MedCave Publications
Inviting Scientific Discovery

Medcave Journal of Environmental Science & Technology

MedCave Publications

Research Article

Open Access

Fungal profile of soil bacterial flora powder (Brown Bio) made from *okara* and rice bran fermentation

Atsushi Sasaki[1], Masahito Takahashi[2] and Shaw Watanabe[2]*

[1]Biosphere Institute, Daikyo-cho, Shinjuku-ku, Japan

[2]Medical Rice Association, Daikyo-cho, Shinjuku-ku, Japan

Corresponding Author: Dr. Shaw Watanabe, MD, Ph.D, 25-3-1004, Daikyo-cho, Shinjuku-ku, Tokyo 160-0015, Japan. Email: watashaw@lifescience.or.jp

Received Date: Aug 19, 2019; **Accepted Date:** Aug 28, 2019; **Published Date:** Aug 31, 2019

Abstract

Special fertilizer, the BrownBio powder was elaborated by mixinga special complex of soil fungi and okara and rice bran. Mixture of fungi contained proteolytic bacteria count; 6 × 105 ~ 2.4 × 107 cells /g dry weight, starch-degrading bacteria count; 1 × 105 ~ 4.6 × 106 cells /g dry weight, and oil-degrading bacteria count; 2 × 104 ~ 3.7 × 106 cells /g dry weight. The number of cellulolytic bacteria was 1.4 × 10 4 to 2.3 × 10 6 cells /g dry weight. Bacterial profile was determined in relation to the resolution of substrates. It was effective when the humus was mixed at work. The humus soil is a soil-like material made up of organic plant matter such as dead leaves and branches of hardwoods. Itworksto increase the overall strength of the soil, such as drainage, breathability, and fertilizer, and regenerates thin soil.

Introduction

The area around a poultry farm smells like ammonia and swarms of flies are always a big problem. There are many ways to remove odors by fermenting chicken dung into fertilizer. Usually, lactic acid bacteria are considered good for fermentation, so chicken dung and lactic acid bacteria are put into a fermenter and fermented. Since lactic acid bacteria are anaerobic bacteria, they are close to septic fermentation, and the odor is also very bad.

However, one poultry farm had no ammonia odor and completely ripe and smooth fertilizer was made from the fermenter. This chicken manure is close to ripe with organic matter with minerals and weakly acidic with no salt, has a lot of phosphorus, and so, it is the best fertilizer. Chinese cabbage made with this chicken manure fertilized well, had a sweet taste and at the same time gave off a strong smell of Chinese cabbage, and had a different taste from others made with ordinally chemical fertilizer. Cow barns that use fermented okara with these bacteria do not smell, as well.

Studies of human intestinal bacteria have shown that a good intestinal environment is made by diversity and symbiosis of bacterial flora. We think that soil fungi have a similar relationship, and Sasaki looked at the soil from various regions in Japan and obtained the same composition of fungus as an odorless chicken farm. This study describes the characteristics of the product, BrownBio, made from soil bacterial flora.

Soil improvers are those that apply soil and change their physical, chemical and biological properties. In Japan, it can be broadly divided into three categories: “fertilizers” defined by the Fertilizer Control Law, “soil conditioners” defined by the Geopower Promotion Law, and other soil conditioners. BrownBio has both fertilizer and soil conditioner properties.

Materials and Methods

Microbiological analysis

Brown Biopowder was made by fermentation of mixture of okara (by-product of tofu) and brown rice bran at a ratio of 8:2. (Hanzo Co. Ltd). Soil bacterial flora for fermentation was selected from various parts of Japan.

The water content of BrownBiopowder was determined from the dry weight of about 10 g of the sample. A 2.5-fold amount of distilled water was added to the sample to form a suspension, and the pH was measured by the glass electrode method. The EC (electric conductivity) of a similar suspension was measured with a Horiba EC meter. The salt concentration conversion value was obtained from the EC value (by automatic conversion of EC meter).

The microbial analyzing methods and their sources are shown in Table 1. Sterile physiological saline was added to 10 g of the sample to make a total volume of 100 mL, and the mixture was dispersed (1,5000 rpm, 15 minutes) using a homogenizer (Ace homogenizer, Nippon Seiki) with a stainless steel blade. A 100-fold diluted suspension was prepared from this dispersion and used as a sample for fluorescent staining. For the culture method, the stock solution to 106 diluted suspension was used and inoculated and cultured by the dilution plate method [1-10].

