,* and *Faecalibacterium prausnitzii* [20]. Omnivorous and animal-based diets conversely show a high protein and fat content and low carbohydrate content. These diets are associated with an increase of bile-tolerant bacteria such as *Bacteroides*, *Alistipes,* and *Bilophia*., butyrate-producing bacteria, specifically the *Clostridium* cluster XVIa [58].

Diverse forms of malnutrition through the life-course are associated with dysbiosis such as lower diversity of gut microbiota in low birth weight, stunting, severe acute malnutrition (6-24 months), sarcopenia, anorexia nervosa (adults), and obesity (adult) [19]. The opposite consequences of energy absorption from the

diet (obesity versus undernutrition) had similar alterations of the gut microbiota such as decreasing diversity, decreasing functionality, resistance to dietary intervention, and increasing of the inflammatory potential [60]. Observational clinical studies on the relationship between gut microbiota with obesity in human subjects showed obesity was associated with phylum-level changes in the microbiota with more abundant on the phylum Firmicutes as compared to Bacteroidetes, reduced bacterial diversity, and altered representation of bacterial genes and metabolic pathways [61]. Gut microbiota is implicated in the regulation of energy metabolism through the digestion of indigestible polysaccharides for the host which fermentation by the microbiota leads to the production of SCFA such as propionate, butyrate, and acetate. SCFA represents 10% of the daily energy supply in humans [20].

Inadequate protein intake in developing countries is prevalent because of the high intake of starchy staple food [10]. Sago is the oldest staple food of native tribes in Papua province. It was recently changed to rice consumption in the past 40 years ago. Sago has a high content of carbohydrates but is low in protein, contrary to rice. People usually consumed sago with low protein intake while the rice was consumed with sufficient protein intake. The first main study found there was it is no difference in body composition and albumin level on moderately low protein intake-sago diet and sufficient protein intake-rice diet. There was also a different predictor on albumin level between moderately low protein intake-sago diet and sufficient protein intake-rice diet of well-nourished adult of lowland, Mimika, Papua [62]. Different predictors might be mediated by different gut

microbiota of both groups. This study aimed to analyze the gut microbiota profile and gut microbiota related to the protein-energy nutritional status of two adult populations with similar hereditary and environmental conditions, one, who consumed sago with a moderately low protein intake, and the other, who consumed rice with sufficient protein intake.

MATERIALS AND METHODS

Study Design and Population

A cross-sectional study was conducted on well-nourished adults of lowlanders with sufficient protein intake-rice diet (*n* =25) and moderately low protein intake-sago diet (*n* = 24) in Mimika Regency, Papua, Indonesia in September 2019 (Figure 2). The sample size was estimated based on the equation of SD between two groups with 95% of CI and 90% of the power of the study. From a study by Greenhill, AR et al [63], the result showed that the standard deviation of the gut microbiota was 0.74 with the mean difference between the groups being 0.70. The estimation of the sample size for each group was 24—25. The protocol was approved by Komite Etik Penelitian Kesehatan (Health Research Ethical Committee), of the Faculty of Medicine, Hasanuddin University, Makassar, Indonesia (Approval Recommendation Number: 554/UN4.6.4.5.31/PP36/2019 and protocol code: UH19070416). This study was also approved by the Ethics Committee of Okayama Prefectural University (Protocol Code Number: 19-55). The inclusion criteria of the participants were men and women lived in the lowland of Mimika Regency, Papua with ages ≥ 20 years, body mass indexes between 18,5—24,9 kg/m2, those who consumed traditional carbohydrate-based food (sago) with low protein intake (< 25 g/d) or who consumed modernized carbohydrate-based food (rice) with sufficient protein intake (≥ 25 g/d). Participants who had taken antibiotics within the preceding six

months, who had taken laxatives, gastric motility medications, prebiotics, or probiotics containing foods or supplements within the preceding month, had a medical history of clinically significant diseases such as cancer, gastrointestinal disorders (irritable bowel syndrome, inflammatory bowel diseases, coeliac diseases, constipation, diarrhea, excessive bloating), autoimmune disorders, diabetes, heart diseases, renal failure or previous gastrointestinal surgery, infectious diseases, smokers or those with high alcohol consumption, were excluded from the study.

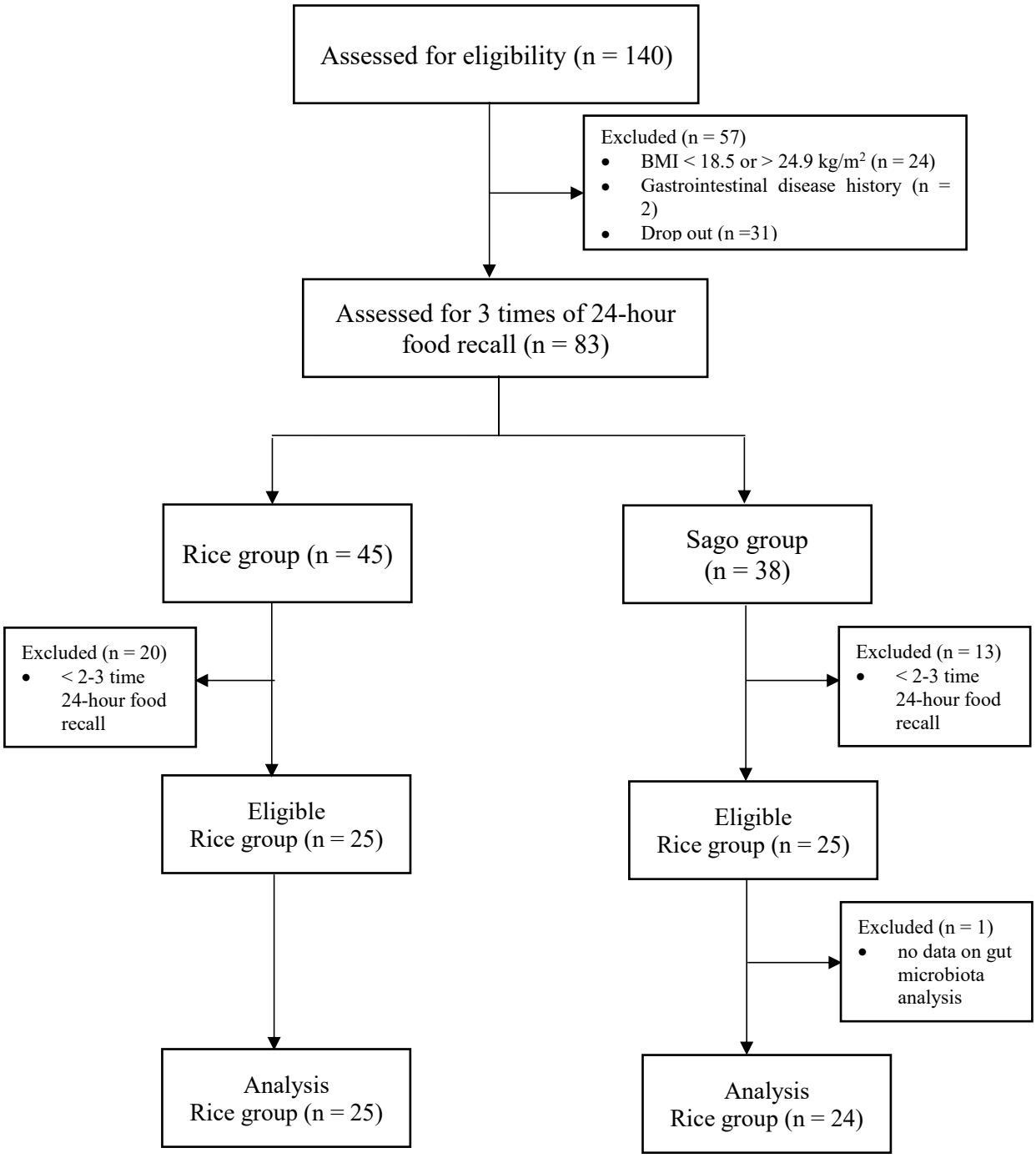


Figure 2. Flow chart of the subjects

A structured questionnaire was used for face-to-face interviews to collect sociodemographic information. The dietary intake was recorded from basically face-to-face interviews of 3-day non-consecutive days (2 days on weekdays and 1day on weekends) of a 24-hour food recall (only one participant had 2-day non-consecutive days (1 day on weekdays and 1 day on weekends)). Energy and macronutrient (including fiber) intake was calculated using the Nutrisurvey 2007 application (www.nutrisurvey.de). Participant height was measured using a mobile stadiometer (SECA 213) and took the average value of two times measurements. Anthropometric measurements with the average value of two times measurements such as body weight, muscle mass, body fat %, bone mass, total body water BMR, and visceral fat were measured using a body composition monitor (TANITA BC 730). Muscle strength was measured using a handgrip dynamometer and took the average value of two times measurements. After blood sampling, serum albumin levels were measured by a subcontractor (Prodia Laboratory, Jayapura, Papua, Indonesia).

Fecal sampling

All participants received a stool collection device with a plastic bag and were taught how to use it the day prior to the fecal collection. Fecal was collected for the first time on defecation of the day and given to the collection center within 5 minutes after defecation. The fecal was then placed in the two-stool collection tubes with a disposable spoon. One tube with a preservation solution and one

without a preservation solution and stored at -20°C. All fecal samples were stayed one night in Papua and sent to Makassar to restore at different temperatures for each tube. The tube with preservation solution was placed at -20°C until deoxyribonucleic acid (DNA) extraction and the tube without preservation solution was placed at -80°C. Microtube with DNA extraction solution and tube without preservation solution was sent to Japan to do fecal microbiota analysis and short-chain fatty acid measurement.

DNA Extraction

DNA was extracted from frozen feces with preservation solution using the fecal DNA extraction kit (ISOSPIN Fecal DNA, Nippon Gene Co., Ltd., Tokyo, Japan), with standard protocol. Frozen stool samples, the size of a grain of rice, were homogenized with 700 µl of lysis buffer FE1 and 10 µl of RNase A. The mixture was mechanically disrupted at 4,200 rpm to 6,800 rpm for 30-45 s at room temperature (20-25°C) using a tabletop cell disruptor, MagNA Lyser (Roche, Germany). The cells were then added 90 µl of lysis buffer FE2 and mixed thoroughly. The cells were then centrifuged at 12,000 × g for 15 min at room temperature (20-25°C). The supernatant (600 µl) was collected and mixed with 240 µl of buffer FB and 240 µl of isopropanol and mixed well by inversion. There was 1080 µl of supernatant, the mixture was done two times. First, the supernatant (540 µl) was centrifuged at 13,000 x g for 30 seconds and then added another (540 µl) and centrifuged at 13,000 x g for 30 seconds. Added 600 µl of buffer FB to the supernatant and centrifuged at 13,000 x g for 1 minute. Added 600 µl of buffer

FW to the supernatant (20-25℃) and centrifuged at 13,000 x g for 1 minute. After dropping 50-100 μl of TE on the membrane, let it stand at room temperature for 3 min. Then, centrifuged at 13,000 x g for 1 minute. The DNA solution was collected in a 1.5 ml microtube. DNA concentration was measured using BioDrop TM μLITE spectrophotometer (Biochrom, UK). The extracted DNA samples were stored at -20℃ until use.

16S rRNA Sequencing

For the analysis of gut microbiota in feces, metagenomic analysis using 16S rRNA sequencing was performed, as previously described [64]. The V3-V4 region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using published primers [65] from DNA samples extracted from feces. The PCR conditions were 95 °C for 3 minutes, followed by 25 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, 68 °C for 1 minute, and a final extension at 68 °C for 5 minutes. The final extension was performed at 68 °C for 5 minutes. The resulting PCR products were purified using Agencourt AMPure XP (Beckman Coulter, Inc., Brea, CA, USA), according to the manufacturer's protocol, and eluted in 50 μL of 10 mM Tris-HCL (pH 8.5). DNA libraries were then prepared using Illumina MiSeq Nextera kit set A (Illumina Inc., San Diego, CA, USA) and sequenced using Illumina MiSeq (Illumina).

Bioinformatics analysis

FASTQ files (files containing sequences and quality scores), obtained by Illumina pair-end amplicon sequencing of 16S rRNA genes, were processed according to previously reported methods [66]. Processed sequence data were analyzed using the QIIME version 1.9.1 pipeline [67]. Operational taxonomic units (OTUs) were generated using USEARCH [68], based on clusters with 97% similarity in the SILVA 128 database. OTUs were analyzed from phylum to genus using the SILVA 128 database [69]. In all samples, 10,000 randomly picked reads were used for analysis.

SCFA and BCFA measurements by HPLC

The quantification of succinic acid, lactic acid, formic acid, acetic acid, propionic acid, iso-butyric acid, n-butyric acid, iso-valeric acid, and n-valeric acid was outsourced to Techno Suruga Laboratory Co., Ltd. for analysis. Frozen samples were approximately ¼ in the volume collection tube put in a bead tube. Then, it was suspended in extract solution, and then heat-treated at 85 °C for 15 minutes. After it was crushing with the beads, then the cell was centrifuged at 18,400 x g for 10 minutes. The supernatant was filtered through a membrane filter with a pore size of 0.20 μm to prepare a sample solution. The concentrations of organic acids in the samples were determined by high-performance liquid chromatography and analyzed using Shimadzu Organic Acid Analysis System (Shimadzu, Kyoto, Japan).

Statistical Analysis

Descriptive data are expressed as the means ± SDs or median (minimum, maximum), and categorical data are expressed as percentages. Statistical significance was defined as a P-value < 0.05. Statistical analyses of the age, nutrient intake, protein-energy nutritional status, and comparisons between groups were performed using IBM SPSS Statistics software version 28.0 (SPSS Inc, Chicago, IL, USA).

The normality of the variables was assessed using Shapiro Wilk tests. Differences in categorical variables were examined by Chi-squared analysis. Differences in continuous variables with parametric and nonparametric distribution were examined using Student's t-test and the Mann-Whitney U test, respectively. The general linear model, Bonferroni was used to adjust with the age of the gut microbiome data.

The resulting data of the gut microbiome were exported as BIOM files and imported to R (version 3.5.0). Diversity analysis was performed using the "phyloseq" R-package [32]. Alpha-diversity indexes (OTU observed, Chao1 Index, Shannon Index, Simpson Index, and Fisher Index) were calculated by the *estimate_richness* function and the ß-diversity index, calculated by unweighted UniFrac distance, was generated using the unifrac function in the "phyloseq" R-package. The dominant bacterial from phylum to genus level was defined as the mean of the distribution of bacterial composition with at least 0.1%.

A linear discriminant analysis (LDA) effect size (LEfSe) using an online version of Galaxy to visualize specific microbial that were significantly

overexpressed in each group as a biomarker. The LDA score threshold was 2, and the alpha value was 0.1 for Kruskal–Wallis and pairwise Wilcoxon, respectively. We also performed Pearson correlation to analyze for selected parameters with the gut microbiota.

