# Wellness Fasting-induced Hyperketosis and Interaction by Intestinal Microbiota

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Abstract—β-hydrobutyrate (BHB), one of representative ketone bodies, increases by fasting. Fasting endogenously produces ketone bodies in the liver, kidney and astrocytes in the brain. We reported the case in whom increased BHB would come from intestinal microbiota by Koda's fasting diet. Relationship between plasma BHB and intestinal microbiota has not well known. Sixty subjects were admitted in the ARSOA Wellness Fasting program. During a 4-day fasting period, they received a combination of very low-energy vegetarian diet, physical exercise, meditation, and lectures about healthy lifestyles. Peripheral blood was collected on days 1 and 4 for routine biochemical analyses and for the determination of serum levels of insulin, glucagon and other hormones. Glucose and BHB were measured every morning by finger tip blood. The stools were collected pre-,mid- and post-fasting, and profiles of microbiota were analyzed by the metanalytic cytotechnicon. All data were collated in an Excel file, and transferred to SPSS for statistical analysis. BHB in the blood increased from 0.3±0.2 mM at the day 1 to 2.0±1.2 mM at the day 4 of fasting, while the glucose level decreased from 5.6±1.6 mM to 3.9±1.3 mM. Increases in plasma BHB were assigned to 4 groups (less than 1.0 mM, 1.0-1.99 mM, 2.0-2.99 mM and more than 3.0 mM). Highest group of BHB showed significant association with family *Enterobacteriaceae* of prefasting microbiota profile. Further analysis of bacterial profile at the species level clarified that 15 species were directly or indirectly related to BHB level, such as *Providencia vermicola, P. rustigianii, P. sneebia, Morganella morganii, M. psychrotolerans, Proteus hauseri, Butyricimonas virosa,.etc*. Five cases with very low *Enterobacter* presence showed lowest plasma BHB increase. Only one case with high BHB level did not show any attributable bacteria. Plasma concentration of BHB was related to *Enterobacteriaceae* directly or indirectly. The latter suggested syntrophic effects on *Enterobacteriaceae*. Known butyrate producing bacteria were suppressed by competitive interaction. It suggested that the metabolic pathway of butyrate synthesis and BHB production seemed to be done by different microbiota. Absorption of BHB from intestinal microbiota could be considered like the BHB supplement use, so dietary effects should be clarified more. Four days of fasting induced hyperketonemia with some metabolic changes. The possible absorption of BHB through intestinal wall could be accepted independent from butyrate production.

**Index Terms**—β-hydroxybutyrate, *Enterobacteriaceae*, fasting, intestinal microbiota, ketone bodies, metabolism, physiology

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## 1.Introduction

The importance of ketone body metabolism in diabetic patients has been well known since 1970s, but recently, β-hydroxybutyrate (BHB) has been identified as a key component of a metabolic signaling pathway. [1-3] However, many questions remain unanswered what determined individual differences in the production and excretion of ketone bodies [4]. Recently, hyperketonemia induced by fasting or ketogenic diet calls attention because of the possibilities for various clinical pharmaceutical effects [5,6].

We hypothesized that 3-β-hydroxybutyrate (BHB) could be the fuel for the basic engine that produces energy in all terrestrial species [7]. However, ATP production from glucose-pyruvic acid pathway seems to be later added as a dominant system in human. We hypothesize the establishment of TCA cycle in mitochondria and enough oxygen supply since two billion years ago would be the key events to promote this change. The efficacy of ATP production from β-oxidation product is 10 ATP molecules, while it is 12.5 molecules from pyruvic acid. So, evolution should select glucose burning system as a booster engine for energy production [7].

The liver and kidney are considered to be the major ketone body producing organs which contain abundant glycogen particles. So, a close relationship between ketone and glucose burning system may be present. This explains why certain level of glucose burning system may be present. This explains why certain level of glucose is steadlily maintained even in the hyper ketogenic state.[8]

BHB shows various pharmacological effects on disease prevention, such as cardiovascular disease, Alzheimer's disease, epilepsy, etc. [9-12] Inhibition of histone deacetylase, stimulation of FOXO, resistance to oxidative stress, protection of mitochondria, stimulation of adiponectin release, suppression of inflammasome, etc. are reported [10-16].

We found that a lady who had lived 18 years by only one glass of vegetable juice a day could live by high BHB which seemed to be produced by the intestinal bacteria.[17] However, individual differences in ketone production and the dynamics of ketogenesis are still unclear. Possibility of BHB absorption from intestinal lumen like butyrate is not known. Therefore, we tried to clarify the occurrence of ketogenesis during fasting by measuring concurrent changes in glucose, ketone bodies, fatty metabolism and various hormonal changes in both blood and urine in relation to the intestinal micarobiota. We recruited volunteer subjects among participants in a comprehensive fasting program, which included a balanced diet, physical activity, and mind exercises to achieve a healthy spiritual life.

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## 2.Subjects and Mehods

Persons who wanted to participate in the program of ARSOA wellness fasting were telephone-interviewed by the expert staff about their health and willing to join the program. All participants were requested to send 1 week dietary record and health sheet, including past medical history, abnormal laboratory data at a health check-up, habitual foods and/or supplements, and any question to the counselor. [18] All participants signed the agreement sheet after precise explanation of the program.

Each participant took 50 ml fermented vegetable juice (*ko-so)*, CellEnergy (multivitamins), and mineral tablet per day. Water and tea were freely available. On the last day, the attention to return to normal diet was taught, and the subjects ate half volume of boiled brown rice and vegetable soup as a brunch. All participants were requested to report their health condition after returning home (6th day and 1 month later).

Every morning participants measured their body composition using the Body Composition Analyzer® (Tanita, Tokyo, Japan), which determines body weight, fat volume, muscle volume, water contents, estimated bone weight, basal metabolic rate and body mass index (BMI), based upon the electric impedance.[19] Blood pressure and pulse rate were measured with the Omron HEM-7021® monitor (Kyoto, Japan).

Blood glucose and BHB were measured every morning with the Freestyle Precision Neo® meter (Abott, Chicago, IL, USA). Daily first urine was checked for urinary pH, glucose, BHB, urobilinogen, proteins, occult blood, bilirubin, nitrate, and relative gravity using the Uropaper III® (Eiken, Tokyo, Japan). A sample of about 10 ml of urine was stored in a frozen tube for quantitative analysis of creatinine, acetoacetate (AcAc), BHB and catecholamines by the Serum Research Laboratory (SRL).[20]

On the 1st and 4th day morning, 7ml venous blood was collected and the frozen sera were sent to the Serum Research Laboratory (SRL, Tokyo) for biological analysis, such as total protein, triacylglyceride (TG) and free fatty acid, glucose and ketone bodies, insulin, glucagon, creatinine, γGTP, asparatic acid aminotransferase (AST) and alanine aminotransferase (ALT) .

Intestinal Bacteria

Fresh feces were collected 3 times; a few days before (pre-) fasting, during (mid-) fasting and about 1 week after (post-) fasting. Fecal samples (approximately 50-100 mg) were sent to Techno Suruga Laboratory , Shizuoka, for the sequence amplicon analysis.[21-27] The stools were suspended in a solution containing100 mM Tris-HCl, pH 9.0, 40 mM Tris-EDTA, pH8.0, and 4 M guanidine thiocyanate and 0.001% bromothymol blue. An aliquot of 0.8 ml of the suspension was homogenized with zirconia beads in a 2.0 ml screw cap tube by FastPrep24 Instrument (MP Biomedicals, Santa Ana, CA) at 5 m/s for 2 min and placed on ice for 5 min. After centrifugation at 5000 × g for 1 min, DNA was extracted from 200 µL of the suspension using an automatic nucleic acid extractor (Precision System Science, Chiba, Japan). MagDEA DNA 200 (GC) (Precision System Science) was used as the reagent for automatic nucleic acid extraction, as previously described.[21]