Genome analysis

Samples (approximately 50-100 mg) were extracted genome DNA, which used for PCR reaction by the Qubit fluorometer. The first step PCR (94C 2min, 94C 30 sec, 50C 30 sec, 72C 30 sec) x23 cycle was done by TaKaRaExTaq HS, and from which 20 ul was refined by AMPure XP beads. 2ul from it was used for the second step PCR (94C 2min, 94C 30 sec, 50C 30 sec, 72C 30 sec) x 8 cycles. The final product was refined by AMPure XP Beads, and the sequence analysis was done by the Miseq. Used softwares were sicklever 1.33 for quality filtering, Fastx toolkit ver. 0.0.13.2 for sequence streaming, FLASH ver. 1.2.10 for pair end connection, USEARCHver. 8.0.1623. i86 linex 64. The number of reads satisfying that the V4 region primer was completely identical, that both strands maintained a length of 130 bp or more, and that both strands were able to bind was 54,0688.

Citation: Sasaki A, Takahashi M, Watanabe S. Fungal profile of soil bacterial flora powder (Brown Bio) made from *okara* and rice bran fermentation. Medcave J Environ Sci Technol. 2019; 1:101.

Table 1: Method of bacteriological analysis.

Purpose	Analyzing method	Reference
Total number of bacteria	Ethidium bromide (EB) fluorescence	1
Total number of live bacteria	CFDA fluorescence	1
Number of Eutrophic bacteria	NA medium (5 days at 30C)	1,3
No. of Undernourished bacteria	DNB medium (3 weeks at 30C)	1,4
Proteolytic bacteria	Gelatin medium	1,7
Amyololytic bacteria	Starch medium	1,8
Oil-degrading bacteria	Tween 20 medium	1,9
Cellulolytic bacteria	CMC medium	1,10
Number of Coliform and *E. coli*	Coliform agar	2,5
Number of *Salmonella*	MLCB medium	2,6

Results

Sample appearance and physicochemical properties

Physicochemical properties of the samples are shown in Table 2. Four samples (A to D) were raw materials of bacteria products, all of which were powder and had a low moisture content of 3.8% to 11.5%. The sample pH was in the range of 4.4 to 6.0 and was slightly acidic. The EC was 3.9 to 7.0 mS / cm, and the salt equivalent concentration was 0.20 to 0.30%, which was presumed to contain a low concentration of salinity.

From the above results, it was clear that these products maintained a low moisture content that was good for product storage by preventing secondary fermentation. A slightly acidic pH indicates that an organic acid is produced during the cultivation under somewhat anaerobic conditions.

Microbial properties of the sample

The microbiological properties of the samples (the number of bacteria by fluorescent staining and culture methods) is shown in Table2, right column. The total number of bacteria in the sample by fluorescent staining was 1.3 x 108 to 7.4 x 109 cells /g.

The total number of viable bacteria was 1.1 × 108 to 4.1 × 109 cells /g dry weight. The viable cell ratio (ratio of total viable bacteria to all bacteria) was 30 to 82%. Numerous bacteria, such as gonococcus and short gonococcus, were observed inside and outside the powder particles. From these results, these 4 samples contained a large number of bacteria, and the viable cell rate was relatively high at 30-82%, so it remained in good condition during storage for 3-9 years. This indicates that the microorganism contained in the samples was preserved.

In particular, samples D have high viable cell ratios of 82% and 73% and low water content of 3.9%, indicating that low water content may be an important factor in the preservation of microorganisms.

Cultivated heterotrophic bacteria

Among heterotrophic bacteria that can be cultured, the number of eutrophic bacteria on SFT agar medium is 6.6 × 106 to 1.3 × 108 cells / g, and the number of undernourished bacteria on DNB medium is 2.1 × 106 to 8.2 × 107 cells /g (Table 3). In every sample, the number of eutrophic bacteria was higher than the number of undernourished bacteria, and it is assumed that many of the culturable bacteria are eutrophic. The colony formation rate (ratio of eutrophic bacteria among all bacteria) was 0.4 to 11%.

A low colony formation rate of 0.4 to 11% indicates that the bacteria contained during storage of the product are in a "viable, but nonculturable state (VNC state)". As for whether or not the bacteria in such a state in the sample are activated rapidly at the time of use and start to grow and exert their functions, it is expected that the possibility is high because the viable bacteria rate is high.