R E S U L T S

Socio-demographic characteristics of the participants represented that the moderately low protein intake-sago diet was older than sufficient protein intake-rice diet (53.44 years vs. 43.28 years, respectively). Men and women were equally distributed for both groups. Almost all of the participants of both the rice group and sago group were Kamoro (Table 9).

Table 9. Socio-demographic characteristics of the study population

	Rice (n = 25)	**Sago (n = 24)**
Age (year)	43.28 ± 13.72	53.44 (25.00, 64.00) *
Gender (%)		
Men	48.0	45.8
Women	52.0	54.2
Ethnic (%)		
Kamoro	92	100
Others	8	0

Variables are presented as mean ± SD, median (minimum, maximum), and as a percentage. Abbreviation: *SD* standard deviation, *%* percentage, *significantly different ($P < 0.05$) by Mann Whitney U test from rice group.

The nutrient intake profile of the study population showed that the energy intake was significantly higher in the sago group than the rice group (1257 kcal vs. 1029 kcal, $P = 0.036$, respectively). The sago group had significantly lower protein intake (20.0 g vs. 36.7 g, $P < 0.001$, respectively) but higher carbohydrate intake (250.2 g vs. 171.7 g, $P < 0.001$, respectively) and fiber intake (5.1 g vs. 3.3 g. $P = 0.001$, respectively) than in the rice group. The amount of energy intake was significantly higher in the sago than in the rice group (24.18 kcal vs. 18.55 kcal, $P = 0.025$, respectively) but lower in the amount of protein intake (0.36 g vs. 0.66 g, $P < 0.001$, respectively) (Table 10).

Table 10. Nutrient intake of the study population

	Rice (n = 25)	**Sago (n = 24)**	***P* value**
Energy intake (kcal)	1029 ± 373	1257 ± 368	0.036[a]
Protein intake (g)	36.7 ± 16.0	20.2 ± 8.0	<0.001[a]
Fat intake (g)	20.0 (4.6; 67.7)	16.6 ± 10.0	0.384
Carbohydrate intake (g)	171.7 (64.9; 319.8)	250.2 ± 77.5	<0.001[b]
Fiber intake (g)	3.3 ± 1.2	5.1 ± 1.9	0.001[a]
The amount of energy intake (kcal/kg/d)	18.55 ± 5.97	24.18 (8.00, 30.67)	0. 025[b]
The amount of protein intake (g/kg/d)	0.66 ± 0.25	0.36 ± 0.14	<0.001 [a]

Variables are presented as mean ± SD, median (minimum, maximum). Abbreviation: *SD* standard deviation, *kcal* kilocalorie, *g* gram, *P* probability. [a] Significant difference from rice group with Independent T-test, [b] Significant different from the rice group with Mann-Whitney U test.

In the protein-energy nutritional status, we found that there were no significant differences in the protein-energy nutritional status of both groups such as BMI, muscle mass (kg), muscle mass (%), muscle strength (kg), and albumin level. There were all within the normal range (Table 11). `

Table 11. Protein-energy nutritional status of the study population

	Rice (n = 25)	**Sago (n = 24)**	***P* value**
BMI (kg/m2)	22.0 ± 1.8	22.0 ± 1.9	0.931
Muscle mass (kg)	37.4 (29.6; 54.4)	36.2 (17.8; 52.1)	0.826
Muscle mass (%)	71.4 ± 7.0	68.6 (30.9; 86.5)	0.390
Muscle strength (kg)	25.7 (15.8; 77.8)	28.7 (16.7; 41.0)	0.944
Albumin (g/dL)	4.1 ± 0.3	4.0 ± 0.3	0.307

Variables presented as means ± SDs, medians (minimum, maximum). Abbreviation: *SD* standard deviation, m^2 meter square, *kg* kilogram, *%* percentage, *g* gram, *dL* deciliter, *P* probability.

The gut microbiota profile showed that the enterotype (densely populated areas in a multidimensional space of community composition) of gut microbiota for both groups dominantly was *Prevotella* (72% for the rice group and 75% for the sago group) (Figure 3a-b). To look at the richness of the gut microbiota, we conducted alpha and beta diversity. The diversity of species within the same individual (alpha-diversity) showed that there was no difference in the diversity of both groups with some different indexes (OTU observed, Chao1, Shannon, Simpson, Fisher) (Figure 4a-e). Similar to the diversity of species interindividual or beta-diversity with principal coordinated analysis (PCoA) based on the Bray-Curtis dissimilarity index also showed that there is no significant difference between groups (Figure 4f).

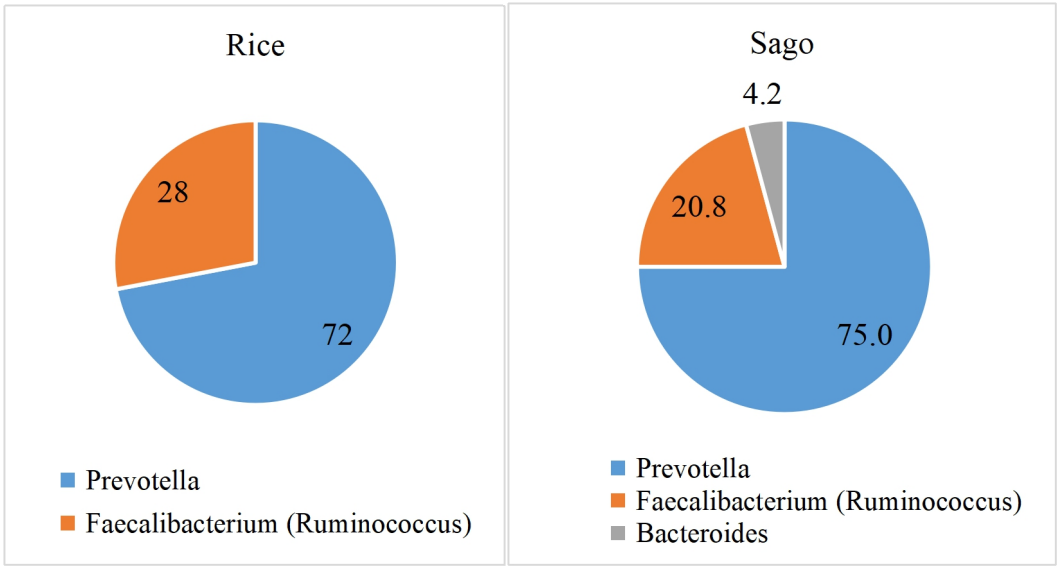
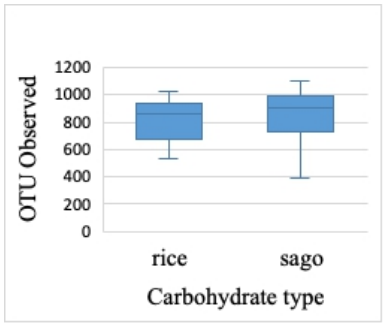
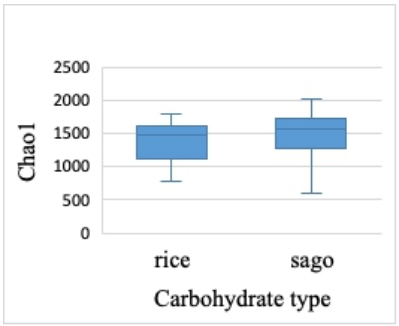


Figure 3. Enterotype of the study population (%) for (a) rice group and (b) for sago group.



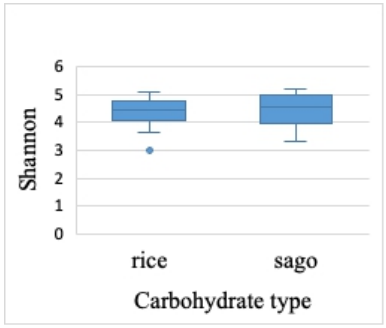


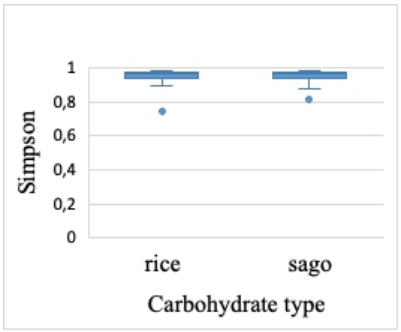
$P = 0.226$

(a)

$P = 0.162$

(b)



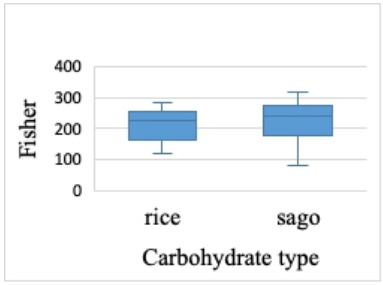


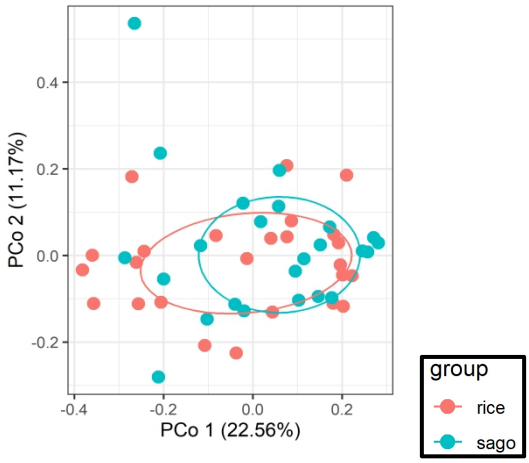
$P = 0.535$

(c)

$P = 0.749$

(d)





$P = 0.377$

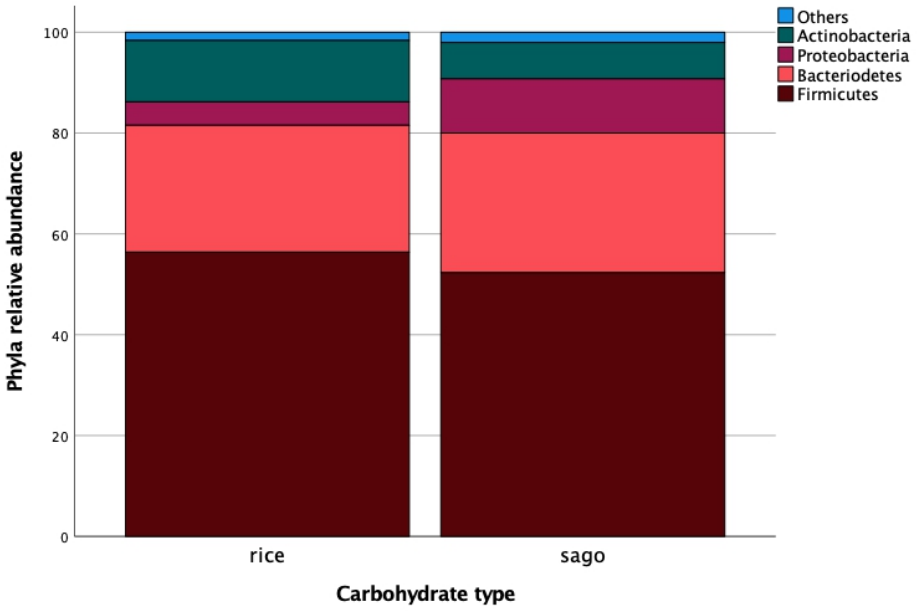
(e)

P = 0.41

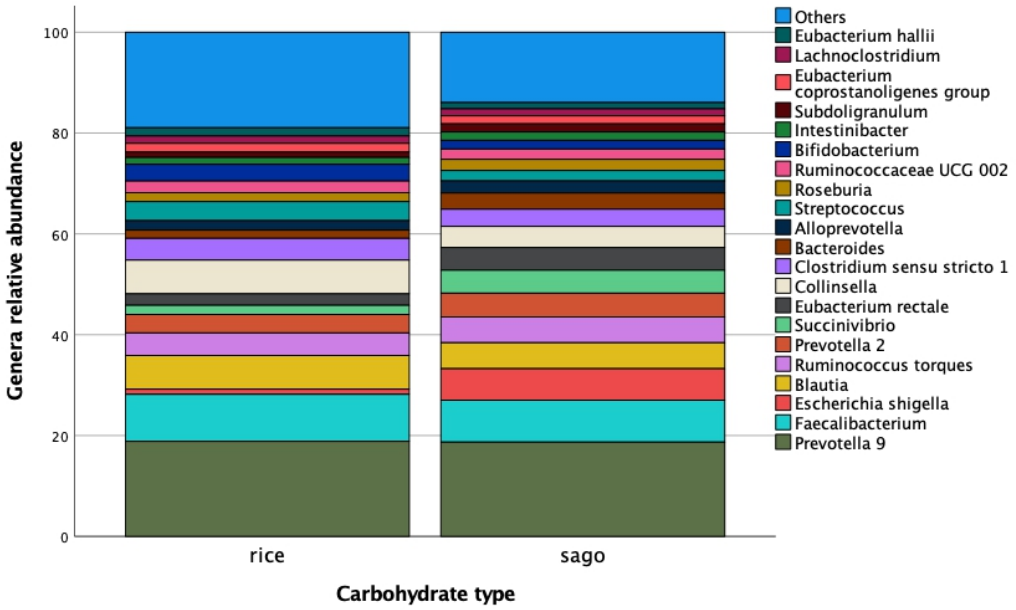
(f)

Figure 4. Differences of gut microbiota diversity ((*Alpha* diversity: a-e) and *Beta*-diversity (f)) between rice and sago groups. (a) OTU observed, (b) Chao1, (c) Shannon, (d) Simpson, (e) Fisher, and (f) principal coordinated analysis (PCoA) based on the Bray-Curtis dissimilarity index.

In the phylum profile of the gut microbiota, we found that the Firmicutes were the highest of phyla for both groups. Proteobacteria was significantly higher in the sago group than in the rice group (P = 0.018, Mann-Whitney U test, respectively) but after adjusting with the age, it showed no significant value (P = 0.116, General Linear Model, Bonferroni, respectively) (Figure 5a). Meanwhile, in the genera profile, *Prevotella* 9 was the highest abundance of the genera for both groups. *Eubacterium rectale*, *Succinivibrio*, and *Subdoligranulum, Prevotella 7* were found significantly higher in the sago group than in the rice group (P = 0.034, Mann-Whitney U test, P = 0.020, Mann-Whitney U test, P = 0.037, student t-test, P = 0.026, Mann-Whitney U test, respectively). *Escherichia-Shigella* in the sago group showed higher than in the rice group, but the statistical test showed it was not significant. The sago group had significantly lower *Collinsella* and *Slackia* compared to the rice group (P = 0.027, Mann-Whitney U test, P = 0.034, Mann-Whitney U test, respectively). *Prevotella 7* and *Slackia* had mean relative abundance < 1% (others) (Figure 5b).