NGS analysis of microbial community structure in feces was performed using a MiSeq (Illumina, San Diego, CA), as previously described. [25-27] The V3-V4 region of 16S rDNA was amplified using the forward primer Pro341 F 5′ -AATGATACGGCGACCACCGA-GATCTACACXXXXXXXXACACTCTTTCCCTACACGAC-GCTCTTCCGATCTCCTACGGGNBGCASCAG-3′ , where Xs represent the sample-specific 8-bp barcode sequences (CTCTCTAT, TATCCTCT, GTAAGGAG, ACTGCATA, AAGGAGTA, CTAAGCCT, CGTCTAAT, TCTCTCCG, TCGACTAG, TTCTAGCT, CCTAGAGT, GCGTAAGA, CTATTAAG, AAGGCTAT, GAGCCTTA and TTATGCGA) ) and the reverse primer Pro806R 5'-CAAGCAGAAGACGGCATACGA-GATZZZZZZZZGTGACTGGAGTTCAGAC-GTGTGCTCTTCCGATCTGACTACNVGGGTATCTAATCC-3', where Zs represent the sample-specific 8-bp barcode sequences (TCGCCTTA, CTAGTACG, TTCTGCCT, GCTCAGGA, AGGAGTCC, CATGCCTA, GTAGAGAG, CAGCCTCG, TGCCTCTT, TCCTCTAC, TCATGAGC, CCTGAGAT, TAGCGAGT and GTAGCTCC). The touchdown PCR method for thermal cycling was used with a GeneAmp PCR system 9700 (ABI, Foster City, CA). The PCR reaction mixture (25 µL) contained 20 ng genomic DNA, 2 ×MightyAmp Buffer Ver.2 (Takara, Otsu, Japan), 0.25 µM of each primer, and 1.25 units of MightyAmp DNA Polymerase (Takara). The PCR reaction and preparation of amplicon pool were performed by the method of Takahashi et al. [27]

Bioinformatics analysis

The determined 16S rDNA sequences were subjected to homology searching using Metagenome@KIN software (World Fusion Co., Ltd., Tokyo, Japan) against the Techno-Suruga

Lab Microbial Identification Database DB-BA10.0 (Techno-Suruga Laboratory), which contains only bacteria with standing in the taxonomic nomenclature. [21]

Statistical analysis

The information obtained from the questionnaire was made into the Excel database. Analysis was carried out as far as possible on the point whether the microbiota influenced ketone production in this study. The complete data of 57 people were provided for analysis

Four groups were categorized by the 4th day serum BHB levels; less than 1 mM, 1.0-1.9 mM, 2.0-2.9 mM, more

than 3.0 mM. It roughly corresponded to the quartile value. Laboratory data at the day 1 and 4 were compared by the paired analysis. Data were checked using an unpaired t-test with Welch's correction for continuous variables or the Mann-Whitney test (two-sided) and Fisher's exact test for categorical variables. Correlation analysis was carried out between BHB level and each profile of microbiota at the phylum level, family level, and species level.

The IBM SPSS software Ver. 24 was used for calculation, and P values less than 0.05 were considered significant. The statistical significance of the decision was shown as * $p < 0.05$, * * $p < 0.01$, * * * $p < 0.001$.

## 3. Results

The number of participants was 60, and 3 of them were excluded from the analysis due to insufficient data. About 20 participants stayed in a mountaneous resort hotel in each time, and received a combination of low-energy vegetarian diet (about 380 kcal intake by fermented vegetable juice per day), physical exercise (2-hour walking, slow training and stretch), meditation, and lectures about healthy life habits in the 4-day program, as previously reported. [18]

Average age of participants was 53.7±18.7 in 3 men and 47.8±11.8 in 54 women. Height and body weight were 116.7±3.2 cm and 64.3±4.1 kg in men and 158.1±5.6 cm and 53.1±8.0 kg in women. Body weight decreased about 1.5±1.3 kg（median 1.7kg）, and fat percent and muscle volume also decreased during 4 day fasting.

The changes of basal metabolic rate (BMR), blood pressure, pulse rate and body temperature are shown in Table 1. Most participants were women, who were working in cometic shops as a sales lady or owners. There were no current smokers. Their lifestyle was healthy and about half of them were ovo- and fish-vegetarians. Although headache, hungry, GI tract distress, emesis, depressive and cold feeling occurred toward the 2nd day, these symptoms dramatically disappeared at the 3rd day, and active and vivid feeling increased on the 4th day and after. Skin condition also became good by fasting.

Glucose decreased, and BHB and acetoacetate (AcAc) increased toward 4th day. Systolic blood pressure decreased 8 mmHg in both males and females, and 4 mmHg in diastolic pressure, which continued at least one month later. On the contrary, the pulse rate increased 9 at median. Body temperature and BMR decreased in both sex. (Table 1). However,

BHB difference only correlated to the body temperature and pulse rate, and glucose decrease correlated with lowered diastolic pressure.

Table 1. Anthropometric, physiological, glucose and BHB change during 4 day-fasting

| | Day 1 | Day 2 | Day 3 | Day 4 | p | After 1 w | After 1 mos |
|---|---|---|---|---|---|---|---|
| body weight (kg) | 54.9 ± 8.3 | 54.4 ± 8.2 | 53.6 ± 8.1 | 53.5 ± 8.1 | *** | 53.5 ± 8.1 | 53.1 ± 7.6 |
| BMI | 21.5 ± 4.2 | 21.5 ± 3.1 | 21.5 ± 3.2 | 21.3 ± 3.2 | 0.502 | 21.5 ± 3.4 | 21.4 ± 3.2 |
| fat % | 29.6 ± 6.8 | 30.3 ± 6.7 | 30.0 ± 6.6 | 29.4 ± 6.8 | 0.454 | 28.1 ± 6.5 | 28.2 ± 5.9 |
| waist circumference | 84.9 ± 8.3 | 84.2 ± 8.2 | 83.1 ± 8.4 | 82.1 ± 8.7 | *** | 82.2 ± 8.6 | 81.3 ± 7.9 |
| BMR (kcal/day) | 1107.0 ± 101.9 | 1090.1 ± 100.0 | 1087.4 ± 95.5 | 1092.4 ± 117.2 | *** | na | na |
| body temp (℃) | 36.5 ± 0.4 | 36.3 ± 0.4 | 36.4 ± 0.3 | 36.3 ± 0.3 | * | 36.3 ± 0.4 | 36.3 ± 0.4 |
| puls (n/min) | 74.5 ± 12.0 | 79.4 ± 11.7 | 81.1 ± 13.6 | 83.5 ± 11.5 | *** | 70.6 ± 8.6 | 70.7 ± 9.8 |
| systolic BP (mmHg) | 121.5 ± 19.2 | 117.5 ± 17.9 | 113.0 ± 16.6 | 110.9 ± 14.4 | *** | 117.1 ± 15.7 | 117.6 ± 24.1 |
| diastolic BP (mmHg) | 80.4 ± 12.7 | 79.1 ± 12.0 | 76.7 ± 11.8 | 75.9 ± 10.9 | *** | 76.9 ± 12.7 | 75.3 ± 9.1 |
| b_glucose (mM) | 5.0 ± 1.6 | 4.6 ± 1.1 | 4.1 ± 1.2 | 3.9 ± 1.3 | *** | na | na |
| b_bHB (mM) | 0.3 ± 0.2 | 0.4 ± 0.3 | 1.2 ± 0.7 | 2.0 ± 1.2 | *** | na | na |

BHB in the plasma increased from 0.3±0.2 mM at the day 1 to 2.0±1.2 mM at the day 4 of fasting, while the glucose level decreased from 5.8±1.9 mM to 4.5±1.5 mM. Increases in plasma BHB were assigned to 4 groups (less than 1.0 mM, 1.0-1.9 mM, 2.0-2.9 mM, and more than 3 mM). The BHB/AcAc ratio in the blood was 5.4±1.3, regardless of the BHB concentration, but the range varied from 3.5 to 11.0. These values returned to the normal range on the 14th-day (10 days after fasting).

Changes of plasma biochemical markers

Changes of biochemical markers between Day 1 and Day 4 are shown in Table 2. Marked increase of free fatty acids and decrease of triacylglycerol, decrease of glucose and insulin, increase of glucagon, slight increase of AST, ALT and creatinine were observed. Increased AcAc and BHB negatively correlated to glucagon and glucose.