Pathogenicity indicator bacteria

Regarding the pathogenicity of the samples, coliforms were not detected. Neither Escherichia coli nor Salmonella was detected in any sample (detection limit: 30 CFU /g dry matter).

In Japan, there are no sanitation standards for microbial materials and compost regarding the measurement results of pathogenic indicator bacteria, but in Europe and the United States, there are standards that fecal coliforms are 1000 /g dry matter or less and Salmonella are not detected (US EPA).Sanitation standards for sludge fertilizers in Germany, fecal Escherichia coli is a microbial indicator.Even in light of these criteria, it is judged that there are no hygiene problems for all specimens examined.

Organic matter-degrading bacteria

The number of various organic matter-degrading bacteria was measured by the dilution plate method. Figure 1 shows examples of colony properties on various media. Table 4 shows the number of organic matter-degrading bacteria in 4 samples. Proteolytic bacteria count was 6 × 105 ~ 2.4 × 107 cells /g dry weight, starch-degrading bacteria count was 1 × 105 ~ 4.6 × 106 cells /g dry weight, and oil-degrading bacteria count was 2 × 104 ~ 3.7 × 106 cells /g dry matter The number of cellulolytic bacteria was 1.4 × 10 4 to 2.3 × 10 6 cells /g dry matter.

The above results indicate that these 4 samples contain various organic-degrading bacteria. However, considering that the number of various organic matter-degrading bacteria in excellent compost is roughly 106-108 cells /g dry matter, the number of various organic matter-degrading bacteria in these 4 samples was particularly high.

Considering that these samples have been storedover a long period of time, it is considered that the number of active bacteria was maintained relatively well.

Presence of individual bacteria in each bacterial group

Whether or not oleolytic bacteria, proteolytic bacteria, saccharide-decomposing bacteria, and cellulose-degrading bacteria are present in the detected microorganisms is ambiguous.The presence or absence of bacteria with distinctive features was examined.

Proteolytic bacteria: A typical protein-degrading bacterium is lactic acid bacteria. About 30 bacteria such as Lactobacillus genus, Leuconostoc genus and Weissella genus are known as lactic acid bacteria.From this specimen, eight families of genus Bifidobacterium,

Table 2: Physicochemical characteristics of soil bacterial flor powder (BrownBio).

Sample number	Passage	Moisture		NaCl (%)		Total number of bacteria	Total number of live bacteria	Living cell	Colony formation
			pH	Eq		EB method	CFBD method	%	%
A	1998	11.52	6.0	0.29		$(7.31\pm0.22)\times10^9$	$(4.14\pm0.02)\times10^9$	56.6	0.40
B	1999	10.89	4.7	0.30		$(4.10\pm0.18)\times10^9$	$(2.85\pm0.11)\times10^9$	69.5	4.5
C	2000	8.02	5.5	0.26		$(1.59\pm0.10)\times10^9$	$(4.84\pm0.23)\times10^8$	30.4	10.8
D	2004	3.88	4.4	0.26		$(1.31\pm0.11)\times10^8$	$(1.08\pm0.00)\times10^8$	82.4	6.1

Table 3: Number of organic material-degrading bacteria in the sample.

Sample Number	Eutrophic bacteria	Undernourished bacteria	Coliform	E. coli	Salmonella
number	NB agar medium	DNB agar medium	Chromo medium	Chromo medium	MLCB medium
A	$(1.66\pm0.24)\times10^7$	$(2.07\pm0.40)\times10^6$	<30	<30	<30
B	$(1.27\pm0.22)\times10^8$	$(8.23\pm2.33)\times10^7$	<30	<30	<30
C	$(5.22\pm1.24)\times10^7$	$(1.57\pm0.37)\times10^7$	<30	<30	<30
D	$(6.59\pm1.59)\times10^6$	$(3.57\pm0.70)\times10^6$	<30	<30	<30

Table 4: Number of organic matter-degrading bacteria in the sample.