(a)



(b)

Figure 5. Microbiota composition in rice and sago groups. Mean relative abundance (%) at phyla (a) and genera (b), others were < 1%

To identify genomic features characterizing the differences between the sago and rice groups, we conducted the linear discriminant analysis (LDA) effect size

(LEfSe). It showed that Proteobacteria, *Succinivibrio*, and *Eubacterium rectale* had effect size >3.5 positively in the sago group while in the sago group only *Collinsella* had effect size > 3.5 negatively (Figure 6).

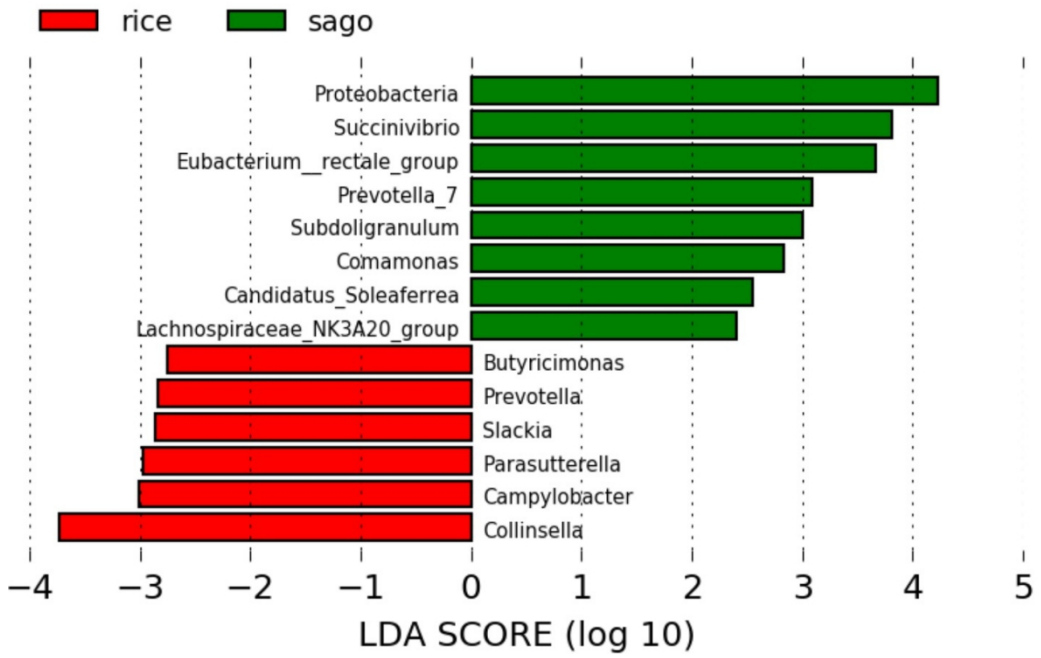
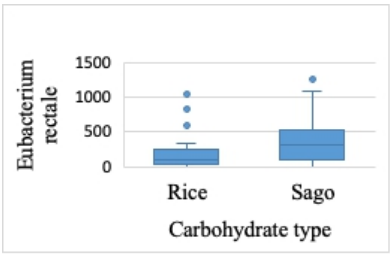


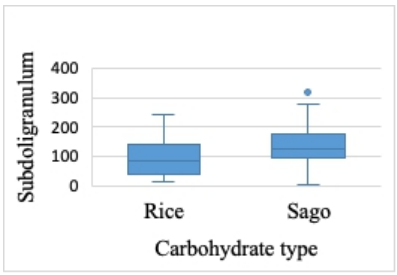
Figure 6. LEfSe analysis between the rice (red) and sago (green) groups. Bar length represents the effect size, which explains the different phenotypes between groups.

Gut microbiota was influenced by age. In our study, we found that there was a significant difference in the age between the sago and rice groups, so we conducted a general linear model, Bonferroni on the significant genera between groups. It showed that *Eubacterium rectale* was the only genera that had a significant difference between the rice and sago group after adjusting with the age (P = 0.017, General Linear Model, Bonferroni, respectively), while *Prevotella* 7 and *Subdoligranulum* tended to be significant (P = 0.055, General Linear Model, P = 0.061, General Linear Model, Bonferroni, respectively). *Succinivibrio*,

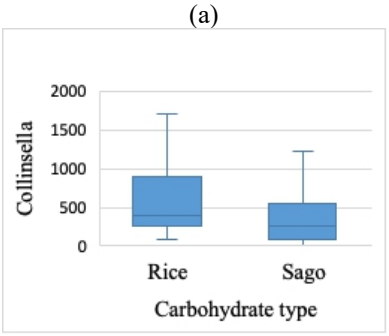
Collinsella, and *Slackia* after adjusting with age showed no significant difference between both groups (P = 0.244, General Linear Model, Bonferroni, P = 0.087, General Linear Model, Bonferroni, P = 0.091, General Linear Model) (Figure 7a-f).



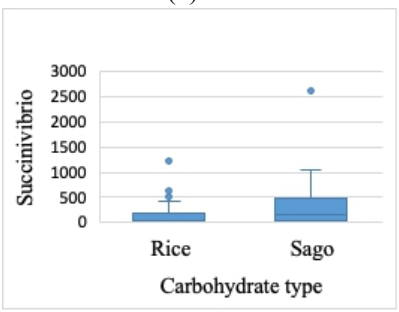
P = 0.034 (Mann-Whitney) adjust with the age, P = 0.017
(a)



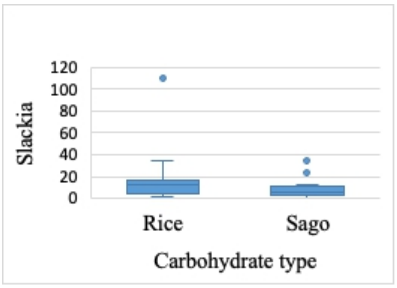
P = 0.037 (Mann-Whitney) adjust with the age, P = 0.061
(b)



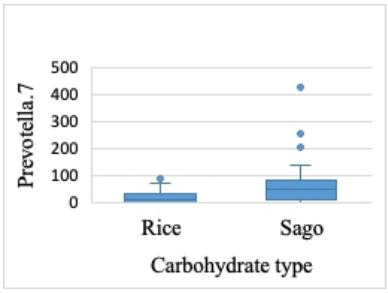
P = 0.027 (Mann-Whitney) adjust with the age, P = 0.087
(c)



P = 0.020 (Mann-Whitney) adjust with the age, P = 0.244
(d)



P = 0.034 (Mann-Whitney)
adjust with the age, *P* = 0.091
(e)



P = 0.026 (Mann-Whitney)
adjust with the age, *P* = 0.055
(f)

Figure 7. Genus of gut microbiota showed significant difference with Mann Whitney U test and adjust with the age with GLM, Bonferroni (a) *Eubacterium rectale,* (b) *Subdoligranulum,* (c) *Collinsella*, (d) *Succinivibrio*, (e) *Slackia*, (f) *Prevotella* 7.

Bacterial fermentation of undigested dietary carbohydrates and to a lesser extent, dietary and endogenous proteins can produce organic acid such as short-chain fatty acids (SCFA) and branched-chain fatty acid (BCFA). Acetic acid was the highest production of SCFA for both groups followed by propionic acid and n-butyric acid. The profile of SCFA showed that there was no significant difference between the sago and rice groups. Iso-valeric acid was the highest of BCFA for both groups and showed no significant difference between groups (Table 12).

Table 12. Short-chain fatty acid and branched-chain fatty acid profile of the study population

	Rice (n = 25)	**Sago (n = 24)**	***P* value**
Acetic acid (mg/g)	3.54 ± 1.54	3.19 ± 1.12	0.207
Propionic acid (mg/g)	2.02 (0.46; 6.25)	2.00 ± 0.91	0.857
n-butyric acid (mg/g)	1.50 ± 0.70	1.19 (0.50; 4.03)	0.439

n-valeric acid (mg/g)	0.40 ± 0.14	0.32 (0.14; 1.64)	0.360
Iso-butyric acid (mg/g)	0.28 ± 0.10	0.19 (0.11; 0.88)	0.245
Iso-valeric acid (mg/g)	0.46 ± 0.18	0.31 (0.14; 1.71)	0.448
SCFA (mg/g)	8.01 ± 3.83	6.81 ± 3.25	0.413
BCFA (mg/g)	0.71 ± 0.29	0.50 (0.19; 2.59)	0.356

Variables presented as means ± SDs, medians (minimum, maximum). Abbreviation: *SD* standard deviation, *m*g milligram, g gram, *P* probability, *SCFA* short-chain fatty acid, *BCFA* branched-chain fatty acid, SCFA is the sum of acetic acid, propionic acid, n-butyric acid, n-valeric acid. BCFA is the sum of iso-butyric acid and iso-valeric acid.

To analyze the correlation between gut microbiota and the amount of protein intake, we conducted a Pearson correlation test for both groups. In the rice group, only *Succinivibrio* was significantly positively correlated with the amount of protein intake. Meanwhile, in the sago group, there were four genera correlated with the amount of protein intake. Only *Bacteroides* had a positive correlation with the amount of protein intake, while *Succinivibrio*, *Collinsella*, and *Faecalibacterium* had a negative correlation (Table 13).

Table 13. The significant correlation between log gut bacteria and the amount of protein intake of the rice and sago diet group[a]

	The amount of protein intake	
	Coefficient correlation	***P* value**
Rice group D:1::Proteobacteria_D:2::Gammaproteobacteria_D:3::Aeromonadales_ D:4::Succinivibrionaceae_D:5::Succinivibrio	0.620	0.003
Sago group D:1::Proteobacteria_D:2::Gammaproteobacteria_D:3::Aeromonadales_ D:4::Succinivibrionaceae_D:5::Succinivibrio	-0.543	0.007
D:1::Bacteroidetes_D:2::Bacteroidia_D:3::Bacteroidales_D:4::Bacteroidaceae _D:5::Bacteroides	0.543	0.007
D:1::Actinobacteria_D:2::Coriobacteriia_D:3::Coriobacteriales_ D:4::Coriobacteriaceae_D:5::Collinsella	-0.499	0.015
D:1::Firmicutes_D:2::Clostridia_D:3::Clostridiales_D:4::Ruminococcaeae_ D:5::Faecalibacterium	-0.429	0.036

Abbreviations : P probability [a] Pearson correlation test, D:1 phylum, D:2 class, D:3 order, D:4 family, D:5 genus.

In relation to gut microbiota with albumin level, we conducted Pearson correlation test. Different gut microbiota was found for both groups. In the rice group, *Clostridium sensu stricto* 1, *Eubacterium rectale*, and *Catenibacterium* had a positive correlation with albumin level. On the other hand, *Bifidobacterium*, *Collinsella*, *Coriobactericeae* uncultured, *Eubacterium hallii*, and *Blautia* had a positive correlation with albumin level in the sago group (Table 14).

Table 14. The significant correlation between log gut bacteria and albumin level of the rice and sago diet[a]

	Albumin	
	Coefficient correlation	***P* value**
Rice group		
D:1::Firmicutes_D:2::Clostridia_D:3::Clostridiales_D:4::Clostridiaceae_ D:5::Clostridium sensu stricto 1	0.510	0.013
D:1::Firmicutes_D:2::Clostridia_D:3::Clostridiales_D:4::Lachnospiraceae_ D:5::Eubacterium rectale group.	0.495	0.016
D:1::Firmicutes_D:2::Erysipelotrichia_D:3::Erysipelotrichales_ D:4::Erysipelotrichaceae_D:5::Catenibacterium	0.531	0.009
Sago group		
D:1::Actinobacteria_D:2::Actinobacteria_D:3::Bifidobacteriales_ D:4::Bifidobactericeae_D:5::Bifidobacterium	0.427	0.047
D:1::Actinobacteria_D:2::Coriobacteriia_D:3::Coriobacteriales_ D:4::Coriobactericeae_D:5::Collinsella	0.662	0.001
D:1::Actinobacteria_D:2::Coriobacteriia_D:3::Coriobacteriales_ D:4::Coriobactericeae_D:5::uncultured	0.426	0.043
D:1::Firmicutes_D:2::Clostridia_D:3::Clostridiales_D:4::Lachnospiraceae_ D:5::Eubacterium hallii group	0.486	0.024
D:1::Firmicutes_D:2::Clostridia_D:3::Clostridiales_D:4::Lachnospiraceae_ D:5::Blautia	0.446	0.029

Abbreviations : P probability, [a] Pearson correlation test D:1 phylum, D:2 class, D:3 order, D:4 family, D:5 genus.

DISCUSSION

The present study showed that *Prevotella* type was enterotype for the moderately low protein intake-sago diets and sufficient protein intake-rice diets. There was no difference in the diversity (alpha and beta) between the sago-moderately low protein intake and rice-sufficient protein intake groups. In linear discriminant analysis effect size (LEfSe) showed that Proteobacteria, *Succinivibrio*, and *Eubacterium rectale* had a positive effect size > 3.5 in the sago group, meanwhile, *Collinsella* had a negative effect size > 3.5 in the rice group. There was a significantly higher abundance of *Eubacterium rectale* in the sago group compared to the rice group after adjusting with age. In the sago group, *Collinsella* had a negative correlation with the amount of protein intake but had a positive correlation with albumin level.

The sago-eating participants were older than the rice-eating participants. A study by Syartiwidya in Riau Province, Indonesia found a similar finding that the people who consumed more sago were older (> 50 years old) compared to those who consumed less sago [23]. Sago is the oldest staple food for local people in the lowland of Papua Province [12]. But there is a decline in sago consumption in the sago-producing areas of Indonesia and a change to rice. The rice policy and unclear sago forest responsibility at national and local levels might be the factors. Better households' income and education will reduce sago consumption and production. The perception of sago as soil food and having lower social status

compared with rice are other factors influencing the decline of sago consumption [16]. Younger local people in Papua and other sago-producing areas are consumed rice than sago. While elder local people still consumed sago as their staple food. Among our study population, Kamoro was the dominant ethnic for both groups. Kamoro ethnic was local people that lived in the coastal areas (lowland) in Papua province. Their lifestyle was influenced by the nature. Sago forest and river have provided them with all the needed of Kamoro people [70]. Because of the same ethnic, their daily living habit was almost similar for the sago and rice groups.