Table 2 Changes of serum biochemical markers on day 1 and 4th day of fasting

| | day 1 | | | day 4 | | | dif | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| n = 53 | mean | | sd | mean | | sd | mean | | sd | p |
| TG (mg/dl) | 99.8 | ± | 60.2 | 56.0 | ± | 20.9 | -45.5 | ± | 58.0 | *** |
| FFA (mg/dl) | 736.2 | ± | 344.8 | 1900.2 | ± | 535. | 1181.4 | ± | 607.3 | *** |
| glucose (mg/dl) | 5.0 | ± | 1.2 | 3.9 | ± | 1.2 | 1.1 | ± | 1.0 | *** |
| insulin (uUI/ml) | 6.2 | ± | 5.3 | 2.8 | ± | 1.7 | -3.5 | ± | 5.2 | *** |
| glucagon (pg/ml) | 153.7 | ± | 33.6 | 183.1 | ± | 60.0 | 26.0 | ± | 63.7 | *** |
| AST (U/L) | 21.4 | ± | 5.2 | 31.5 | ± | 8.9 | 10.0 | ± | 6.5 | *** |
| ALT (U/L) | 14.3 | ± | 6.8 | 16.1 | ± | 7.5 | 1.8 | ± | 3.3 | ** |
| $\gamma$_GTP (U/L) | 28.3 | ± | 32.1 | 27.1 | ± | 28.3 | -1.6 | ± | 5.4 | 0.113 |
| Cre (mg/dl) | 0.6 | ± | 0.1 | 0.7 | ± | 0.1 | 0.1 | ± | 0.1 | *** |
| eGFR (ml/min/1.73m$^2$) | 84.4 | ± | 14.8 | 74.0 | ± | 15.0 | -10.1 | ± | 10.3 | *** |
| AcAc (mM) | 0.0 | ± | 0.0 | 0.3 | ± | 0.2 | -0.3 | ± | 0.2 | *** |
| BHB (mM) | 0.1 | ± | 0.1 | 1.8 | ± | 1.1 | -1.7 | ± | 1.1 | *** |
| total keton (mM) | 0.0 | ± | 0.0 | 2.2 | ± | 1.3 | -2.1 | ± | 1.3 | *** |

TG; tryglyceride, FFA; free fatty acids, AST; ALT $\gamma$_GTP, Cr; creatinine, eGFR; estimated glomelular filtration rae, AcAc; acetoacetate, BHB; $\beta$-hydroxybutyrate

Changes of microbiota profiles by fasting

Our analyzing system of microbiota profile recognized 600 species from about more than 24000 taxons. Number of species more than 0.01% was 129 species. Rejected hits were 29.6% and undetermined rate was 9.4%. These were summarized to 60 genus, 29 families, 14 orders, 11 classes and 5 phylum. (Supplementary Table 1) We selected bacteria which presented more than 0.01% or maximum value of 0.1% for further analysis. Most common bacteria in phylum was *Fermicutes* (36-40%), next *Bacteroidetes* (24%), *Actinobacteria* (11-16%), and then *Proteobacteria* (0.7-1.9) and *Verrucomicrobia* (0.602%.). When compared bacterial profiles in 3 different times, pre-, mid- and post-fasting, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* showed significant changes by fasting (Table3).

Table 3. Phylum profile of microbiota before, during and after fasting

| time | Pre | | | Mid | | | Post | | | ANOVA |
|---|---|---|---|---|---|---|---|---|---|---|
| phylum | mean | sd | median | mean | sd | median | mean | sd | median | p |
| Firmicutes | 40.31 | 8.12 | 40.57 | 36.88 | 11.03 | 38.61 | 41.09 | 9.38 | 40.71 | 0.057 |
| Bacteroidetes | 24.06 | 10.93 | 23.19 | 24.11 | 11.17 | 24.80 | 24.05 | 7.99 | 23.86 | 0.999 |
| Actinobacteria | 11.70 | 7.59 | 10.04 | 16.63 | 12.52 | 13.85 | 11.87 | 10.73 | 8.73 | 0.024* |
| Proteobacteria | 6.65 | 6.51 | 3.94 | 3.90 | 4.77 | 1.58 | 7.04 | 7.74 | 3.61 | 0.026* |
| Verrucomicrobia | 0.69 | 1.71 | 0.02 | 1.93 | 3.88 | 0.01 | 0.70 | 1.84 | 0.01 | 0.024* |
| Fusobacteria | 0.38 | 1.46 | 0.00 | 0.31 | 1.16 | 0.00 | 0.18 | 0.69 | 0.00 | 0.68 |
| Lentisphaerae | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | nd |
| Spirochaetes | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | nd |
| Synergistetes | 0.00 | 0.01 | 0.00 | 0.01 | 0.03 | 0.00 | 0.00 | 0.02 | 0.00 | nd |
| Rejected hit | 16.20 | 7.76 | 15.80 | 16.24 | 9.70 | 14.36 | 15.06 | 6.69 | 15.43 | 0.699 |

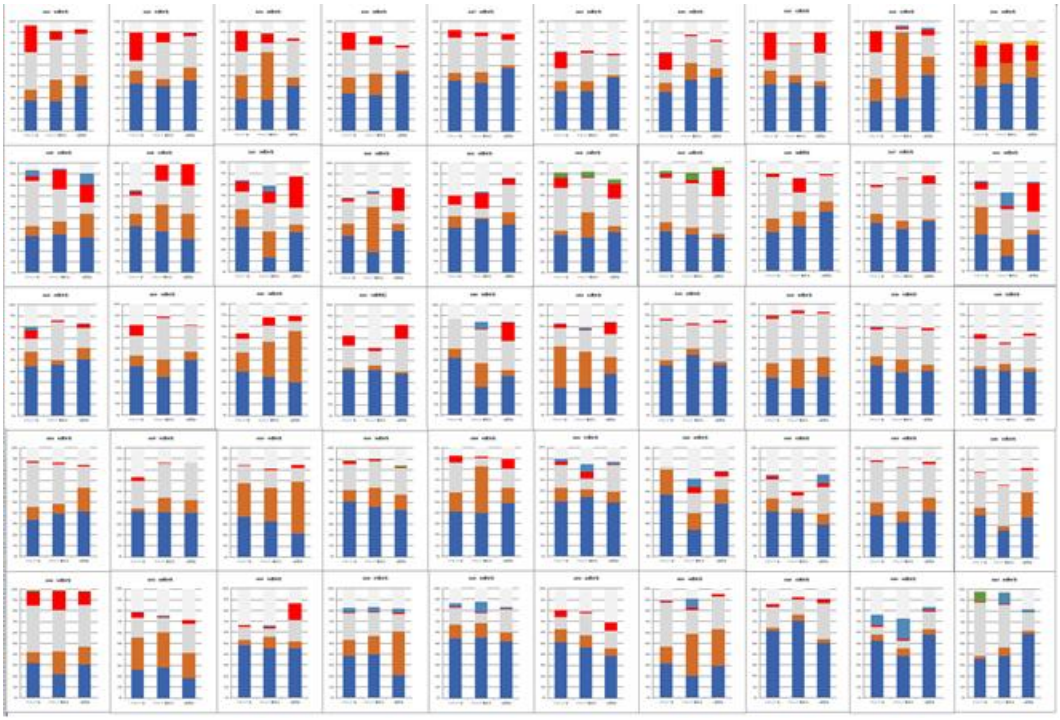

Fig. 1. Changes of individual bacterial profiles in pre-, mid- and post-fasting. (left to right column). Upper 2 colums are high BHB level. Phylum is shown from the bottom; Firmcutes (blue), Actinobacteria (brown), Bacteroidetes (grey), Proteobacteria (red), Verrucomicrobia (green), Fusobacteria (violet), Spirochaetes (sky grey), Synergistetes (yellow), Lentisphaerae (pale grey) , and rejected (white).