Sample number	Proteolytic bacteria	Amylolytic bacteria	Oil-degrading bacteria	Cellulolytic bacteria
A	$(5.96\pm0.77)\times10^5$	$(1.62\pm0.45)\times10^5$	$(1.98\pm0.24)\times10^4$	$(1.89\pm1.30)\times10^4$
B	$(2.39\pm0.39)\times10^7$	$(3.56\pm0.75)\times10^6$	$(1.17\pm0.02)\times10^6$	$(2.34\pm0.18)\times10^6$
C	$(1.45\pm0.06)\times10^6$	$(3.34\pm0.55)\times10^5$	$(1.41\pm0.18)\times10^5$	$(1.41\pm0.18)\times10^5$
D	$(1.11\pm0.12)\times10^6$	$(1.07\pm0.16)\times10^5$	$(3.21\pm0.57)\times10^5$	$(1.38\pm0.60)\times10^4$

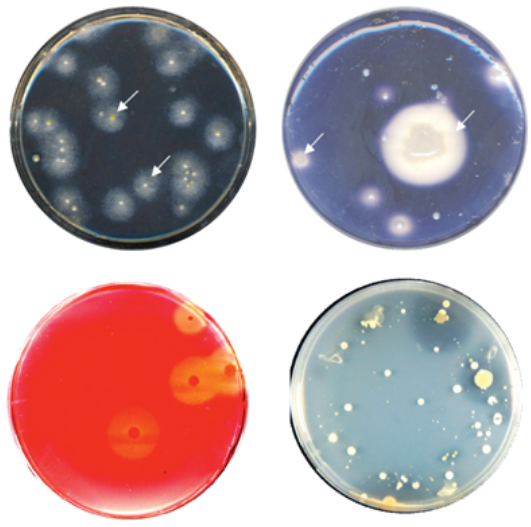

Figure 1: (left)* For Tween 20 degradation around the colony Formation of white cloudy zone of calcium salt (right) Starch-degrading bacteria on starch medium* On medium that became dark purple by Lugol To form a zona pellucida around the colony. (left) Cellulolytic bacteria on CMC medium Congo red dyes orange Zona pellucida around the colonies on the collected medium Forming. (right) Proteolytic bacteria on gelatin medium * On medium turbid due to gelatin precipitant Due to the degradation of gelatin around the colony Forms a zona pellucida.

Desemzia, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, and Weissellawere detected.In addition, it is known that the protein resolution of the genus Bacillus is also high.

Amylolytic bacteria: Glycolytic bacteria are a broad taxon. There is many bacteria belonging to Bifidobacteriaand Enterobacteria, and in this detection, many genera and species with glycolytic ability were included.

Oil-degrading bacteria: Actinomycetes, Nocardia genus and some Bacillus genera are known as oil-degrading bacteria.From this specimen, related Nocardioidesand Nocardiopsis genera were detected. In the genus Bacillus, Bacillus subtilis, which was actually used for the preparation of oil decomposition, was detected.

Cellulolytic bacteria: Cellulose, the most abundant polysaccharide in nature, is a rich source of renewable energy and sustains soil nutrients. Among the microorganisms known to degrade cellulose, bacteria are less studied compared to fungi.Cellulomonas, genus Cellvibrio, Cytophaga, Bacillus, Clostridium, etc. are known as cellulolytic bacteria.Bacteroides genus contained in human intestinal bacteria was discovered from this sample, but the detected amount is small and it seems difficult to exist as a living bacterium because it is a non-spore bacterium.

Discussion

Soil bacteria have not been well studied like human microbiota. In the human intestine variety and co-living with certain combination of bacteria are known to make good intestinal environment [11-14]. It seemed to be influenced by the dietary habit and geographical location [15].

Table 5: Bacterial profile of BrownBio (top 100).