Prevotella was the enterotype for both groups in our study population. A similar finding was reported in a study in Papua New Guinea, where *Prevotella* predominated over *Bacteroides*. This is represented by their diet of high in carbohydrates with a variety of sources but low in protein [63]. A comparison study of the health status of Japanese and Asians indicated by gut microbiome research found that urban and rural areas in Yogyakarta Indonesia had *Prevotella* type that reflects of high consumption of rice. Meanwhile Japanese had *Bacteroides/Bifidobacterium* type [71]. *Prevotella* species have been correlated with plant-rich diets, abundant in carbohydrates and fibers [72]. The genus *Prevotella* was underrepresented in Americans with a Western diet compared to Africans with a plant-based diet [73]. Bacteroides enterotype is common in people following a Western diet that is rich in fat and protein. *Prevotella* enterotype is common in people who consume a lot of fiber/polysaccharides [20, 58, 59]. The *Prevotella* genus seems to be a discriminatory taxon between high and low carbohydrate diets, or rural and metropolitan diets, in the gut microbiota of

children and adults [20]. Comparison of long-term and short-term dietary data showed that only the long-term diet was correlated with enterotype clustering in the cross-sectional study. In the interventional study, changes were significant and rapid, but the magnitude of the changes was modest and not sufficient to switch individuals between the enterotype clusters associated with protein/fat and carbohydrates [74]. Both groups in our population study either the rice group or the sago group had higher portions of carbohydrate intake from total calories than other macronutrients (the rice group: 60% and the sago group: 79%, respectively). *Prevotella* enterotype in our study population represents the long-term dietary pattern of a plant-based diet (polysaccharides/fiber). At the genera level, *Prevotella* 9 was the highest genus found in both groups in our data. A similar finding from a study on gut microbiota profile of Indonesian stunted children and children with normal nutritional status showed that *Prevotella* 9 was the most abundant genus, and it was significantly lower in stunted children than in normal children. Their carbohydrate intake was 57% in normal and stunted groups. *Prevotella* was known to be associated with long-term dietary fibre intake [75]. It was similar to our carbohydrate intake in our population with > 50% of carbohydrate intake.

A recent study found that the diversity of the sago and rice groups showed no significant difference. A study on two traditional societies of Papua New Guinea, which is one located in highland and the other in lowland showed there is no significant difference in alpha-diversity as well as beta-diversity. All subjects have a similar lifestyle (live in a traditional setting) and consume traditional staple

foods (sweet potato, taro, and plantain) [76]. Human studies showed that the influence of diet on gut microbiota diversity was mainly based on the abundance or lack of the three main dietary components: fats, carbohydrates, and proteins. These dietary products serve as an energy source for the microorganisms which compose the intestinal flora and have a profound impact on the gut microbiota. Most differences in gut microbiota are found in the phylum and genus level rather than species. Microbiota in people who lived within the same area who are in contact with one another appears to have similar diversity [77]. Kamoro ethnic was the dominant ethnic among our study participants. A study on the impact of ethnicity on the adult gut microbiota in a multi-ethnic community from a single district in southern peninsular Malaysia showed that ethnicity exhibited the largest effect size across participants. The influence of ethnicity on the gut microbiota was retained even after controlling for all demographic, dietary factors, other covariates which were significantly associated with the gut microbiome. Ethnicity, therefore, serves as a proxy for lifestyle and dietary variations across different ethnic groups living in the same community [78]. Ethnicity and socio-cultural practices could have an effect on the modulation of gut microbiota within inhabitants of the same geographical area. Gut microbiota composition differs according to diet and eating habits which are closely related to geographical location and ethnic diversity [77]. Our population was two adult populations with similar hereditary and lifestyle (local people of Papua with Kamoro ethnic) and environmental conditions (living in the same areas), the difference only was in the source of staple food and the amount of protein intake. The diversity of our

populations showed no difference because of similar lifestyles and living in the same areas.

Firmicutes were the dominant phylum found in both groups. A study in Papua New Guinea that consumed traditional staple food (sweet potato and sago starch) showed that Firmicutes were higher than Bacteroidetes. However, their methods did not comprehensively detect all bacteria within the Firmicutes and Bacteroidetes [63]. Firmicutes is a fibrolytic gut bacteria found in the human colon and rumen that had carbohydrate-active enzymes involved in plant structural polysaccharide degradation [79]. Carbohydrates that reach the gut are RS, non-starch polysaccharides, and oligosaccharides. Starch is one of the most common carbohydrates in rice, wheat, root vegetables, fruits, beans, and the like. There are four types of indigestible RS based on the food sources of the carbohydrate sources [59]. The source of carbohydrates of our population study was sago and rice. It was classified as RS-3 (retrograded cooled gelatinized starch).

Proteobacteria was found significantly higher in moderately low protein intake-sago diets compared to sufficient protein intake-rice diets. But after adjusting with the age, it showed no significance. An animal study with different dietary protein levels (high protein diet, medium protein diet, and low protein diet) by Zhao, Y, et al in 2020 found that a reduction of 4% of dietary protein level (low protein diet) increases the number of Proteobacteria. Proteobacteria was the nitrogen metabolism-associated bacteria and all ammonia-oxidizing bacteria belong to Proteobacteria. When the amount of nitrogen-containing substances that

enter the hindgut sharply decreased, the nitrogen metabolism-associated bacteria will compete for the limited nitrogen sources. Therefore, more nitrogenous compounds would be utilized by Proteobacteria in the group with a 4% reduction in the dietary protein [80]. A cross-sectional study on the age-related changes in gut microbiota composition from newborn to centenarian showed an increase in the relative abundance of Proteobacteria in subjects over 70 years old. The change in the relative abundance of Proteobacteria was opposite to the Firmicutes. It was suggested that nutrients in the gut might play an important role in changing the gut microbiota composition with the age [81]. The moderately low protein intake-sago diet showed a significant higher of Proteobacteria representing the nitrogen metabolism-associated bacteria (ammonia-oxidizing bacteria). Increasing age over 70 years showed the increased relative abundance of Proteobacteria. Meanwhile, our mean age of sago-eating people was 53.44 years with the highest age being 64 years. We assumed the influence of age might be not very strong as the dietary intake.

In the genera profile, Eubacterium *rectale*, *Succinivibrio*, *Subdoligranulum,* and *Prevotella* 7 were higher in the sago group but lower in *Collinsella* and *Slackia. Eubacterium rectale* was found to be higher in the sago group compared to the rice group. *Eubacterium rectale* was human amylolytic species that fermented resistant starch [79]. High intake of resistant starch increase *Eubacterium rectale* in vivo [82]. A study on fermentation RS-3 derived from sago and rice starch with *Eubacterium rectale* showed that sago had higher RS-3 than rice [26]. *Eubacterium rectale* was incapable of degrading resistant

starches unless they are heat-treated to denature some of the crystal structure. A study on the dietary interventions with three fermentable fibers (resistant starch from potatoes (RPS), resistant starch from maize, and inulin chicory root) and an accessible corn starch control found that RPS has the greatest increase in total SCFAs, including butyrate. *Eubacterium rectale* was one of the most abundant butyrate producers in the human gut. *Eubacterium rectale* were more likely to yield higher butyrate concentrations compare to *Ruminococcus bromii* or *Clostridium chartatabidum*. *Eubacterium rectale* was the only butyrate producer whose abundance increased with a primary degrader and was associated with higher fecal butyrate [83]. Sago and rice were classified as RS-3 and *Eubacterium rectale* was increased when intake of RS was high. Sago produced more RS-3 than rice with *Eubacterium rectale* fermentation. This gut microbiota was the main producer of butyrate. The higher abundance of *Eubacterium rectale* in a moderately low-protein intake-sago diet was from sago that contains RS-3. This gut microbiota produced butyrate (source energy in colonocytes). However, in the SCFA profile, there was no significant difference in butyrate level between the groups.

Succinivibrio was higher in the moderately low protein intake-sago diets than in the sufficient protein intake-rice diet. However, after adjusting with the age it showed no significance. A gut microbiota study comparison between children living in rural and urban Burkina Faso and Italy showed that microbiota of rural children retains *Prevotella*, *Treponema*, and *Succinivibrio* that assigned to ferment fiber and polysaccharides from vegetables. Differently with children living in

urban Burkina Faso showed the gut microbiota progressively similar to Italian children (animal protein, fat and sugar rich foods) [84]. A study of animals reported that a 4% reduction in the dietary protein level (low protein diet) evidently enhanced the abundance of *Succinivibrio*. The high content of corn and wheat bran in a low protein diet boosts the abundance of *Succinivibrio*, which can degrade starch and hemicelluloses and produce acetate and succinate [80]. The concentration of dietary protein is a primary factor affecting protein fermentation and intestinal microbe composition. A low protein diet is associated with low concentrations of ammonia, plasma urea nitrogen, and SCFA contents in ileal digesta. Ammonia in the gut is not derived from host urea activity, but from proteolytic and microbial activity and *Succinivibro* was one of the amino acid fermenting bacteria [85]. An animal study on the analysis of nitrogen metabolism of *Succinivibrio dextrinosolvens* from dairy cow rumen showed that activities of urease and glutamine synthase and glutamate dehydrogenase were significantly different in the nitrogen and growth phase. There were 1246 differentially expressed genes (DEGs) were identified in all nitrogen. Among DEGs, 33 were related to nitrogen metabolism. Their expression correlated with nitrogen sources and the intensity of enzyme activity [86]. The increase of *Succinivibrio* in moderately low protein intake-sago diet can be because of the higher portion of sago (starch) in the diet and the nitrogen metabolism-related microbiota.

The genus *Subdoligranulum* and *Prevotella* 7 were found significantly higher in the sago group. The genus *Subdoligranulum* was belong to the Ruminococcaceae family and is closely related to the *Faecalibacterium* genus. It

was discovered together with the administration of *Akkermansia muciniphila* in obese and diabetic mice. Obese humans with a mild improvement of metabolic parameters during caloric restriction had a significantly lower abundance of *Subdoligranulum* than the subgroup with a better metabolic response to the caloric restriction. It was found also negatively correlated with C-reactive protein (CRP), fatty liver index, and Homeostasis Model Assessment-Insulin Resistance (HOMA-IR). Moreover, *Subdoligranulum* was shown to produce butyrate, which has a health potential effect. However, the animal study showed there was no significant difference in metabolic parameters of obesity and diabetes after supplementation with *Subdoligranulum variabile* with the increasing of the abundance of this gut microbiota [87]. The study of *Prevotella* 7 in humans was rarely found. A study in animals for improving taxonomic of rumen bacterial 16S r RNA sequences using revised SILVA taxonomic framework found that *Prevotella* 7 was previously fell into *Prevotella* classification with *Prevotella* 2, *Prevotella* 6, and *Prevotella* 9. Recently with finer-scale subdivision, it was classified with the given unique designation [88]. *Subdoligranulum* and *Prevotella* 7 were recently be found in other studies. Higher of *Subdoligranulum* might be beneficial to the sago group for metabolic-related inflammation.

Collinsella in the sago group had a lower abundance than the rice group. A study of overweight and obese pregnant women found that *Collinsella* abundance was correlated negatively with dietary fiber intake. Low dietary fiber may enable the overgrowth of *Collinsella* and alter the overall fermentation pattern in gut microbiota [89]. A study to relate feeding behavior with gut microbiota in healthy

young overweight adults found that higher relative abundance of *Collinsella* were related to less healthy eating behavior. Lower fiber intake was associated with *Collinsella* [90]. In our study, the fiber intake of the sago group was significantly high than the rice group. However, there is no correlation was found between *Collinsella* and fiber intake.

Serum albumin levels in the sago group did not differ from those in the rice group and fell within the normal range. In linear discriminant analysis, it showed that Proteobacteria, *Succinivibrio*, and *Eubacterium rectale* had effect size > 3.5 positively in the sago group. Meanwhile, in the rice group, *Collinsella* had an effect size > 3.5 negatively. In the correlation of gut microbiota with the amount of protein intake and albumin level, *Collinsella* in the sago group was found to be correlated with those variables (negatively with the amount of protein intake and positively with albumin level). Based on our working hypotheses, in the moderately low protein intake-sago group there was an adaptive mechanism to prevent deficiency protein symptoms with higher of Proteobacteria and *Succinivibrio* that can produce SCFA (acetate and succinate) and *Eubacterium rectale* as the source of energy of colonocytes (butyrate-producing bacteria with higher of RS-3) and decrease *Collinsella* (decreasing inflammatory/proteolysis). These roles will maintain the integrity of the gut barrier function. Then, we assumed higher AA and SCFA reach the systemic circulation. These conditions will maintain albumin within in normal range in the moderately low protein intake-sago diet (Figure 8).

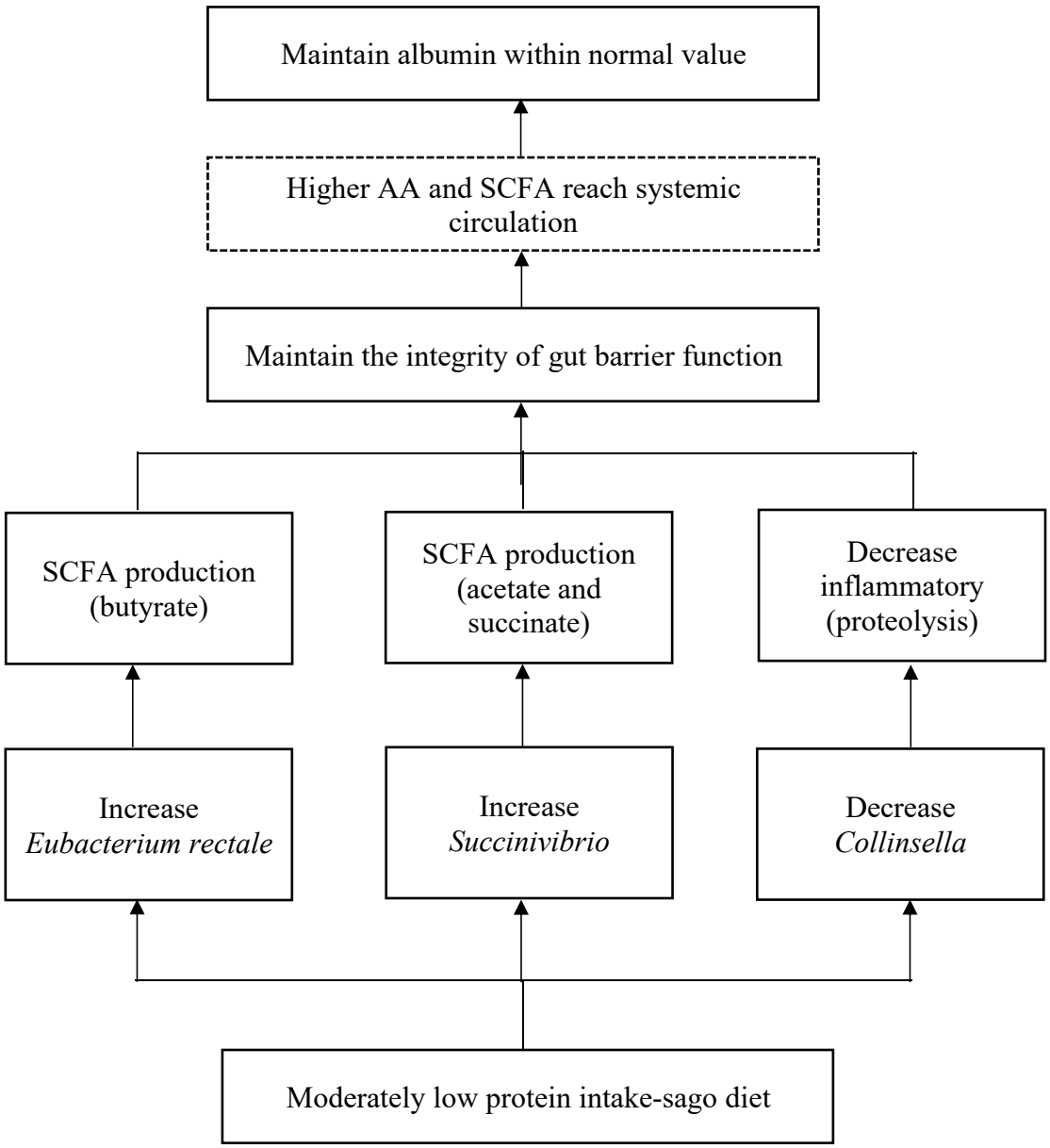


Figure 8. Working hypotheses on how moderately low protein intake-sago diet maintain albumin within normal value mediated by gut microbiota