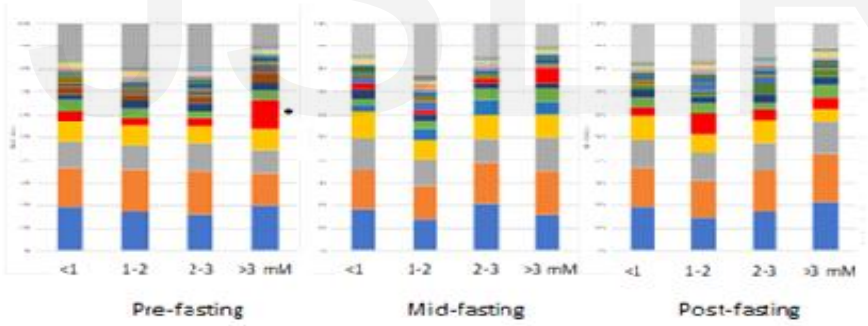


Fig.2. Bacterial family profile of pre-, mid- and post-fasting by four BHB levels at 4th day. Enterobacteriaceae at prefasting profile showed significant association with high BHB group. Column shows Bacteroidaceaae (blue), Lachnospiraceae (brown), Ruminococcaceae (grey), Bifidobacteriaceae (yellow), Enterobacteriaceae (red), Eubacteriaceae (green), Prophyromonadaceae (dark blue), Coriobacteriaceae (moss green), Prevotellaceae (light blue), Streptococcaceae (dark brown), Veiolonellaceae (violet), Acidaminococcaceae (black), Rikenellaceae (cyan), Peptostreptococcaceae (right brown), Ersipelotrichaceae (grey), Verrucomicnobiaceae (yellaw), Sutterrelbaceae (sky blue), Desulfovibrionaceae (green grey), ge Flovonifractorincetae sedis (blue), Clostridaceae (red brown), Fusobacteriaceae (violet grey), Bacillacieae (right brown), Enterococcaceae (sky blue), Lactobacillaceae (dark blue), Pasteurellaceae (light blue), Not determined (right brown), Actinomycetaceae (grey), Oscilospiracease (light brown), Brucellaceae (sky blue), ge intestinimonas incertage sedis (grey), Micrococcacease (dark blue), Peptoniphilaceae (brown), Pseudomanodaceae, Leuconostocaceae, ge Pseudoflavonifracter incertae sedis, ge Gemilla ircetae sedis, Synergistaceae, Succinivitrionaceae, and rejectd hit (top: grey)

Comparison of profiles at the family level by BHB category showed significant correlation between prefasting proteobacteria and 4th day BHB level. Such difference was disappeared in the feces during fasting, and it became rather balanced after fasting. Individual response showed various type in high BHB producers but the presence of proteobacteria was common (Fig. 2).

In comparison of profiles at the species level, significant difference by BHB level was recognized in *Morganella morganii, Proteus hauseri,* and *Providencia rustigianii* (Table 4). Bacterial profiles of individuals are listed in supplementary table 2. (Suppl. Table 2)

Table 4. Profile of microbiota by BHB level

| BHB degree | <1.0 mM | (n=16) | 1.0-1.99 | (n=17) | 2.0-2.99 | (n=12) | >3.0 mM | n=10 | p |
|---|---|---|---|---|---|---|---|---|---|
| Bacili % | mean | sd | mean | sd | mean | sd | mean | sd | |
| Faecalibacterium prausnitzii | 5.37 | 4.46 | 5.45 | 3.18 | 5.05 | 3.62 | 4.54 | 3.35 | 0.932 |
| Bacteroides vulgatus | 3.72 | 8.24 | 3.67 | 5.57 | 3.78 | 4.21 | 4.57 | 6.37 | 0.985 |
| Bacteroides dorei | 3.75 | 6.37 | 2.81 | 3.82 | 2.20 | 3.67 | 3.62 | 5.57 | 0.508 |
| Bacteroides uniformis | 3.02 | 2.44 | 2.41 | 2.28 | 3.86 | 2.37 | 2.78 | 1.55 | 0.392 |
| Blautia wexlerae | 3.17 | 2.60 | 2.60 | 1.70 | 3.28 | 2.72 | 1.82 | 1.37 | 0.377 |
| Bifidobacterium adolescentis | 2.68 | 4.32 | 4.35 | 4.67 | 3.07 | 3.13 | 2.23 | 2.40 | 0.842 |
| Eubacterium rectale | 2.46 | 3.42 | 1.94 | 2.98 | 0.54 | 0.47 | 1.72 | 2.04 | 0.302 |
| Bifidobacterium longum | 2.41 | 2.34 | 1.55 | 1.25 | 0.79 | 0.67 | 1.41 | 0.93 | 0.057 |
| Ruminococcus bromii | 2.13 | 2.34 | 2.71 | 3.58 | 3.60 | 3.77 | 1.69 | 3.50 | 0.539 |
| Collinsella aerofaciens | 1.83 | 2.52 | 2.94 | 4.60 | 3.12 | 3.66 | 3.59 | 1.97 | 0.598 |
| Subdoligranulum variabile | 1.79 | 0.98 | 1.32 | 1.20 | 1.87 | 1.77 | 1.37 | 1.59 | 0.621 |
| Fusicatenibacter saccharivorans | 1.68 | 1.38 | 1.79 | 1.28 | 2.01 | 1.93 | 1.86 | 1.58 | 0.955 |
| Bacteroides plebeius | 1.42 | 2.56 | 2.40 | 4.77 | 0.40 | 0.91 | 1.96 | 4.70 | 0.522 |
| Blautia luti | 1.27 | 1.08 | 1.66 | 1.57 | 2.30 | 2.81 | 0.64 | 0.77 | 0.149 |
| Morganella morganii | 1.29 | 3.04 | 0.00 | 0.00 | 1.76 | 4.12 | 3.87 | 6.80 | 0.016 |
| Ruminococcus obeum | 0.90 | 0.79 | 1.76 | 1.77 | 1.21 | 1.01 | 0.55 | 0.62 | 0.072 |
| Parabacteroides distasonis | 0.78 | 0.73 | 1.24 | 2.00 | 1.48 | 1.45 | 2.55 | 2.40 | 0.089 |
| Megamonas funiformis | 0.77 | 1.58 | 0.55 | 1.56 | 1.84 | 4.30 | 3.09 | 6.65 | 0.306 |
| Citrobacter freundii | 0.60 | 1.47 | 0.32 | 0.72 | 0.13 | 0.38 | 0.60 | 1.13 | 0.591 |
| Citrobacter werkmanii | 0.40 | 0.68 | 0.58 | 1.22 | 0.00 | 0.00 | 1.23 | 3.64 | 0.412 |
| Clostridium xylanolyticum | 0.45 | 0.64 | 0.32 | 0.34 | 0.44 | 0.72 | 0.06 | 0.08 | 0.259 |
| Bacteroides caccae | 0.41 | 0.60 | 0.45 | 0.62 | 0.38 | 0.61 | 0.04 | 0.09 | 0.288 |
| Ruminococcus lactaris | 0.33 | 0.52 | 0.05 | 0.06 | 0.21 | 0.27 | 0.63 | 0.61 | 0.007 |
| Megasphaera elsdenii | 0.28 | 0.89 | 0.73 | 1.63 | 0.32 | 1.06 | 0.16 | 0.50 | 0.579 |
| Streptococcus sinensis | 0.27 | 0.48 | 0.06 | 0.09 | 0.04 | 0.07 | 0.08 | 0.11 | 0.083 |
| Eubacterium ventriosum | 0.25 | 0.65 | 0.07 | 0.09 | 0.18 | 0.24 | 0.23 | 0.31 | 0.575 |
| Roseburia inulinivorans | 0.22 | 0.59 | 0.25 | 0.48 | 0.14 | 0.31 | 0.77 | 0.94 | 0.072 |
| Flavonifractor plautii | 0.16 | 0.18 | 0.18 | 0.20 | 0.20 | 0.20 | 0.37 | 0.46 | 0.216 |
| Bacteroides finegoldii | 0.11 | 0.34 | 0.02 | 0.07 | 0.07 | 0.15 | 0.41 | 1.27 | 0.372 |
| Alistipes shahii | 0.11 | 0.13 | 0.13 | 0.14 | 0.15 | 0.17 | 0.01 | 0.02 | 0.08 |
| Clostridium bolteae | 0.09 | 0.13 | 0.15 | 0.16 | 0.25 | 0.46 | 0.35 | 0.49 | 0.217 |
| Bifidobacterium dentium | 0.02 | 0.03 | 0.00 | 0.01 | 0.00 | 0.01 | 0.00 | 0.00 | 0.09 |
| Proteus hauseri | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.91 | 4.02 | 0.023 |
| Providencia vermicola | 0.00 | 0.00 | 0.02 | 0.08 | 0.47 | 1.09 | 1.65 | 5.20 | 0.262 |
| Providencia rustigianii | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.09 | 0.19 | 0.022 |
| Veillonella tobetsuensis | 0.00 | 0.01 | 0.00 | 0.01 | 0.00 | 0.00 | 0.02 | 0.03 | 0.035 |
| Veillonella parvula | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.02 | 0.002 |
| Solobacterium moorei | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.01 | 0.004 |
| Corynebacterium argentoratense | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.081 |
| Kluyvera georgiana | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.083 |
| Pectobacterium aroidearum | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.023 |
| Total % of bacili more than 0.01% species | 44.17 | | 44.46 | | 45.15 | | 52.48 | | |