1	Bacillus	207829	39.23		51	Caldicoprobacter	105	0.02
2	Aneurinibacillus	56610	10.69		52	Methylobacterium	103	0.02
3	Paenibacillus	44362	8.37		53	Herbinix	101	0.02
4	Acetobacter	38968	7.36		54	Brevundimonas	93	0.02
5	Brevibacillus	37892	7.15		55	Sphingomonas	90	0.02
6	Ureibacillus	26782	5.06		56	Comamonas	88	0.02
7	Lactobacillus	25261	4.77		57	Desulfotomaculum	84	0.02
8	Weissella	15249	2.88		58	Staphylococcus	78	0.01
9	Thermobacillus	12590	2.38		59	Streptomyces	77	0.01
10	Klebsiella	10860	2.05		60	Thermoanaerobacterium	77	0.01
11	Enterococcus	6565	1.24		61	Novibacillus	76	0.01
12	Pseudomonas	6437	1.21		62	Glutamicibacter	75	0.01
13	Corynebacterium	5624	1.06		63	Pelagibacterium	73	0.01
14	Acinetobacter	4479	0.85		64	Anoxybacillus	71	0.01
15	Nocardiopsis	2812	0.53		65	Lamia	65	0.01
16	Dietzia	2734	0.52		66	Rhizobium group	62	0.01
17	Tepidimicrobium	2185	0.41		67	Gordonia	62	0.01
18	Micrococcus	1999	0.38		68	Anaeroporak	56	0.01
19	Pediococcus	1825	0.34		69	Sphincobacterium	54	0.01
20	Aeribacillus	1620	0.31		70	Thermobifida	53	0.01
21	Geobacillus	1400	0.26		71	Elizuhethkingia	52	0.01
22	Clostridium	1198	0.23		72	Lysinibacillus	52	0.01
23	Brachymonas	1044	0.20		73	Melghirimyces	51	0.01
24	Caldibacillus	1033	0.19		74	Flavobacterium	49	0.01
25	Streptococcus	1026	0.19		75	Sedimentubacier	48	0.01
26	Paracoccus	1015	0.19		76	Chitinophaga	46	0.01
27	Empedobacier	884	0.17		77	Gracilibacillus	43	0.01
28	Cohnella	740	0.14		78	Ulvibacter	43	0.01
29	Pseudogracilibacillus	643	0.12		79	Arcticibacter	42	0.01
30	Symbiobacterium	637	0.12		80	Bacteroides	42	0.01
31	Caproiciproducens	516	0.10		81	Bifidobacierium	35	0.01
32	Virgibacillus	463	0.09		82	Muroides	34	0.01
33	Lactococcus	450	0.08		83	Planifilum	32	0.01
34	Anaerocolumna	422	0.08		84	Saccharibacillus	32	0.01
35	Kroppenstedtia	378	0.07		85	Enhydrobacter	31	0.01
36	Kurthia	366	0.07		86	Aliererythrobacter	30	0.01
37	Brachybacterium	287	0.05		87	Fonticella	30	0.01
38	Macrococcus	241	0.05		88	Leucobacter	26	0.00
39	Anaerosalibacter	217	0.04		89	Nocardioides	26	0.00
40	Stenotrophomonas	213	0.04		90	Ruminiclostridium	24	0.00
41	Oceanobatillus	190	0.04		91	Desulfovibrio	23	0.00
42	Nesterenkonia	187	0.04		92	Jeotgalicoccus	23	0.00
43	Deinococcus	186	0.04		93	Saccharomonospora	23	0.00
44	Rummeliibacillus	183	0.03		94	Lentibacillus	22	0.00
45	Leuconostoc	156	0.03		95	Pseudoxanthomonas	21	0.00
46	Aeromicrobium	145	0.03		96	Rufibacter	21	0.00
47	Chryseobacterium	133	0.03		97	Cupriavidus	18	0.00
48	Bogoriella	123	0.02		98	Brevibacterium	16	0.00
48	Escherichia-Shigella	122	0.02		99	Cutibacterium	16	0.00
						Total taxa	527281	99.52

Citation: Sasaki A, Takahashi M, Watanabe S. Fungal profile of soil bacterial flora powder (Brown Bio) made from *okara* and rice bran fermentation. Medcave J Environ Sci Technol. 2019; 1:101.

Table 6: Bacterial profile of BrownBio powder at species level (top 10).

Order	Name (species)	taxon	%
1	*Aneurinibacilllusthermoaerophilua*	55823	46.0
2	*Weissellaparaesenteroides*	15202	12.5
3	*Lactobacillus salivarius*	14333	11.8
4	*Paenibacillusbarengoltzii*	13181	10.9
5	*Pseudomonas aeruginosa*	6427	5.3
6	*Carynebacteriumvariabile*	5556	4.6
7	*Lactobacillus fermentum*	4192	3.5
8	*Lactobacillus casei*	2189	1.8
9	*Pediococcusacidilactici*	1825	1.5
10	*Geobacillusthermoparaffinivorans*	665	0.5
		119393	52.4

As a result of microbiological analysis of 4 samples of BrownBiopowder, it was found that these samples contain many bacteria including various organic matter-degrading bacteria. In particular, the high viability rate of 30 to 82% despite the long storage period of several years to nine years indicates that the product storage management is easy.