Butyrate was one of the important SCFA produced in humans. Besides as an energy source for the epithelial cells, it also influences a wide array of cellular

functions affecting colonic health [91]. Through its functions as a histone deacetylase inhibitor (HDI), butyrate may have anti-diarrhetic, anti-oxidant, anti-carcinogenic, and anti-inflammatory functions [92]. In ulcerative colitis, some studies showed a reduction in inflammation and clinical symptoms after luminal administration of butyrate or stimulation of luminal butyrate production by the ingestion of dietary fiber. There are several mechanisms for the anti-inflammatory activity of butyrate but the most frequently studied was by suppressing the nuclear factor kappa B (NF-$_K$B) activation [91]. In a randomized, double-blind, cross-over with 16 healthy human subjects showed with butyrate treatment, there was significantly higher glutathione and lower uric acid concentrations compared to placebo [93]. In vitro studies with human colon cancer cell lines found that butyrate is involved in repair after mucosal damage through an increase in the rate of cell migration. Efficient repair of superficial injuries and mucosal ulcers is important in maintaining and re-establishing the epithelial barrier. Butyrate is also shown to affect several components of the colonic defense barrier leading to enhanced protection against luminal antigens [91]. Even though a recent study showed that butyric acid was the least produced among the three major abundant of SCFA of the moderately low protein intake-sago group, but butyrate might have an important role in maintaining protein-energy nutritional status within the normal range.

Succinivibrio dextrinosolvens was often the predominant isolate from the rumen when the diet of the animal is high in starch. In vitro study showed that the end product was the formation of acetate and succinate. Acetate is readily

absorbed from the rumen and subsequently used in fatty acid metabolism. Succinate is thought to be an important intermediate for propionate formation [94]. Acetate and succinate were SCFA that can be a potential source of energy for the moderately low protein intake-sago diet.

A study by Menni, C et al found that *Collinsella* was the mediated gut microbiota for the association between vegetable intake and lymphocyte counts. A habitual diet high in vegetables, but not fruits, is linked to a lower inflammatory profile for white blood cells. This is might be mediated by the nitrate content in the vegetable. Dietary nitrate content has been related to increased nitric oxide production. Nitric oxide production has been postulated as a possible molecular mechanism for the cardiometabolic risk reduction seen with the vegetable-rich Mediterranean diet [95]. Another study by Chen, J et al showed that the abundance of *Collinsella* correlated strongly with the high level of the production of chemokines and IL-17A. *Collinsella* contributes to hyper-permeability of the gut by reducing the expression of the tight junction protein ZO-1 directly, as well as by producing specific metabolites [96]. *Collinsella* in a study on nonalcoholic steatohepatitis (NASH) patients showed strongly associated with NASH. This genus also positively correlated with fasting levels of triglycerides and total cholesterol but negatively correlated with high-density lipoprotein cholesterol. This genus may influence lipid metabolism in the host [97]. Higher The decreasing *Collinsella* in the sago group was assumed to make higher nitrate intake. Higher nitrate intake was the adaptation mechanism on the moderately low

protein intake of the sago group to maintain albumin levels within the normal range.

Our study found that there were some different genera between the sago moderately low protein intake-sago diets and the sufficient protein intake-rice diets. Those genera were shown might be an adaptive mechanism for the sago group who consumed moderately low protein intake to maintain protein-energy nutritional status within the normal range.

This study was the first study on gut microbiota in local people of Papua with their traditional lifestyle and eating habits. Improving the methodology with specific indicators on the protein-energy nutritional status will make a better understanding of how gut microbiota affect their energy-protein nutritional status based on their traditional eating habits.

Gut microbiota that found the difference in the moderately low protein intake-sago diet will have valuable information for the geriatric population who were consumed low protein intake. Decreasing the high risk of malnutrition in the elderly population might be achieved by improving the gut microbiota composition of the elderly population.

Chapter IV

Changes in Intestinal Environment and Defecation Associated with Ingestion of Rice Koji Amazake in Middle-aged and Elderly People

Constipation was one of the diseases that occur frequently in the late-stage elderly and has a great influence on the quality of life of the elderly [98]. A leaky gut syndrome caused by constipation had been studied in recent years. In leaky gut syndrome, the barrier function of the intestinal mucosa is reduced, and food substances, intestinal bacteria, and pathogens invade the body through the intestine. This is will cause chronic inflammation, increased protein catabolism, and decreased muscle mass [99, 100].

As a diet for chronic constipation, it is recommended to take dietary fiber, lactic acid bacteria foods, and fermented foods. Among fermented foods, amazake was not only improved the intestinal environment but also rich in vitamins and amino acids. It has been reported that bowel movements were improved and the number of bowel movements is significantly increased [101-104].

However, the mechanism of improvement of constipation by ingestion of rice-koji amazake regarding the involvement of the intestinal bacterial flora has not been clarified. The purpose of the study was to conduct a 6-week drinking test of rice-koji amazake in middle-aged and elderly people and to analyze the changes in the intestinal environment and constipation symptoms before and after taking rice-koji amazake.

MATERIALS AND METHODS

Test food

The test food for this test was rice koji amazake (Koji Sweet, Marumi Koji Main Store, Japan), which is a natural sweetener made from rice. Table 1 shows the component analysis values per 35 g of test food. Quantitative analysis of sugar has been performed on this product so far, and isomaltose, panose, and isomaltotriose have been detected, and the component analysis values per 35 g of test food are shown in Table 15.

Table 15. Nutritional components per 35 g of the test food,” Koji Sweet”

Characteristic	Quantity / 35g
Energy	76.7 kcal
Protein	1.2 g
Fat	0.1 g
Carbohydrates	18.0 g
Sugar	17.4 g
Isomaltose	0.83 g
Panose	0.07 g
Isomaltotriose	0.06 g
Soluble dietary fiber	0.1 g
Insoluble dietary fiber	0.5 g
Sodium	10.1 mg
Water	15.7 g
Ash	0.035 g

Subjects ingested about 35 g per day for 6 weeks.

Subjects and examination schedule

The subjects of this study were 32 middle-aged and elderly people (9 males and 23 females) aged 55 to 92 years (71.2 ± 1.1 years) living in the community, and the characteristics of the subjects where height was 156.2 ± 1.3 cm and weight was 56.4 ± 1.7 kg with BMI 23.2 ± 0.7 kg / m2. They were recruited from community health project participants who were middle-aged and elderly people and received home-visit medical care. All subjects understood the research content and the advantages and disadvantages of participating in the research before participating in the research and signed the research participation agreement. The protocol of this study was approved by the Okayama Prefectural University Ethics Committee (No.17-73) before the study was conducted in accordance with the Declaration of Helsinki.

Regarding the test schedule, the test food intake period was set to 6 weeks in consideration of changes in the intestinal bacterial flora. We investigated the bacterial flora. According to the previous study [105], 35 g (2 tablespoons) of the test food per day was diluted about 3 times with plain hot water or water, and about 150 mL of amazake was ingested daily. As a general rule, the intake time of the test food was at breakfast. Throughout the study period, diet and medication were instructed to be maintained as before participation in the study.

Defecation status survey

The defecation habits were surveyed in the form of a questionnaire before and after the intervention by a self-administered questionnaire survey. The survey items were constipation evaluation using the Bristol stool scale (BSS) [106], and

the Japanese version of the constipation evaluation scale (CAS) [107]. BSS is a stool property score that classifies the shape of stool into 7 levels in order from hard stool to loose stool [108]. In this study, each item 1 to 7 was scored to 1 to 7 points, and stratified analysis was also performed with 1 to 3 points as hard stool, 4 points as a normal stool, and 5 to 7 points as loose stool. CAS consists of eight items related to constipation [107]. Subjective symptoms are evaluated, each category is evaluated as 0 to 2 points, and the total score is a maximum of 16 points. When the tendency of constipation becomes stronger, the score becomes high, and a total score of 5 points or more is judged as constipation.

DNA extraction from feces

Before and after the intervention, stool was collected using a stool collection kit (test container type A white screw cap type, code; 0-1762-01, AS ONE). After collection, feces were stored at -20 ° C. DNA extraction was performed using the QIAamp DNA Stool Mini Kit (Qiagen, Venlo, Netherlands).

Real time PCR

Adjust the DNA solution to a final concentration of 10 ng / μL and use the Step One Plus Real-Time PCR System (Thermo Fisher Scientific, Tokyo, Japan) according to the SYBR Premix Ex Taq II (Takara Bio Inc., Shiga, Japan) protocol. Then, real-time PCR analysis was performed. Using specific primers targeting the 16S rRNA gene, two genera of Bacteroidetes and Firmicutes and their ratio Firmicutes / Bacteroidetes ratio (F / B ratio), *Clostridium coccoides* group, *Clostridium leptum* subgroup, *Clostridium ramosum* subgroup, *Bacteroides fragilis* group, *Atopobium* cluster, *Prevotella*, *Eubacterium cylindroides* group,

Bifidobacterium, *Desulfovibrio*, *Akkermansia*. The results were corrected by the ΔΔCt method, and the relative values were calculated.

Statistical analysis

The measured values in this study are shown as mean ± standard error. Two-way ANOVA and multiple comparisons were performed among the three groups of hard stool, normal stool, and loose stool using the BSS scores before and after the amazake intervention. For CAS before and after the amazake intervention, two-way ANOVA and multiple comparisons were performed between the non-constipation group and the constipation group. For BSS and CAS, a paired t-test (two-sided test) was used for gender, and Wilcoxon signed-rank sum test (two-sided test) was used for age. Intragroup comparison of gut microbiota was analyzed by Mann-Whitney-U test or Wilcoxon signed-rank sum test (two-sided test). The significance level was set to less than 5%.

RESULTS

BSS was 3.8 ± 0.2 points before the intervention and 4.0 ± 0.1 points after the intervention, and there was a significant interaction before and after the intervention (F (2, 29) = 66.17, $P < 0.05$). The stool shapes evaluated by BSS before the intervention were hard stools in 7 patients (21.9%), normal stools in 19 patients (59.4%), and loose stools in 6 patients (18.7%). The changes before and after are shown in Figure 9. The BSS of patients with hard stool increased significantly from 2.1 ± 0.3 points to 3.6 ± 0.2 points before and after the intervention ($P < 0.01$). The BSS of patients with loose stools decreased significantly from 5.2 ± 0.2 points to 4.7 ± 0.3 points before and after the intervention ($P < 0.01$). A comparison of men and women before and after the amazake intervention was performed to examine the differences by gender, and no significant changes were observed between men (n = 9) and women (n = 23) ($P > 0.05$).

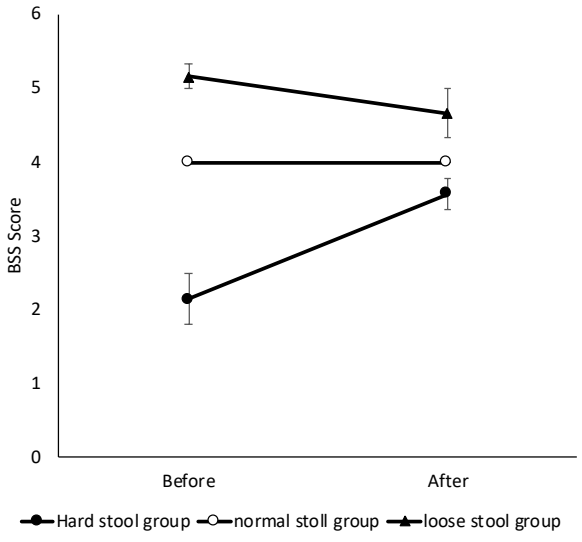


Figure 9. BSS Score of hard stool group, normal stool group, and loose stool group
Data are expressed by the mean and standard error of estimation (Means ± SE). There is a significant difference within-group (** P <0.01).

CAS was 2.8 ± 0.5 points before the intervention and 1.8 ± 0.5 points after the intervention, and there was a significant interaction before and after the intervention (F (1, 30) = 39.50, $P < 0.05$). As a result of classification according to the CAS before the intervention, 7 patients were judged to be constipated (constipation group) (21.9%), and 25 patients were judged to be non-constipated (non-constipation group) (78.1%). Regarding the age of each group, the constipation group was 72.0 ± 2.3 years old, and the non-constipation group was 71.0 ± 1.3 years old, and there was no significant difference when comparing the groups ($P > 0.05$). Figure 10 shows the changes before and after the intervention in each group. The CAS in the constipation group was 7.7 ± 0.9 points before the intervention and 4.7 ± 1.6 points after the intervention, showing a significant difference ($P < 0.01$). The CAS in the non-constipation group was 1.4 ± 0.3 points before the intervention and 1.0 ± 0.3 points after the intervention, showing no significant difference. A comparison of men and women before and after the amazake intervention was performed to examine the differences by gender, and no significant changes were observed between men (n = 9) and women (n = 23) ($P > 0.05$).