## Syntrophic or competitive interaction of bacteria for BHB production

Further analysis of bacterial profile at the species level clarified that 7 species directly related to BHB level, such as *Providencia vermicola, Providencia rustigianii, Providencia sneebia, Morganella morganii, Morganella psychrotolerans, Proteus hauseri, and Butyricimonas virosa.* (Table 5). They showed syntrophic growth among them. *Butyricimonas virosa* coexisted with *Providencia rustigianii* (CC=1.000***) and *Morganella morganii* (CC=0.763***).[28] *Bacteroides finegoldii and Parabacteroides distasonis* in *Bacteroidaceae*

Table 5. Correlation of microbiota which show syntrophic and/or competitive interaction for BHB production

| | | Proteobacteria | | | | | | Bac-teroides | | | Fermicutes | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 相関 | BHB | P | P | P | P | P | P | Porphy-lo | B | B | F | F | F | F | F | F | F | F |
| | 4thday ~~BHB~~ | Provi-dencia rustigi-anii | P.sneeb ia | P.vermi cola | Mor gane lla mor ganii | Morga-nella psy-chrotol-erans | Proteus hauseri | Butyr-ici-monas virosa | Bac-teroides fi-negoldii | Parab-acteroid es dista-sonis | Clos-tridium bolteae | Clos-tridium la-valense | Entero-coccus avium | Megam onas rupel-lensis | Alli-sonella his-tamini-formans | Fla-vonifrac tor plautii | Rose-buria faecis | Blautia faecis |
| BHB at 4day | 1 | .400** | .399** | .418** | .332* | .399** | .281* | .400** | .318* | .360** | .282* | .399** | .399** | .293* | .285* | .272* | -.288* | -.350** |
| Providencia rustigi-anii | .400** | 1 | -0.026 | -0.035 | .753** | -0.026 | -0.038 | 1.000** | -0.042 | .442** | -0.011 | -0.026 | -0.026 | -0.026 | -0.038 | 0.042 | -0.156 | -0.098 |
| Providencia sneebia | .399** | -0.026 | 1 | .972** | -0.051 | 1.000** | -0.026 | -0.026 | .937** | 0.045 | 0.176 | 1.000** | 1.000** | -0.019 | -0.026 | 0.237 | -0.109 | -0.114 |
| Providencia vermicola | .418** | -0.035 | .972** | 1 | 0.055 | .972** | -0.035 | -0.035 | .907** | 0.013 | 0.147 | .972** | .972** | -0.025 | -0.035 | 0.198 | -0.142 | -0.125 |
| Butyricimonas virosa | .400** | 1.000** | -0.026 | -0.035 | .753** | -0.026 | -0.038 | 1 | -0.042 | .442** | -0.011 | -0.026 | -0.026 | -0.026 | -0.038 | 0.042 | -0.156 | -0.098 |
| Morganella morganii | .332* | .753** | -0.051 | 0.055 | 1 | -0.051 | 0.064 | .753** | -0.081 | 0.22 | -0.123 | -0.051 | -0.051 | -0.051 | 0.063 | -0.05 | -0.158 | -0.092 |
| M. psychrotolerans | .399** | -0.026 | 1.000** | .972** | -0.051 | 1 | -0.026 | -0.026 | .937** | 0.045 | 0.176 | 1.000** | 1.000** | -0.019 | -0.026 | 0.237 | -0.109 | -0.114 |
| Proteus hauseri | .281* | -0.038 | -0.026 | -0.035 | 0.064 | -0.026 | 1 | -0.038 | -0.042 | -0.155 | -0.101 | -0.026 | -0.026 | -0.026 | .994** | -0.136 | 0.193 | -0.126 |
| Providencia rettgeri | 0.043 | -0.047 | -0.033 | 0.161 | .327* | -0.033 | -0.047 | -0.047 | -0.052 | -0.156 | -0.125 | -0.033 | -0.033 | -0.033 | -0.046 | -0.168 | -0.075 | -0.073 |
| Hafnia paralvei | 0.145 | -0.048 | -0.034 | -0.045 | 0.02 | -0.034 | .813** | -0.048 | -0.054 | -0.169 | -0.129 | -0.034 | -0.034 | -0.034 | .813** | -0.175 | .317* | .272* |
| Klebsiella variicola | 0.085 | -0.054 | -0.038 | -0.051 | -0.031 | -0.038 | .359** | -0.054 | -0.051 | -0.204 | -0.146 | -0.038 | -0.038 | -0.038 | .361** | -0.144 | 0.228 | -0.067 |
| Enterobacter asburiae | 0.05 | -0.065 | -0.045 | -0.06 | -0.044 | -0.045 | .319* | -0.065 | -0.062 | -0.238 | -0.158 | -0.045 | -0.045 | -0.045 | .316* | -0.162 | 0.218 | -0.045 |
| Enterobacter hormaechei | -0.087 | -0.05 | -0.035 | -0.043 | -0.084 | -0.035 | -0.05 | -0.05 | 0.001 | -0.17 | -0.129 | -0.035 | -0.035 | -0.035 | -0.05 | -0.171 | .405** | 0.058 |
| Enterobacter kobei | -0.074 | -0.054 | -0.038 | -0.046 | -0.104 | -0.038 | -0.054 | -0.054 | -0.041 | -0.152 | -0.129 | -0.038 | -0.038 | -0.038 | -0.054 | -0.177 | .339* | -0.054 |
| Kluyvera cryocre-scens | -0.021 | -0.058 | -0.041 | -0.054 | -0.112 | -0.041 | -0.058 | -0.058 | -0.047 | -0.072 | 0.071 | -0.041 | -0.041 | .390** | -0.058 | 0.01 | -0.107 | -0.144 |
| Klebsiella oxytoca | -0.007 | -0.033 | -0.023 | -0.031 | -0.064 | -0.023 | -0.033 | -0.033 | 0.026 | -0.116 | -0.089 | -0.023 | -0.023 | -0.023 | -0.033 | -0.12 | .289* | 0.052 |
| Citrobacter freundii | -0.019 | -0.078 | 0.033 | 0.016 | -0.144 | 0.033 | -0.078 | -0.078 | 0.015 | -0.066 | -0.11 | 0.033 | 0.033 | .380** | -0.078 | -0.073 | -0.081 | -0.129 |
| Citrobacter sedlakii | -0.121 | -0.026 | -0.019 | -0.025 | -0.051 | -0.019 | -0.026 | -0.026 | -0.029 | -0.058 | -0.016 | -0.019 | -0.019 | -0.019 | -0.026 | -0.095 | 0.008 | -0.085 |
| Escherichia/Shigella | -0.008 | -0.036 | -0.025 | -0.039 | -0.09 | -0.025 | 0.014 | -0.036 | -0.047 | -0.12 | .385** | -0.025 | -0.025 | -0.025 | -0.058 | 0.015 | 0.059 | 0.054 | -0.062 |
| Bacteroides fi-negoldii | .318* | -0.042 | .937** | .907** | -0.081 | .937** | -0.042 | -0.042 | 1 | 0.018 | 0.142 | .937** | .937** | -0.029 | -0.042 | 0.231 | -0.106 | -0.088 |
| Parabacteroides distasonis | .360** | .442** | 0.045 | 0.013 | 0.22 | 0.045 | -0.155 | .442** | 0.018 | 1 | .289* | 0.045 | 0.045 | 0.25 | -0.155 | .483** | -0.191 | -0.11 |
| Clostridium bolteae | .282* | -0.011 | 0.176 | 0.147 | -0.123 | 0.176 | -0.101 | -0.011 | 0.142 | .289* | 1 | 0.176 | 0.176 | 0.068 | -0.101 | .705** | -0.243 | -.272* |
| Clostridium la-valense | .399** | -0.026 | 1.000** | .972** | -0.051 | 1.000** | -0.026 | -0.026 | .937** | 0.045 | 0.176 | 1 | 1.000** | -0.019 | -0.026 | 0.237 | -0.109 | -0.114 |
| Enterococcus avium | .399** | -0.026 | 1.000** | .972** | -0.051 | 1.000** | -0.026 | -0.026 | .937** | 0.045 | 0.176 | 1.000** | 1 | -0.019 | -0.026 | 0.237 | -0.109 | -0.114 |
| Megamonas rupellensis | .293* | -0.026 | -0.019 | -0.025 | -0.051 | -0.019 | -0.026 | -0.026 | -0.029 | 0.25 | 0.068 | -0.019 | -0.019 | 1 | -0.026 | 0.073 | -0.109 | -0.114 |
| Allisonella histamini-formans | .285* | -0.038 | -0.026 | -0.035 | 0.063 | -0.026 | .994** | -0.038 | -0.042 | -0.155 | -0.101 | -0.026 | -0.026 | -0.026 | 1 | -0.136 | 0.184 | -0.13 |
| Flavonifractor plautii | .272* | 0.042 | 0.237 | 0.198 | -0.05 | 0.237 | -0.136 | 0.042 | 0.231 | .483** | .705** | 0.237 | 0.237 | 0.073 | -0.136 | 1 | -.292* | -0.211 |
| Bacteroides faecich-inchillae | 0.226 | 0.098 | .676** | .633** | 0.13 | .676** | -0.043 | 0.098 | .683** | 0.103 | 0.134 | .676** | .676** | -0.081 | -0.043 | .323* | 0.021 | -0.057 |
| Bacteroides stercoris | 0.184 | -0.106 | .315* | .292* | -0.156 | .315* | -0.106 | -0.106 | .286* | .374** | 0.235 | .315* | .315* | -0.074 | -0.105 | .400** | -0.164 | 0.028 |
| Bacteroides xylani-solvens | 0.157 | .448** | -0.048 | -0.064 | .285* | -0.048 | -0.069 | .448** | -0.076 | .362** | 0.205 | -0.048 | -0.048 | 0.071 | -0.069 | 0.139 | -0.098 | -0.064 |
| Hungatella hathewayi | 0.179 | -0.059 | .415** | .392** | -0.114 | .415** | -0.059 | -0.059 | .367** | .354** | 0.179 | .415** | .415** | -0.041 | -0.059 | .322* | -0.142 | 0.09 |