Soil fungi are microorganisms that live in soil called "soil microorganisms". It has the potential to have a positive or negative effect on plant growth, such as bacteria, molds, actinomycetes (soil enzymes), and nematodes. There are about 100 million soil fungi in a teaspoon of soil, and the microorganisms squeeze and coexist with each other, increasing and decreasing depending the environmental condition.

In gardening and kitchen gardens, it is important to adjust the balance of biased soil fungi with soil conditioners.

Okara is a by-product of soy milk and tofu, and is a squeezed soybean meal. Since the demand for food is significantly lower than supply and the quality deteriorates quickly, many are disposed of as industrial waste, and the processing costs are a major burden on soymilk and tofu manufacturers. Due to the global health boom, the soymilk market in Europe and the US is expanding rapidly, but as in Japan, the problem of industrial waste has become a serious problem throughout the world.

Recycling okara, which is a nutrient-rich material, and commercializing it leads to reductions in disposal costs and corporate image, so drying process from okara and recycling as a product are major themes globally.Okara powder is heat driedto innactivte enzymes and can be stored for long periods at room temperature.

Rice bran is decomposed slowly and exerts a fertilizer effect, so it is divided into "slow-acting" among fertilizers and is often used as a fertilizer that is mixed into the soil before planting seedlings.

Our preliminary study showed that the BrownBio powder was effective when the humus was mixed at work. The humus soil is a soil-like material made up of plant organic matter such as dead leaves and branches of hardwoods. It works to increase the overall strength of the soil, such as drainage, breathability, and fertilizer, and regenerates thin soil. This is categorized to "special fertilizer" by the Fertilizer Control Law and that is subject to the application of the Promotion Law in Japan.

References

1. Gun Haruaki, Koichi Koshida, Koichi Inoue, Takashi Someya, 2005 *Journal of Japanese Society of Soil Fertilizer*, 76: 401-410
2. Gun Haruaki, Koshida Junichi, Moriyama Noriko, Wang Yutan, YuzoTakezo, Inoue Koichi, et al, 2005 *Journal of Japanese Society of Soil Fertilizers*, 76: 865-874
3. Soil Microbial Society: New soil microbiological experiment method, 15-23, Yokendo, Tokyo, 1992
4. Soil Microbiology Society: New Soil Microbial Experiments, 35-41, Yokendo, Tokyo, 1999.
5. Frampton EW, Restaino L. and Blaszko L, 1988 Evaluation of β-glucuronidase substrate 5-bromo-4-chloro-3-indol-β-D-glucuronide (X-GLUC) in a 24 hour direct plating method for Escherichia coli. *Journal of Food Protection* 51; 402- 404.
6. Edited by the Pharmaceutical Society of Japan: Hygiene Testing Methods / Comments, p.88-89, Kanehara Publishing Co., Tokyo, 2000.
7. Hankin L, Sands DC, Hill DE, 1974 Relation of land use to some degradative administrative activities of soil bacteria.Soil Science, 118: 38-44.
8. Cowan ST. Guide to Identification of Medical Bacteria, Translated by Toshikazu Sakazaki, Modern Publishing, 335, Tokyo.
9. Sierra G, 1956 A simple method for the detection of lipolyticactivity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. *Antonie van Leeuwenhoek* 23; 15-22.
10. Suyama, K., Yamamoto, H., Naganawa, T., Iwata, T. And Komada, H, 1993 A plate count method for aerobic cellulose decomposers in soil by congo red staining. *SoilSci.PlantNutr* 39; 361-365.
11. Hisada T, Endoh K, Kuriki K, 2015 Inter- and intra-individual variations in seasonal and daily stabilities of the human gut microbiota in Japanese. *Arch Microbiol* 197; 7: 919-934.
12. Watanabe S, Utada I, Hisada T, Mochizuki J, Mizuno S, et al, 2018 Wellness fasting-induced hyperketosis and interaction by intestinal microbiota. *Int J Sci Engineer Res* 9; 7: 1833-1844.
13. Ishikawa E, Matsuki T, Kubota H, Makino H, Sakai, et al. Diversity in gut bacterial community of school-age children in Asia. *Sci Rep* 5: 8397.
14. Hirakawa A, Aoe S, Watanabe S, Hisada T, Mochizuki J, et al, 2019 The Nested Study on the Intestinal Microbiota in Genki Study with Special Reference to the Effect of Brown Rice Eating. *J Obes Chronic Dis* 3; 1: 1-13.
15. De Filippo, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, et al. 2010 Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci* USA 107; 33: 14691-14696.