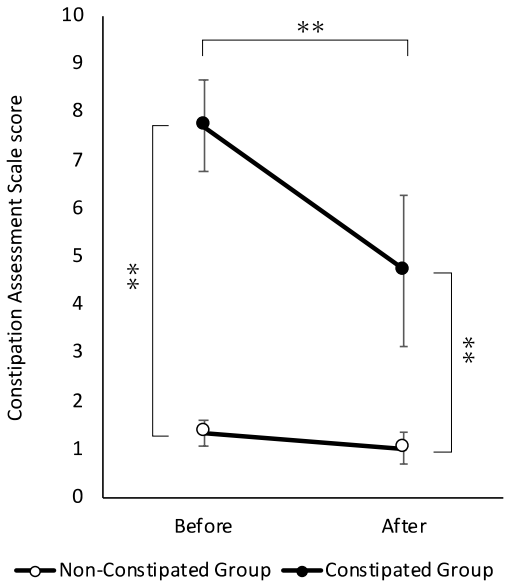


Figure 10. Constipation Assessment Scale Score of Non-Constipated group and Constipated Group
Data are expressed by the mean and standard error of estimation (Means ± SE).
There is a significant difference within-group (** P <0.01).

Firmicutes were 1.00 ± 0.14 before the intervention and 0.82 ± 0.22 after the intervention, which decreased significantly before and after the intervention (P < 0.05). As a result, the F / B ratio was 3.03 ± 1.11 before the intervention and 1.26 ± 0.25 after the intervention, which decreased significantly before and after the intervention (P < 0.05) (Figure 11).

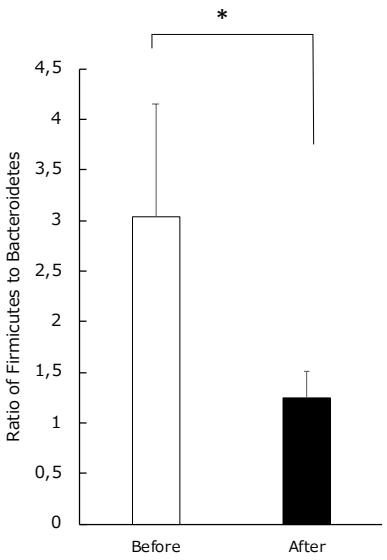


Figure 11. The ratio of Firmicutes to Bacteroides before and after intervention
Data are expressed by the mean and standard error of estimation (Means ± SE).
There is a significant difference within-group (* P <0.05).

The genus and species levels were 1.00 ± 0.23 before the intervention of the *Clostridium leptum* subgroup and 1.67 ± 0.91 after the intervention, showing an increasing trend (P < 0.1) (Figure. 12a). The *Bacteroides fragilis* group was 1.00 ± 0.15 before the intervention, 6.09 ± 4.56 after the intervention (Figure. 12b), 1.00 ± 0.24 before the intervention by *Desulfovibrio* (Figure. 12c), and 0.53 ± 0.12 after the intervention, showing significant changes (P < 0.05) was confirmed. In addition, significant changes were confirmed in the F / B ratio, Firmicutes, and *Bacteroides fragilis* group even in the non-constipation group.

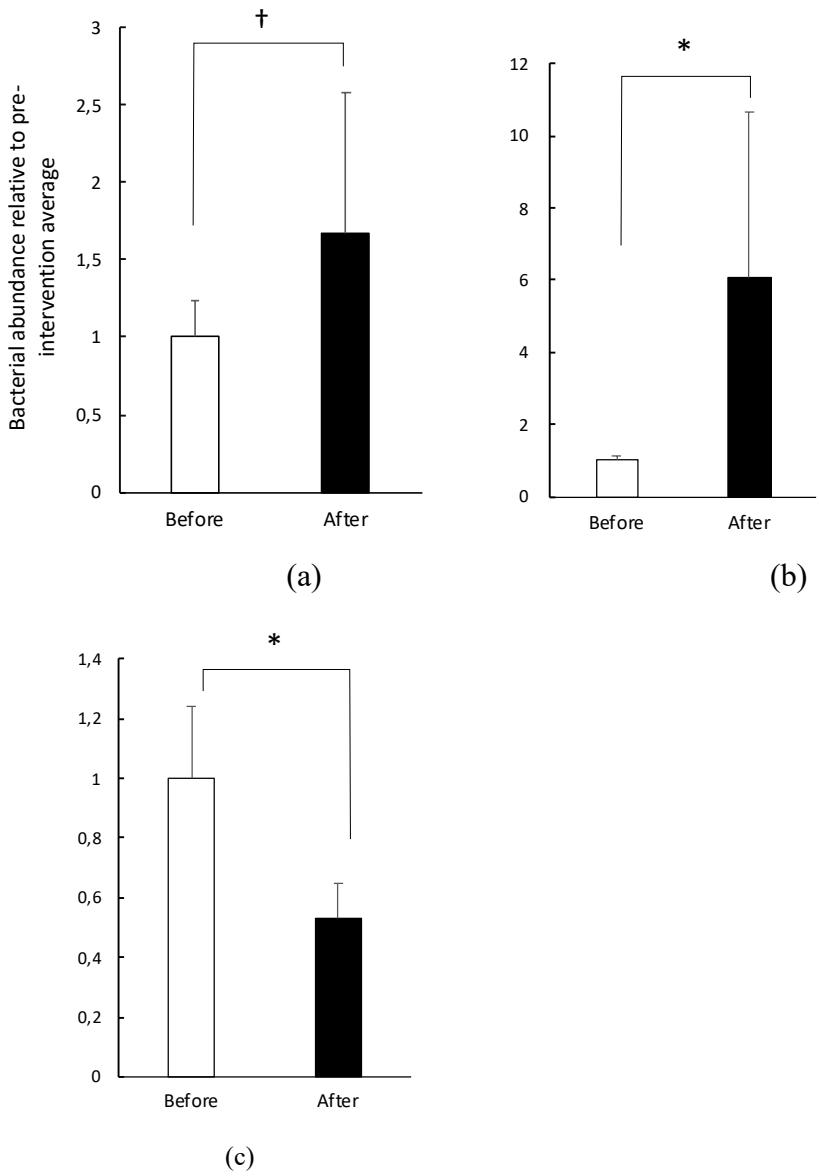


Figure 12. Bacterial abundance relative to pre-intervention average (a) *Clostridium leptum* subgroup, (b) *Bacteroides fragilis* group, (c) *Desulfovibrio*

Data are expressed by the mean and standard error of estimation (Means ± SE). There is a significant difference within-group († $P < 0.1$, * $P < 0.05$) a) *Clostridium.leptum* subgroup, b) *Bacteroides fragilis* group, c) *Desulfovibrio*

A comparative study before and after the sweet drink intervention showed that the constipation group was 1.00 ± 0.55 before the *Bifidobacterium* intervention and the non-constipation group was 1.00 ± 0.58, and the constipation group was 3.20 ± 1.25 and the non-constipation group was 1.53 ± 1.38 after the

intervention (Figure 13a). Before the intervention, the constipation group was 1.00 ± 0.69, and after the intervention, the constipation group was 3.63 ± 2.84, and the non-constipation group was 2.12 ± 2.06 (Figure 13b).

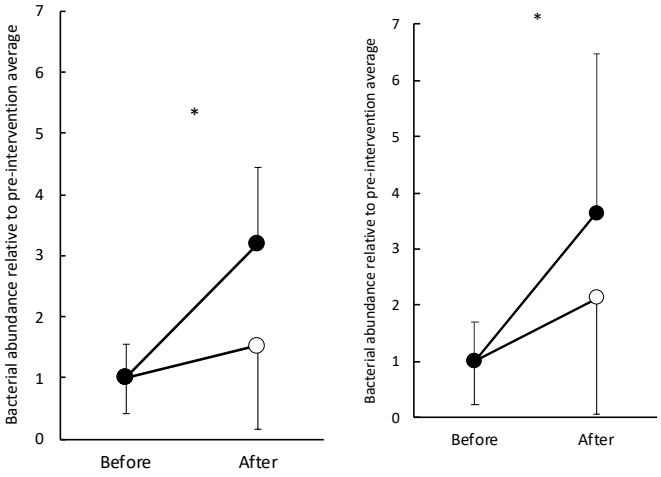


Figure 13. Bacterial abundance relative to pre-intervention average (a) *Bifidobacterium,* (b) *Akkermansia*

Data are expressed by the mean and standard error of estimation (Means ± SE).
There is a significant difference within-group (*$P < 0.05$)
a) *Bifidobacterium*, b) *Akkermansia*

DISCUSSION

This study is based on the hypothesis that improvement of the intestinal environment is involved in the effect of amazake intake on improving constipation. The study found there were changes in the intestinal environment and constipation symptoms before and after the 6-week drinking test of rice koji amazake in middle-aged and elderly people with constipation symptoms. There were improvements in CAS and BSS and changes of *Bifidobacterium* and *Akkermansia*. These results support previous studies that show amazake intake has an effect on relieving constipation. Our study found that there was no age difference between the constipation group and the non-constipation group, and there was no gender difference in the constipation-improving effect of amazake. The originality of this study was that in middle-aged and elderly people with constipation symptoms, both the intestinal environment and constipation symptoms changed before and after ingestion of amazake.

In this study, changes in the intestinal bacterial flora due to ingestion of rice koji amazake were confirmed, but this study is the first report on changes in the intestinal bacterial flora in humans. There are several reports on the functional components of rice koji amazake, and among them, oligosaccharides are mentioned as substances that affect intestinal bacterial flora. In addition, among oligosaccharides, glucose is a constituent of sugar, and the molecule has at least one binding mode such as an α -1,6 bond, α -1,2 bond, α -1,3 bond, and the degree of polymerization is 2 to 2. Oligosaccharides mainly composed of 6 are

collectively called isomaltooligosaccharides. The amazake in present study contains isomaltose (2.37 g / 100 g), panose (0.20 g / 100 g), and isomaltotriose (0.17 g / 100 g) among the isomaltose oligosaccharides. This isomaltooligosaccharide is contained in fermented foods such as miso, soy sauce, and sake, and acts as a prebiotic. It is known that oligosaccharides are hydrolyzed by intestinal bacteria and produce short-chain fatty acids such as acetic acid, butyric acid, and propionic acid. This short-chain fatty acid lowers the intestinal pH and has an intestinal regulating effect [109]. A study by Yen, CH et al found that after receiving 10 g of isomaltooligosaccharide daily to 13 constipation patients, there were increased significantly of *Bifidobacterium*, *Bacteroides*, and *Lactobacillus*, decreased of *Clostridium*, and increased stool volume and defecation frequency [110]. The amount of amazake used in this study was 35 g, and it is considered that the intake of isomaltooligosaccharide was lower than that in the previous study, but a significant increase in *Bifidobacterium* in the constipation group (n = 7) was observed in this study (Figure 13a). It is suggested that the function of such isomaltooligosaccharides contributed, and as a result, the stool properties were improved, and the constipation symptoms were improved. Also, elderly people have decreased masticatory and swallowing functions, and it is difficult to eat a diet high in dietary fiber. A low fiber diet contributes to constipation, but isomaltooligosaccharide supplementation has been reported to have a beneficial effect on relieving constipation in the elderly [111].

Dysbiosis will occur when feces were in the intestine for a long time due to constipation. There is a changing of bacteria such as an increase in bacteria

belonging to Firmicutes and *Clostridium* and a decrease in *Bifidobacterium*. It has also been reported that *Bifidobacterium* is reduced in patients with irritable bowel syndrome compared with healthy subjects and that *Clostridium leptum* subgroup is less in inflammatory bowel disease [112]. The proglutamyl peptide, which is present in fermented foods and has been reported to show significant physiological function when administered orally at a relatively low dose in colitis in an animal study [113]. *Desulfovibrio* has strongly active LPS and has been suggested to be involved in the inflammatory response. In this study, it was shown that *Desulfovibrio* was reduced by amazake intervention, suggesting that the influence of proglutamyl peptide contained in amazake may be partly involved in this mechanism. The *Bacteroides. fragilis* group has polysaccharide A (PSA) on the cell surface, which has been reported to exert T cell-dependent and independent immunostimulatory activity [114,115]. It has been reported that in the colon, Treg cells are also induced in the *Clostridium leptum* subgroup, which suppresses gastrointestinal inflammation and systemic allergic reactions [116]. The increase in *Bacteroides fragilis* group and *Clostridium leptum* subgroup due to amazake drinking in this study suggests that the inflammatory reaction of the intestinal tract may be suppressed. On the other hand, *Akkermansia,* whose increase was confirmed in this study, is greatly involved in glucose metabolism, lipid metabolism, and intestinal immunity promotes intestinal mucus secretion and strengthens the barrier function. It has also been reported that polyphenols increase *Akkermansia* [117,118]. The present study showed that the intervention

with rice-koji amazake for 6 weeks improved constipation and dysbiosis in middle-aged and elderly.

Chapter V

Conclusion Remark

Protein-energy nutritional status in moderately low protein intake-sago diets showed there was no significant difference compared to sufficient protein intake-rice diets of well-nourished lowlanders in Papua, Indonesia. Different predictors on albumin between the two groups suggest there is an adaptive mechanism to maintain albumin levels within the normal range. We assumed that there was decreasing protein turnover (protein synthesis, amino acid oxidation, protein degradation) with maintained post-absorptive whole-body protein and basal muscle protein synthesis as the mechanism of long-term low protein intakes. Lower MCV level was the inducing factor of adaptive mechanism in the moderately low protein intake-sago diet.

Gut microbiota had an important role in adaptation to moderately low protein intake-sago diet to maintain protein-energy nutritional status within a normal value. The increase of *Eubacterium rectale*, an increase of *Succinivibrio*, and a decrease of *Collinsella* maintained the albumin level within the normal range. Maintaining gut barrier function through producing SCFA (butyrate, acetate, and succinate), and reducing inflammation (proteolysis) were the suggestive pathways on maintaining albumin level in the normal value in moderately low protein intake. The mechanism of adaptation was more understandable through the difference in the gut microbiota profile of both groups.

The rice-koji amazake improves constipation symptoms in the constipation group of middle-aged and elderly. Rice-koji amazake can act as a prebiotic that improves dysbiosis through decreasing *Desulfovibrio*, increasing *Bacteroides fragilis* in the constipated and non-constipated group, and increasing

Bifidobacterium, and *Akkermansia* in the constipated group. Lowering inflammation improves gut function and is expected to improve the protein-energy nutritional status of the elderly later on. Another intervention study (sub-study) in undernourished hospitalized elderly found that amazake given for 6 weeks improves dysbiosis and increases geriatric nutritional risk index (GNRI) and albumin level but there is no improvement of BSS and CAS because of laxative use. The high risk of malnutrition in the elderly can be prevented by modifying gut microbiota profiles to maintain gut barrier function. Based on our study (main study and sub-study), protein-energy nutritional status can be affected by gut microbiota composition.

REFERENCES

[1] United Nations. Department of Economic and Social Affairs. World Population Ageing 2019 Highlights. United Nations, New York, 2019; pp. 1–13.

[2] Kido Y. The Issue of Nutrition in an Aging Society. *J Nutr Sci Vitaminol.* 2015;61 Suppl:S176-S177.