| Blautia stercoris | 0.207 | -0.091 | .453** | .426** | -0.091 | .453** | -0.035 | -0.091 | .391** | -0.165 | .342* | .453** | .453** | -0.064 | -0.034 | 0.193 | -0.102 | -0.237 |
| Blautia glucerasea | 0.075 | -0.056 | .272* | 0.245 | -0.139 | .272* | -0.084 | -0.056 | 0.232 | 0.042 | .574** | .272* | .272* | -0.059 | -0.084 | .268* | -.267* | -.310* |
| Clostridium leptum | 0.03 | .452** | -0.083 | -0.102 | .327* | -0.083 | -0.118 | .452** | -0.123 | 0.25 | -0.03 | -0.083 | -0.083 | -0.044 | -0.118 | 0.058 | 0.053 | -0.124 |
| Clostridium nexile | -0.128 | -0.038 | -0.048 | -0.064 | -0.107 | -0.048 | -0.068 | -0.038 | -0.076 | .427** | 0.065 | -0.048 | -0.048 | 0.013 | -0.068 | 0.214 | -0.154 | -0.074 |
| Clostridium ra-mosum | -0.026 | -0.085 | .325* | .297* | -0.141 | .325* | -0.085 | -0.085 | .272* | -0.089 | 0.119 | .325* | .325* | -0.011 | -0.084 | 0.091 | -0.247 | -.278* |
| Odoribacter laneus | 0.068 | -0.026 | -0.019 | 0.144 | .331* | -0.019 | -0.026 | -0.026 | -0.029 | -0.088 | -0.071 | -0.019 | -0.019 | -0.019 | -0.026 | -0.095 | -0.109 | -0.048 |
| Parabacteroides goldsteinii | 0.053 | -0.06 | .320* | .298* | -0.115 | .320* | -0.06 | -0.06 | .392** | -0.016 | 0.265 | .320* | .320* | -0.042 | -0.06 | 0.027 | -0.129 | -0.166 |
| Parabacteroides merdae | -0.001 | 0.255 | -0.077 | -0.101 | .325* | -0.077 | -0.11 | 0.255 | -0.11 | 0.055 | 0.119 | -0.077 | -0.077 | -0.077 | -0.11 | .444** | -0.063 | -0.016 |
| Parasutterella excrementihominis | 0.207 | .290* | -0.07 | -0.093 | 0.235 | -0.07 | -0.1 | .290* | -0.111 | .390** | .274* | -0.07 | -0.07 | -0.07 | -0.1 | .267* | -0.011 | -0.09 |
| Ruminococcus bromii | 0.022 | .346** | -0.107 | -0.108 | 0.243 | -0.107 | -0.153 | .346** | -0.139 | 0.064 | 0.028 | -0.107 | -0.107 | -0.107 | -0.152 | -0.173 | -0.221 | 0.087 |
| Ruminococcus torques | 0.24 | -0.084 | .363** | .322* | -0.198 | .363** | -0.134 | -0.084 | .298* | 0.222 | .666** | .363** | .363** | -0.067 | -0.134 | .602** | -0.021 | -0.125 |
| Anaerostipes butyraticus | 0.068 | -0.01 | -0.029 | -0.039 | -0.056 | -0.029 | -0.042 | -0.01 | -0.047 | -0.057 | .569** | -0.029 | -0.029 | -0.029 | -0.042 | 0.03 | -0.152 | -0.145 |
| Anaerotruncus colihominis | 0.079 | -0.054 | -0.038 | -0.05 | -0.104 | -0.038 | -0.054 | -0.054 | -0.06 | 0.111 | .315* | -0.038 | -0.038 | -0.038 | -0.054 | .412** | -0.162 | 0.043 |
| Odoribacter splanchnicus | -0.218 | -0.061 | -0.154 | -0.159 | -0.158 | -0.154 | -0.22 | -0.061 | -0.147 | -0.117 | -0.16 | -0.154 | -0.154 | -0.154 | -0.219 | -0.099 | 0.137 | .277* |
| Faecalibacterium prausnitzii | -0.137 | -0.083 | -0.178 | -0.149 | -0.01 | -0.178 | -0.028 | -0.083 | -0.168 | -0.098 | -.410** | -0.178 | -0.178 | -0.049 | -0.029 | -.394** | .421** | .415** |
| Acidaminococcus intestini | -0.137 | -0.076 | -0.053 | -0.071 | -0.088 | -0.053 | -0.076 | -0.076 | -0.075 | -0.138 | 0.047 | -0.053 | -0.053 | 0.046 | -0.076 | 0.115 | -0.075 | -0.028 |
| Adlercreutzia equolifaciens | -0.121 | -0.026 | -0.019 | -0.025 | -0.051 | -0.019 | -0.026 | -0.026 | -0.029 | -0.058 | -0.016 | -0.019 | -0.019 | -0.019 | -0.026 | -0.095 | 0.008 | -0.085 |
| Alistipes shahii | -0.262 | -0.143 | -0.1 | -0.076 | -0.029 | -0.1 | -0.143 | -0.143 | -0.088 | -0.134 | -.275* | -0.1 | -0.1 | -0.1 | -0.143 | -0.194 | -0.068 | -0.02 |
| Asaccharobacter celatus | -0.156 | -0.066 | -0.046 | -0.062 | -0.128 | -0.046 | -0.066 | -0.066 | -0.009 | -0.102 | -0.079 | -0.046 | -0.046 | -0.046 | -0.066 | -0.101 | -0.035 | -0.155 |
| **Roseburia faecis** | -.288* | -0.156 | -0.109 | -0.142 | -0.158 | -0.109 | 0.193 | -0.156 | -0.106 | -0.191 | -0.243 | -0.109 | -0.109 | -0.109 | 0.184 | -.292* | 1 | .402** |
| **Blautia faecis** | -.350** | -0.098 | -0.114 | -0.125 | -0.092 | -0.114 | -0.126 | -0.098 | -0.088 | -0.11 | -.272* | -0.114 | -0.114 | -0.114 | -0.13 | -0.211 | .402** | 1 |
| **Bifidobacterium longum** | -0.259 | 0.146 | -0.137 | -0.169 | -0.001 | -0.137 | 0.032 | 0.146 | -0.013 | 0.083 | -0.105 | -0.137 | -0.137 | -0.061 | 0.034 | -0.013 | 0.039 | 0.004 |
| Bacteroides faecis | 0.15 | -0.069 | -0.048 | -0.064 | -0.132 | -0.048 | -0.069 | -0.069 | -0.051 | 0.104 | 0.236 | -0.048 | -0.048 | .651** | -0.068 | 0.142 | -0.159 | -0.154 |
| Bacteroides fragilis | 0.244 | -0.009 | 0.158 | 0.136 | -0.092 | 0.158 | -0.075 | -0.009 | 0.116 | .343* | 0.215 | 0.158 | 0.158 | .371** | -0.075 | .348** | -0.252 | 0.065 |
| Bacteroides vulgatus | 0.078 | -0.122 | 0.246 | 0.229 | -0.12 | 0.246 | 0.113 | -0.122 | 0.205 | .361** | 0.107 | 0.246 | 0.246 | -0.086 | 0.113 | 0.231 | -0.107 | -0.194 |
| Bacteroides nordii | 0.021 | -0.03 | -0.021 | -0.028 | -0.059 | -0.021 | -0.03 | -0.03 | -0.034 | .356** | 0.004 | -0.021 | -0.021 | 0.141 | -0.03 | 0.223 | -0.126 | -0.131 |
| Blautia luti | -0.181 | -0.166 | -0.117 | -0.085 | -0.108 | -0.117 | -0.002 | -0.166 | -0.072 | -.315* | -0.216 | -0.117 | -0.117 | -0.117 | -0.002 | -0.223 | 0.032 | 0.107 |
| B.coprocola | -0.02 | -0.069 | -0.049 | -0.064 | -0.08 | -0.049 | -0.069 | -0.069 | -0.054 | -0.031 | -0.074 | -0.049 | -0.049 | .447** | -0.069 | 0.001 | 0.007 | 0.045 |
| Bacteroides dorei | -0.046 | 0.037 | -0.087 | -0.111 | -0.038 | -0.087 | -0.124 | 0.037 | -0.119 | -0.024 | .401** | -0.087 | -0.087 | 0.092 | -0.124 | .327* | -0.099 | -0.065 |
| B. eggerthii | -0.208 | -0.051 | -0.036 | -0.048 | -0.099 | -0.036 | -0.051 | -0.051 | 0.224 | -0.067 | -0.087 | -0.036 | -0.036 | -0.036 | -0.051 | -0.04 | -0.053 | -0.007 |
| B. ovatus | -0.079 | 0.013 | -0.089 | -0.113 | 0.002 | -0.089 | -0.098 | 0.013 | -0.116 | -0.038 | .424** | -0.089 | -0.089 | 0.02 | -0.098 | .289* | -0.232 | -0.218 |
| Barnesiella intestini-hominis | -0.21 | -0.133 | -0.093 | -0.076 | -0.075 | -0.093 | -0.133 | -0.133 | -0.066 | -0.118 | 0.18 | -0.093 | -0.093 | -0.093 | -0.133 | 0.094 | 0.051 | 0.168 |
| Bifidobacterium adolescentis | -0.117 | 0.037 | -0.113 | -0.125 | -0.032 | -0.113 | -0.133 | 0.037 | -0.127 | 0.009 | -0.155 | -0.113 | -0.113 | -0.113 | -0.133 | -0.114 | 0.164 | 0.029 |
| B.bifidum | -0.002 | 0.258 | -0.071 | -0.094 | 0.112 | -0.071 | -0.101 | 0.258 | -0.095 | 0.043 | -0.131 | -0.071 | -0.071 | -0.071 | -0.101 | -0.095 | -0.055 | 0.09 |
| Bilophila wadsworthia | 0.206 | 0.189 | 0.117 | 0.11 | 0.076 | 0.117 | -0.165 | 0.189 | 0.123 | 0.265 | .304* | 0.117 | 0.117 | 0.022 | -0.164 | .328* | -0.234 | -0.121 |
| Blautia hansenii | 0 | -0.06 | 0.186 | 0.167 | -0.116 | 0.186 | -0.06 | -0.06 | 0.15 | -0.057 | .520** | 0.186 | 0.186 | -0.042 | -0.06 | .292* | -0.248 | -0.207 |
| Blautia schinkii | 0.07 | -0.03 | -0.021 | -0.028 | -0.057 | -0.021 | -0.03 | -0.03 | -0.033 | -0.064 | .584** | -0.021 | -0.021 | -0.021 | -0.03 | 0.039 | -0.123 | -0.128 |
| Blautia wexlerae | -0.224 | -0.161 | -0.173 | -0.2 | -0.145 | -0.173 | 0.048 | -0.161 | -0.076 | -0.08 | -.303* | -0.173 | -0.173 | 0.035 | 0.049 | 0.011 | 0.193 | .459** |