[3] Miyake, M.; Ogawa, Y.; Yoshida, Y.; Imaki, M. Seven-year large cohort study for the association of serum albumin level and aging among community dwelling elderly. *Journal of Analytical Bio-Science* 2011, 34, 281-286.

[4] Gomi, I.; Fukushima, H.; Shiraki, M.; Miwa, Y.; Ando, T.; Takai, K.; Moriwaki, H. Relationship between serum albumin level and aging in community-dwelling self-supported elderly population. *J. Nutr.Sci.Vitaminol.* 2007, 53, 37–42.

[5] Cabrerizo S, Cuadras D, Gomez-Busto F, Artaza-Artabe I, Marín-Ciancas F, Malafarina V. Serum albumin and health in older people: Review and meta-analysis. *Maturitas*. 2015;81(1):17-27.

[6] Boirie Y, Morio B, Caumon E, Cano NJ. Nutrition and protein energy homeostasis in elderly. *Mech Ageing Dev*. 2014;136-137:76-84.

[7] Ni Lochlainn M, Bowyer RCE, Steves CJ. Dietary Protein and Muscle in Aging People: The Potential Role of the Gut Microbiome. *Nutrients*. 2018;10 (7):929.

[8] Kim M, Benayoun BA. The microbiome: An emerging key player in aging and longevity. *Translational Medicine of Aging*. 2020;4 :103–16.

[9] de Marco Castro E, Murphy CH, Roche HM. Targeting the Gut Microbiota to Improve Dietary Protein Efficacy to Mitigate Sarcopenia. *Front Nutr*. 2021;8:656730.

[10] Ghosh, S.; Suri, D.; Uauy, R. Assessment of protein adequacy in developing countries: quality matters. *Food Nutr Bull*. 2013, 34, 244-246.

[11] Ruel, M.T.; Haris, J.; Cunningham K. Diet Quality in Developing Countries. In Diet Quality: An Evidence-Based Approach, V.R.Preedy et.al, Eds.;Springer Science+Business Media, New York, USA, 2013; Volume 2, pp. 239-260.

[12] Hiroshi E; Tokada Y; Johnson; Dennis V. Sago Palm Multiple Contributions to Food Security and Sustainable Livelihoods, Springer Nature: Singapore, Singapore, 2018; pp.10-62.

[13] Bantacut, T. Sagu: Sumberdaya untuk Penganekaragaman Pangan Pokok. *Pangan* 2011, 20, 27–40.

[14] Girsang, W. Sago Food Product Development for Food Security in Small Islands, Maluku, Indonesia. *Int. J. Sci. Eng Res*. 2017, 8, 704-712.

[15] Metaragakusuma, A.; Katsuya, O.; Bai, H. An Overview of The Traditional Use of Sago for Sago-based Food Industry in Indonesia. *KnE Life Sciences*, 2016, 3, 119-124.

[16] Girsang, W. Socio-Economic Factors That Have Influenced the Decline of Sago Consumption in Small Islands: A Case in Rural Maluku, Indonesia. *South Pacific Studies*. 2014, 34, 99-116.

[17] Fearnside, P. Transmigration in Indonesia: Lessons from Its Environmental and Social Impacts. *Environ Manage* . 1997,21, 553–570.

[18] Rice Policy Analysis in Indonesia: Then and Now**.** Available online at https://www.researchgate.net/publication/344250375. (Accessed on 3 August 2021).

[19] Wells JC, Sawaya AL, Wibaek R, Mwangome M, Poullas MS, Yajnik CS, Demaio A. The double burden of malnutrition: aetiological pathways and consequences for health. Lancet. 2020 ;395(10217):75-88.

[20] Tidjani Alou M, Lagier J-C, Raoult D. Diet influence on the gut microbiota and dysbiosis related to nutritional disorders. *Human Microbiome Journal*. 2016;1:3–11.

[21] Turua, U.; Hadi, S.; Juanda, B.; Murniningtyas, E. Ekologi dan Budaya Petani Asli Papua dalam Usaha Tani di Kabupaten Keerom. *Sosiohumiora*. 2014, 16, 234-241.

[22] Ministry of Agriculture's Food Security Agency of Indonesia (Badan Ketahanan Pangan Kementerian Pertanian). Indeks Ketahanan Pangan Indonesia (Indonesian Food Security Index). Ministry of Agriculture of Indonesia: Jakarta, Indonesia, 2018; pp.8-13.

[23] Syartiwidya; Martianto, D.; Sulaeman, A.; Tanziha, I.; Rimbawan. Preference for Sago and Nutrient Intake among Communities Consuming Sago in Kepulauan Meranti District, Riau Province, Indonesia. *J. Gizi Pangan*, 2019, 14, 91-98.

[24] Directorate General of Public Health Directorate of Public Nutrition of Indonesia (Direktorat Jenderal Kesehatan Masyarakat. Direktorat Gizi Masyarakat). Tabel Komposisi Pangan Indonesia (Indonesian Food Composition Table). Ministry of Health of Indonesia: Jakarta, Indonesia, 2018; pp.9-15.

[25] Okuda T.; Kajiwara, N.; Date, C.; Sugimoto, K.; Rikimaru, T.; Fujita, Y.; Koishi, H. Nutritional Status of Papua New Guinea Highlanders. *J. Nutr.Sci.Vitaminol*. 1981, 27, 319-331.

[26] Purwani, E.P.; Purwadaria, T.; Suhartono, M.T. Fermentation RS3 derived from sago and rice starch with Clostridium butyricum BCC B2571 or Eubacterium rectale DSM 17629. *Anaerobe*. 2012, 18, 55-61.

[27] Research and Health Development Unit of Ministry of Heatlh of Indonesia (Badan Penelitian dan Pengembangan Kesehatan). Laporan Nasional RISKESDAS 2018 (Report of National Research and Basic Health 2018). Ministry of Health of Indonesia, Jakarta, Indonesia, 2018; pp. 562.

[28] Research and Health Development Unit of Ministry of Heatlh of Indonesia (Badan Penelitian dan Pengembangan Kesehatan). Laporan Provinsi Papua RISKESDAS 2018 (Report of Papua Province Research and Basic Health 2018). Ministry of Health of Indonesia, Jakarta, Indonesia, 2018; pp. 411.

[29] Fikawati S, Syafiq A, Ririyanti R, Gemily S. Energy and protein intakes are associated with stunting among preschool children in Central Jakarta, Indonesia: a case-control study. *Mal J Nutr*, 2021, 27, 081–091

[30] Millward, D.J. An adaptive metabolic demand model for protein and amino acid requirements. *Br J Nutr* 2003, 90, 249-260.

[31] Young, V.R.; Marchini, J.S. Mechanisms and nutritional significance of metabolic responses to altered intakes of protein and amino acids, with reference to nutritional adaptation in humans. *Am J Clin Nutr*. 1990, 51, 270-289.

[32] Huang, P.C.; Lee, N.Y.; Chen, S.H. Evidences suggestive of no intestinal nitrogen fixation for improving protein nutrition status in sweet potato eaters. *Am J Clin Nutr* 1979, 32, 1741-1750.

[33] Naito, Y.I;, Morita, A; Natsuhara, K.; Tadokoro, K.; Baba, J.; Odani, S.; Tomitsuka, E.; Igai, K.; Tsutaya, T.; Yoneda, M.; Greenhill, A.R.; Horwood, P.F.; Soli, K.W.; Phuanukoonnon, S.; Siba, P.M.; Umezaki, M. Association of protein intakes and variation of diet-scalp hair nitrogen

isotopic discrimination factor in Papua New Guinea highlanders. *Am J Phys Anthropol*, 2015, 158, 359-370.

[34] Morita, A.; Natsuhara, K.; Tomitsuka, E.; Odani, S.; Baba, J.; Tadokoro, K.; Igai, K.; Greenhill, A.R.; Horwood, P.F.; Soli, K.W;, Phuanukoonnon, S.; Siba, P.M.; Umezaki, M. Development, validation, and use of a semi-quantitative food frequency questionnaire for assessing protein intake in Papua New Guinean Highlanders. *Am J Hum Biol* 2015, 27, 349-357.

[35] Yang, S-H. Relationship Between Mean Corpuscular Volume and Liver Function Test. *Korean J Clin Lab Sci* 1996, 28, 134–139.

[36] Higashida, K.; Inoue, S.; Nakai, N. Iron deficiency attenuates protein synthesis stimulated by branched-chain amino acids and insulin in myotubes. *Biochemical and Biophysical Research Communications* 2020, 531, 112–117.

[37] Hoffenberg, R.; Black, E.; Brock, J.F. Albumin and gamma-globulin tracer studies in protein depletion states. *J Clin Invest* 1966, 45, 143-152.

[38] James, W.P.T.; Hay, A.M. Albumin metabolism: effect of the nutritional state and the dietary protein intake. *J Clin Invest* 1968, 47, 1958–1972.

[39] Pain, V.M.; Clemens, M.J.; Garlick, P.J. The effect of dietary protein deficiency on albumin synthesis and on the concentration of active albumin messenger ribonucleic acid in rat liver. *Biochem J* 1978, 172, 129–135.

[40] Motil, K.J.; Matthews, D.E.; Bier, D.M.; Burke, J.F.; Munro, H.N.; Young, V.R. Whole-body leucine and lysine metabolism: response to dietary protein intake in young men. *Am J Physiol* 1981, 240, 712-721.

[41] Levitt, D.G.; Levitt, M.D. Human serum albumin homeostasis: a new look at the roles of synthesis, catabolism, renal and gastrointestinal excretion, and the clinical value of serum albumin measurements. *Int J Gen Med*. 2016, 9, 229-255.

[42] Caso, G.; Scalfi, L.; Marra, M.; Covino, A.; Muscaritoli, M.; McNurlan, M.A.; Garlick, P.J.; Contaldo, F. Albumin synthesis is diminished in men consuming a predominantly vegetarian diet. *J Nutr* 2000, 130, 528-533.

[43] Arques, S. Serum albumin and cardiovascular disease: State-of-the-art review. *Ann Cardiol Angéio*l 2020, 69, 192–200.

[44] Hirata, T.; Arai, Y.; Yuasa, S.; Abe, Y.; Takayama, M.; Sasaki, T., Kunitomi, A.; Inagaki, H.; Endo, M.; Morinaga, J.; Yoshimura, K.;

Adachi, T.; Oike, Y.; Takebayashi, T.; Okano, H.; Hirose, N. Associations of cardiovascular biomarkers and plasma albumin with exceptional survival to the highest ages. *Nat Commun*. 2020, 11, 3820.

[45] Garibotto, G.; Picciotto, D.; Saio, M.; Esposito, P.; Verzola, D. Muscle protein turnover and low-protein diets in patients with chronic kidney disease. *Nephrol Dial Transplant* 2020, 35, 741–751.

[46] Hursel, R.; Martens, E.A.; Gonnissen, H.K.; Hamer, H.M.; Senden, J.M.; van Loon L.J.; Westerterp-Plantenga M.S. Prolonged Adaptation to a Low or High Protein Diet Does Not Modulate Basal Muscle Protein Synthesis Rates - A Substudy. *PLoS One* 2015, 10, e0137183.

[47] Mosoni, L.; Malmezat, T.; Valluy, M-C.; Houlier M-L.; Mirand, P.P. Muscle and Liver Protein Synthesis Adapt Efficiently to Food Deprivation and Refeeding in 12-Month-Old Rats. *J Nutr*. 1996, 126, 516–22.

[48] Visscher, C.; Middendorf, L.; Günther, R.; Engels, A.; Leibfacher, C.; Möhle, H.; Düngelhoef, K.; Weier, S.; Haider, W.; Radko, D. Fat content, fatty acid pattern and iron content in livers of turkeys with hepatic lipidosis. *Lipids Health Dis*. 2017, 16, 98.

[49] Siddique, A.; Nelson, J.; Aouizerat, B.; Yeh, M.M.; Kowdley, K.V. Iron Deficiency in Patients with Nonalcoholic Fatty Liver Disease Is Associated with Obesity, Female Gender, and Low Serum Hepcidin. *Clin Gastroenterol and Hepatol* 2014, 12, 1170-1178.

[50] Britton, L.J.; Subramaniam, V.N.; Crawford, D.H. Iron and non-alcoholic fatty liver disease. *World J Gastroenterol* 2016, 22, 8112–8122.

[51] Du, F.; Higginbotham, D.A.; White B. D. Food intake, energy balance and serum leptin concentrations in rats fed low-protein diets. *J Nutr* 2000, 130, 514-21.

[52] Pezeshki, A.; Zapata, R.; Singh., A.; Yee, N.J.; Chelikani, P.K. Low protein diets produce divergent effects on energy balance. *Sci Rep* 2016, 6, 25145.

[53] Miyatani, S.; Okuda, T.; Koishi, H. Basal metabolism of Papua New Guinea highlanders. *Japanese J. Phys. Fit. Sports Med*. 1988, 37, 296–302.

[54] Maurer, J.; Taren, D.L.; Teixeira, P.J.; Thomson, C.A.; Lohman, T.G.; Going, S.B.; Houtkooper, L.B. The psychosocial and behavioral characteristics related to energy misreporting. *Nutr Rev*. 2006, 64, 53-66.

[55] Olendzki, B.C.; Ma, Y.; Hébert, J.R.; Pagoto, S.; Merriam, P.; Rosal, M.; Ockene, I.S. Underreporting of energy intake and associated factors in a Latino population at risk of developing type 2 diabetes. *J Am Diet Assoc*. 2008, 108, 1003-1008.

[56] Sawaya, A.L; Tucker, K.; Tsay, R.; Willett, W.; Saltzman, E.; Dallal, G.E.; Roberts, S.B. Evaluation of four methods for determining energy intake in young and older women: comparison with doubly labeled water measurements of total energy expenditure. *Am J Clin Nutr*. 1996, 63, 491-499.

[57] Yamamoto, M.; Adachi, H.; Enomoto, M.; Fukami, A.; Nakamura, S.; Nohara, Y.; Sakaue, A.; Morikawa, N.; Hamamura, H.; Toyomasu, K.; Fukumoto, Y. Lower albumin levels are associated with frailty measures, trace elements, and an inflammation marker in a cross-sectional study in Tanushimaru. *Environ. Health Prev. Med*. 2021, 26, 25.

[58] Conlon, M. A., & Bird, A. R. (2014). The impact of diet and lifestyle on gut microbiota and human health. *Nutrients*, *7*(1), 17–44.

[59] Kashtanova DA, Popenko AS, Tkacheva ON, Tyakht AB, Alexeev DG, Boytsov SA. Association between the gut microbiota and diet: Fetal life, early childhood, and further life. *Nutrition*. 2016;32(6):620-627.

[60] Plovier H, Cani PD. Microbial Impact on Host Metabolism: Opportunities for Novel Treatments of Nutritional Disorders?. *Microbiol Spectr*. 2017; 5(3).