and some *Fermicutes*, such as *Clostridium bolteae, C. lavense, Enterococcus avium, Megamonas repellensis, Allisonella histamini-*

*formans* and *Flavonifractor plautii* were indirectly associated with *Enterobacter families,* except for *Flavonifractor plauti*. (Table 5)

*Roseburia faecis* and *Blautia faecis* were negatively associated with BHB concentration, but they showed syntrophic correlation with *Providencia rettgeri, Hafnia paralvei*, and other enterobacters. Five cases with very low *Enterobacter* profile showed lowest BHB level. Only one case with high BHB level showed no positive association to proteobacteriae, but broad association was pre-
sent, such as *Megamonas funiformis, Prevotella copr*i and *P. stercorea, Bifidobacterium adolescentis, Faecalibacterium prausnizii,* etc. (Table 5)

These association was confirmed by individual level (Suppl. Table 2). *Enterobactereciae* species were recognized in 20/22 high bHB producers, 13/17 moderate bHB producers and 15/16 low producers. Two in high BHB, 4 in middle BHB, and 1 in low BHB did not have *enterobacteriacea* species, and in these cases, *Clostridium bolteae, C. lavalense, Enterococcus avium, Hunatella hathewayi, Megamonas rupellensis and Ruminococcus bromii* seemed to be related to BHB production.

Cooperation (syntrophic) or suppression (competitive) of bacilli was suggested by correlation network among individual species (Table 5). Generally BHB producing bacteria suppressed butyrate producing bacteria. Sumary relationship between BHB level and network of microbiota is shown in Table 6.