[61] Mishra AK, Dubey V, Ghosh AR. Obesity: An overview of possible role(s) of gut hormones, lipid sensing and gut microbiota. *Metabolism*. 2016; *65* :48-65.

[62] Syauki AY, Ogawa A, Simanjuntak URP et al. Protein-Energy Nutritional Status of Moderately Low Protein Intake-Sago Diets Compared to Sufficiently Protein Intake-Rice Diets in Well-Nourished Lowlanders in Papua, Indonesia [version 1; peer review: 1 approved with reservations]. F1000Research 2022, 11:138

[63] Greenhill AR, Tsuji H, Ogata K, et al. Characterization of the gut microbiota of Papua New Guineans using reverse transcription quantitative PCR. *PLoS One*. 2015;10(2):e0117427.

[64] Hosomi, K.; Murakami, H.; Natsume-Kitatani, Y.; Tanisawa, K.; Hirata, S.; Suzuki, H.; Nagatake, T.; Nishino, T.; Mizuguchi, K.; Miyachi, M.; et al. Method for preparing DNA from feces in guanidine thiocyanate

solution affects 16S rRNA-based profiling of human microbiota diversity. *Sci. Rep.* 2017, 7, 1–10.

[65] Klindworth, A.; Pruesse, E.; Sheweer, T.; Peplies, J.; Quast, C.; Horn, M.; Glockner, F.O. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 2013, 4*1*, e1.

[66] Mohsen, A.; Park, J.; Kawashima, H.; Chen, Y.A.; Natsume-Kitatani, Y.; Mizuguchi, K. Auto-q Qiime analysis automating script. *Zenodo* 2018, 10, 1.

[67] Caporaso, J.G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.; Costello, E.K.; Fierer, N.; Pena, A.G.; Goodrich, J.K.; Gordon, J.I.; et al. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 2010, 7, 335–336.

[68] Edgar, R.C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010, 26, 2460–2461.

[69] Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glockner, F.O. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* 2013, 41, 590–596

[70] Hapsari, W. Tambelo (Bactronophorus Thoracites): Local traditional food of Kamoro people in Hiripao district. (Pangan lokal tradisional orang Kamoro di Kampung Hiripao). *Papua*, 2013.

[71] Nakayama, J. Health status of Japanese and Asians indicated by gut microbiome research. *Journal for the Integrated Study of Dietary Habits.* 29(3):137-140.

[72] Precup G, Vodnar DC. Gut *Prevotella* as a possible biomarker of diet and its eubiotic versus dysbiotic roles: a comprehensive literature review. *Br J Nutr*. 2019;122(2):131-140.

[73] Graf D, Di Cagno R, Fåk F, et al. Contribution of diet to the composition of the human gut microbiota. *Microb Ecol Health Dis*. 2015;26:26164.

[74] Wu GD, Chen J, Hoffmann C, et al. Linking long-term dietary patterns with gut microbial enterotypes. *Science*. 2011;334(6052):105-108.

[75] Surono IS, Widiyanti D, Kusumo PD, Venema K. Gut microbiota profile of Indonesian stunted children and children with normal nutritional status. *PLoS One*. 2021;16(1):e0245399.

[76] Martínez I, Stegen JC, Maldonado-Gómez MX, et al. The gut microbiota of rural papua new guineans: composition, diversity patterns, and ecological processes. *Cell Rep*. 2015;11(4):527-538.

[77] Senghor B, Sokhna C, Ruimy R, Lagier J-C. Gut microbiota diversity according to dietary habits and geographical provenance. Human Microbiome Journal. 2018 1;7.

[78] Dwiyanto J, Hussain MH, Reidpath D, et al. Ethnicity influences the gut microbiota of individuals sharing a geographical location: a cross-sectional study from a middle-income country. *Sci Rep*. 2021;11(1):2618.

[79] Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. Microbial degradation of complex carbohydrates in the gut. *Gut Microbes*. 2012;3(4):289-306.

[80] Zhao Y, Tian G, Chen D, et al. Dietary protein levels and amino acid supplementation patterns alter the composition and functions of colonic microbiota in pigs. *Anim Nutr*. 2020;6(2):143-151.

[81] Odamaki T, Kato K, Sugahara H, et al. Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. *BMC Microbiol*. 2016;16:90.

[82] Cockburn D, Orlovsky N, Foley M, Kwiatkowski K, Bahr C, Maynard M, et al. Molecular details of a starch utilization pathway in the human gut symbiont Eubacterium rectale. Molecular Microbiology. 2014 Nov 1;95.

[83] Baxter, N. T., Schmidt, A. W., Venkataraman, A., Kim, K. S., Waldron, C., & Schmidt, T. M. (2019). Dynamics of Human Gut Microbiota and Short-Chain Fatty Acids in Response to Dietary Interventions with Three Fermentable Fibers. *mBio*, *10*(1), e02566-18.

[84] De Filippo C, Di Paola M, Ramazzotti M, et al. Diet, Environments, and Gut Microbiota. A Preliminary Investigation in Children Living in Rural and Urban Burkina Faso and Italy. *Front Microbiol*. 2017;8:1979.

[85] Zhao J, Zhang X, Liu H, Brown MA, Qiao S. Dietary Protein and Gut Microbiota Composition and Function. *Curr Protein Pept Sci*. 2019;20(2):145-154.

[86] Hailemariam S, Zhao S, Wang J. Complete Genome Sequencing and Transcriptome Analysis of Nitrogen Metabolism of *Succinivibrio dextrinosolvens* Strain Z6 Isolated From Dairy Cow Rumen. *Front Microbiol*. 2020;11:1826.

[87] Van Hul M, Le Roy T, Prifti E, et al. From correlation to causality: the case of *Subdoligranulum*. *Gut Microbes*. 2020;12(1):1-13.

[88] Henderson G, Yilmaz P, Kumar S, et al. Improved taxonomic assignment of rumen bacterial 16S rRNA sequences using a revised SILVA taxonomic framework. *PeerJ*. 2019;7:e6496.

[89] Gomez-Arango LF, Barrett HL, Wilkinson SA, et al. Low dietary fiber intake increases Collinsella abundance in the gut microbiota of overweight and obese pregnant women. *Gut Microbes*. 2018;9(3):189-201.

[90] Medawar E, Haange SB, Rolle-Kampczyk U, et al. Gut microbiota link dietary fiber intake and short-chain fatty acid metabolism with eating behavior. *Transl Psychiatry*. 2021;11(1):500.

[91] Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther*. 2008;27(2):104-119.

[92] Bedford A, Gong J. Implications of butyrate and its derivatives for gut health and animal production. *Anim Nutr*. 2018;4(2):151-159.

[93] Hamer HM, Jonkers DM, Bast A, et al. Butyrate modulates oxidative stress in the colonic mucosa of healthy humans. *Clin Nutr*. 2009;28(1):88-93.

[94] O'Herrin S, Kenealy W. Glucose and carbon dioxide metabolism by Succinivibrio dextrinosolvens. *Applied and environmental microbiology*. 1993; *59* :748–55.

[95] Menni C, Louca P, Berry SE, et al. High intake of vegetables is linked to lower white blood cell profile and the effect is mediated by the gut microbiome. *BMC Med*. 2021;19(1):37.

[96] Chen J, Wright K, Davis JM, et al. An expansion of rare lineage intestinal microbes characterizes rheumatoid arthritis. *Genome Med*. 2016;8(1):43.

[97] Astbury S, Atallah E, Vijay A, Aithal GP, Grove JI, Valdes AM. Lower gut microbiome diversity and higher abundance of proinflammatory

genus *Collinsella* are associated with biopsy-proven nonalcoholic steatohepatitis. *Gut Microbes*. 2020;11(3):569-580.

[98] Japan Gastroenterological Society Related Study Group: Chronic Constipation Diagnosis and Treatment Study Group. Definition of constipation, Chronic constipation clinical practice guideline. 2017. Tokyo

[99] Kato T, Honda Y, Kurita Y, Iwasaki A, Sato T, Kessoku T, Uchiyama S, Ogawa Y, Ohkubo H, Higurashi T, Yamanaka T, Usuda H, Wada K, Nakajima A: Lubiprostone improves intestinal permeability in humans, a novel therapy for the leaky gut: A prospective randomized pilot study in healthy volunteers. *PLoS One*, 2017, 12: e0175626.

[100] Rennie MJ: Anabolic resistance: the effects of aging, sexual dimorphism, and immobilization on human muscle protein turnover. *Appl Physiol Nutr Metab*, 2009. 34: 377-81.

[101] Shota Takano V. Diet, exercise, and physical therapy for chronic constipation. *Journal of the Japanese Society of Colorectal and Anal Diseases*, 2019. 72: 621-27.

[102] Nagao Y, Sata M. Effect of a Late Evening Snack of Amazake in Patients with Liver Cirrhosis: A Pilot Study. *Journal of Nutrition & Food Sciences*, 2013. 3: 1000223.

[103] Yumi Uehara, Kazumi Yanagisawa, Izumi Yoshida, Kazuhiko Uchiyama, Aya Suwa, Osamu Kashimura: Constipation improving effect of rice koji amazake on dialysis patients. *Journal of Japanese Society for Dialysis Therapy*, 2017. 50: 506.

[104] Mitsuhito Sakurai, Masatoshi Kubota, Akira Iguchi, Toru Shigematsu, Toshio Yamaguchi, Atsushi Kurahashi, Yoshifumi Oguro, Toshikazu Nishiwaki, Kotaro Aihara, Shinji Sato: Fermentation of lactic acid bacteria and aspergillus on the defecation status of healthy adults with relatively few defecations Impact of Amazake. Proceedings of the 65th Annual Meeting of the Japanese Society of Food Science and Technology. 2018: 81.

[105] Sumiyoshi K, Nakao M: Effect of Amazake Ingestion on Constipation. *Japanese Journal of Nursing Art and Science*, 2017. 16: 36-40.

[106] Lewis SJ, Heaton KW: Stool form scale as a useful guide to intestinal transit time. *Scand J Gastroenterol*, 1997. 32: 920-4.

[107] McMillan SC, Williams FA: Validity and reliability of the Constipation Assessment Scale. *Cancer Nurs*, 1989. 12: 183-8.

[108] O'Donnell LJ, Virjee J, Heaton KW: Detection of pseudodiarrhoea by simple clinical assessment of intestinal transit rate. *BMJ*. 1990. 300: 439-40.

[109] Mitsuoka T: Research in Intestinal Flora and Functional Foods. *Biosci Microflora*, 2002. 15: 57-89.

[110] Yen CH, Tseng YH, Kuo YW, Lee MC, Chen HL: Long-term supplementation of isomalto-oligosaccharides improved colonic microflora profile, bowel function, and blood cholesterol levels in constipated elderly people--a placebo-controlled, diet-controlled trial. *Nutrition*, 2011. 27: 445-50.

[111] Chen HL, Lu YH, Lin JJ, Ko LY: Effects of isomalto-oligosaccharides on bowel functions and indicators of nutritional status in constipated elderly men. *J Am Coll Nutr*, 2001. 20: 44-9.

[112] Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, Taniguchi T, Takeda K, Hori S, Ivanov, II, Umesaki Y, Itoh K, Honda K: Induction of colonic regulatory T cells by indigenous Clostridium species. *Science*, 2011. 331: 337-41.

[113] Kenji Sato, Tamami Kiyono: Modified Peptides in Foods: Structure and Function of Pyroglutamyl Peptides. Foods & food ingredients journal of Japan: *FFI Journal*, 2017. 222: 216-22.

[114] Yoshinori Umezaki: Gut Bacteria and Bacterial Species Development Special Feature / Gut-Brain Axis-Microbiome-Gut-Brain Axis-, 2014. 22: 155-61.

[115] Tomohisa Musino, Takanori Kanai: Comprehensive study on Treg, Th17, Th17 / Th1, Th1 cell production induction, competitiveness, and plasticity in intestinal chronic inflammatory bowel disease. *Journal of the Japanese Society of Clinical Immunology*, 2012. 35: 399-411.

[116] Li YN, Huang F, Cheng HJ, Li SY, Liu L, Wang LY: Intestine-derived Clostridium leptum induces murine tolerogenic dendritic cells and regulatory T cells in vitro. *Hum Immunol*, 2014, 75: 1232-8.

[117] Naito Y, Uchiyama K, Takagi T: A next-generation beneficial microbe: Akkermansia muciniphila. *J Clin Biochem Nutr*, 2018. 63: 33-35.

[118] K: Contribution of gut microbiota to etiology of human diseases outside of the gut. モダンメディア, 2014. 60: 356-68.

Acknowledgment

First and foremost, praises and greatest gratitude to Allah Subhana wa' ta ala for His blessing throughout my research work to complete the research successfully.

This dissertation becomes a reality with the kind support and help of many individuals. I would like to extend my sincere gratitude to all of them.

I would like to acknowledge and give my warmest gratitude to my supervisor Prof. Yasuyuki Irie, MD, Ph.D. for giving me the opportunity to do research and providing invaluable guidance throughout this research. His dynamism, vision, sincerity, and motivation have deeply inspired me. I would also like to appreciate him for his patience, empathy, and immense knowledge, I successfully overcame difficulties and learned a lot.

I cannot express enough gratitude for the feedback offered by Prof. Toshiko Yamamoto, Ph.D., Prof. Hiromi Yamashita, Ph.D., Prof. Koichi Tanaka, Ph.D., Prof. Kazuko Sumiyoshi, Ph.D., and Prof. Jinro Takato, Ph.D. to make my dissertation better. I offer my sincere appreciation for their continued support, insightful comments, and encouragement.

I owe my deepest gratitude to Prof. Dr. dr. Nurpudji A. Taslim, MPH, Sp.GK (K) as my teacher and senior colleague in the Nutrition Department, Faculty of Medicine, Hasanuddin University for her support, encouragement to pursue and finish my Ph.D. study.

I would like to express my special gratitude to Rikako Inoue, Ph.D., Aki Ogawa, Ph.D., and all Irie Lab members for all kind assistance and support while I pursue my doctoral course. I am not just getting friends, but also I got family here.

A warm of gratitude is also owed to Prof. Jun Kunisawa, Ph.D., Prof. Kenji Mizuguchi, Ph.D., Koji Hosomi, Ph.D., and Jonguk Park, Ph.D. from National

Institutes of Biomedical Innovation, Health, and Nutrition (NIBIOHN) for their support, time, and knowledge in completion of my research.

I am highly indebted to all teachers in the Department of Nutritional Science, Faculty of Health and Welfare Science, Okayama Prefectural University, and Satoko Hiramatsu for their help, support, and kind advice.

Lastly, I am extremely grateful to my parents (my late father and my mother) for their love, prayers, caring and sacrifices for educating and preparing for my future. Also, I express my gratitude to my late sister, my sister, my brother-in-law, and my nephews and niece for their support, prayer, and love.