Table 6. Microbiota with direct and indirect correlation with serum BHB level

| | Primary correlation | CC with BHB | phylum | Secondary relationship | CC with name1 |
|---|---|---|---|---|---|
| Direct correlation | *Providencia vermicola* | .418** | P | | |
| | *Providencia rustigianii* | .400** | P | | |
| | *Providencia sneebia* | .399** | P | | |
| | *Morganella morganii* | .332* | P | | |
| | *Morganella psychrotolerans* | .399** | P | | |
| | *Proteus hauseri* | .281* | P | | |
| Indirect correlation | *Butyricimonas virosa* | .400** | B | *Providencia rustigianii* | 1.000*** |
| | *Clostridium lavalense* | .399** | F | *E.coli/shigela* | .385** |
| | *Enterococcus avium* | .399** | F | *P. sneebia, P.vermicola, Morganella* | 1.000*** |
| | *Parabacteroides distasonis* | .360** | B | *providencia* | .990*** |
| | *Bacteroides finegoldii* | .318* | B | *providencia, butyricomonas* | .442*** |
| | *Megamonas rupellensis* | .293* | F | *Kluyvera, citrobacter* | .390*** |
| | *Allisonella histaminiformans* | .285* | F | *proteus, hafnia* | .994** |
| | *Clostridium bolteae* | .282* | F | *E.coli/shigela* | .385** |
| | *Flavonifractor plautii* | .272* | F | many | .600*** |
| Negative correlation | Roseburia faecis | -.288* | F | *enterobacter, hafnia, krebsiella* | .405*** |
| | Blautia faecis | -.350** | F | many | .400-.500*** |
| | Rejected hit | -.285* | | | |

Effects of Dietary habit on bacterial profile

From semiquantitative food frequency questionnaire before participation in the wellness fasting, water soluble and insoluble dietary fiber intake, fat and protein intake were individually calculated. Protein intake showed significant correlation with *Acidaminococcus intestinalis* (CC=0.24*), fat intake with *Blautia faecis* (CC=0.326*), *Dialister inivisis* (CC=0.394**), and *Hafnia paraluei* (CC=0.343*). Dietary fibers positively correexcept for *Hafnia arluei.* Competitive and trophic interaction lated with *Fusobacterium mortiferum* (CC=0.343**), *Lactonifactor longoviformis* (CC=0.269*), *Eubacterium eligens* (CC=0.262*) and negatively correlated *Bifidobacterium adolescence* (CC=-0.288*), *B. breve* (CC=-0.284*), *Citrobacter sedlakii* (CC=-0.274*), and *Blautia luti* (CC=-0.247*). Both protein intake and dietary fiber intake did not show significant correlation with Enterobacter family,

seemed to be present.

## 4. Discussion

In the fasted state, acetyl CoA is provided from $\beta$-oxidation of fatty acids and overflowed acetyl CoA makes acetoacetyl CoA, and ketone bodies are produced via 3'-hydroxy-3 methyl glutaryl CoA. [27] In bacteria, synthesis and degaradation of ketone bodies is similar, but relationship between butyrate metabolism is obscure. (Fig. 3)

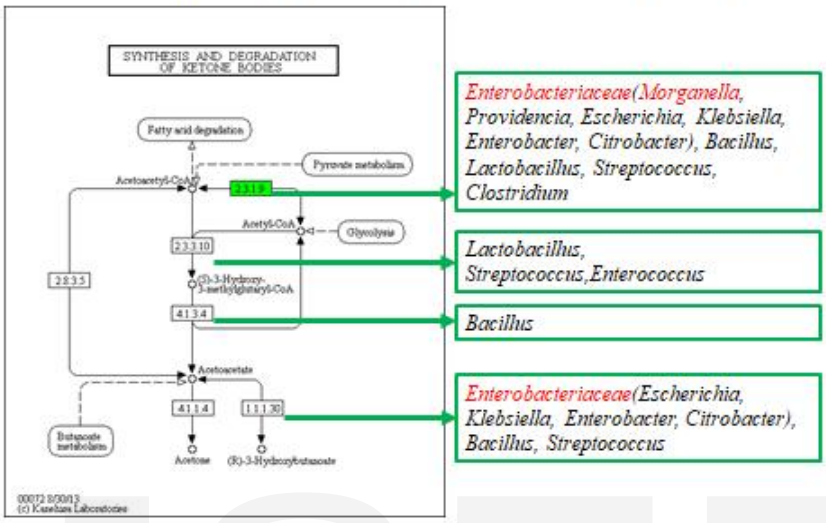


Fig. 3. Synthesis and degradation of ketone bodies
Reference pathyway and bacteria with key enzyme.

Cahill [29] studied the glucose metabolism of people who let themselves fast for 40 days, and he found that in the starving human adult, BHB and aceto-acetate were produced in the liver from long-chain fatty acids and BHB could be the energy source in the brain and other tissues. A rise of BHB blood concentration to approximately 6 mM was characteristic. Approximately all of the lactate, pyruvate, glycerol, and amino acid carbons which are removed by the liver and kidney are converted into glucose, as evidenced by substrate balances across these organs to keep the basic level. [30]

Cahill's research was integrative rather than reductionism, but he opened the unique insight on the metabolic adaptation of humans to starvation. During starvation, extremely low insulin levels facilitate acyl-CoA entry into mitochondria, producing excess amounts of acetyl-CoA that cannot be metabolized in the Krebs cycle and are diverted towards the synthesis of ketone bodies. v BHB is considered to be produced in the liver, kidney and astrocytes in the brain, but our case report suggested the involvement of intestinal microbiota. Fasting caused hyperketonemia, but the degree was different by individuals. [4] We tried to clarify the involvement of microbiota for production of ketone bodies in this study.

Mitsuo Koda [31] developed fasting dietary therapy and confirmed beneficial effects for many patients with intractable diseases. About 900-1000 kcal/day by unpolished brown rice, green vegetable paste and *tofu* constitute the basic regimen of Koda's therapy. We found the successful patient who had recovered from spino-cerebellar degeneration at a young age by Koda's dietary therapy.[17] She had been living on only one glass of fresh vegetable juice per day for 18 years since her acute episode at age 20. Her ketone bodies, especially BHB in the blood, were more than 3 mM, so the main energy should come from ketone bodies. Her biochemical changes coincided with the metabolic adaptation to yield BHB, as shown by elevated aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatine kinase (CK). High aspartate was a reflection of above metabolic change. Increased BHB was observed in two participants among five, and both of them had *Bifidobacteriacae* in the fecal bacteria. [17] Some *Bacteroides* had xylanase or cellulose activities, so these species may play an important role in fiber degradation in a strict vegetarian. Hayashi, et al. [32] had previously analyzed the fecal bacteria of Mori 15 years ago. They found that *Clostridium* and *Bacteroides* were the dominant groups. They also found many *Bifidobacterium* by direct culture.

Our previous study in wellness fasting and BHB production by Pass analysis showed that the decreased BMR and insulin significantly contributed to the increase in BHB and AcAc. [4,5] From our experience of Mori's case, we considered the different gut microbiome should influence the production of BHB.

Comparison of our cases with other Japanese microbiota

Nishijima et al. [33] analyzed gut microbiota of the Japanese by comparing the metagenomic data obtained from 106 Japanese individuals with those from 11 other nations. They found that the composition of the Japanese gut microbiome showed more abundant in the phylum Actinobacteria, in particular in the genus *Bifidobacterium*, than other nations. Hisada et al. [22] reported predominance of *Fermicutes* and our case showed the similar proportion. However, *Proteobacteria* and *Verrucobacteria* may be influenced by fasting and their dietary habit as vegetalian.

Numerous animal models and human studies consistently demonstrated that gut microbiota can modulate host energy homeostasis and adiposity through different mechanisms. [34-39] Dietary change or fasting could change microbiota quickly within a short period [40,41]. Some people could not raise ketone concentration in the blood as expected even by the ketogenic diet. A metabolic homeostasis would be different in these people, and need further study.

Relationship with butyrate producing bacteria:

Butyrate-producing bacteria may represent a functional group, rather than a coherent phylogenetic group, within the microbial community of the human large intestine. Butyrate formation can play a special role in bacterial energy metabolism [42-46] and this implies that certain features of energy metabolism and microbial ecology may be shared between phylogenetically distinct groups of butyrate-producing bacteria. Numerically, two of the most important groups appear to be *Faecalibacterium prausnitzii*, which belongs to the *Clostridium leptum* cluster, and *Eubacterium rectale/Roseburia* spp., which belong to the *Clostridium coccoides* cluster of firmicute bacteria. After reduction to butyryl-CoA, butyrate can be formed either with the enzymes phosphotransbutyrylase and butyrate-kinase via butyry-l-phosphate or with the enzyme butyryl-CoA : acetate CoA-transferase, which utilizes acetate as a co-substrate and generates acetyl-CoA. (Fig. 4)

Butyrate-producing human gut isolates, covering a phylogenetically wide range of representatives of clostridial cluster XIVa and some cluster IV and XVI strains, indicated that the butyryl-CoA : acetate CoA-transferase route is far more prevalent in this ecosystem than the butyrate kinase route.[44]

Could synthesis of BHB come from butyrate? The current data suggested *Enterobactericeae* mainly correlated to the BHB production, but several other species in *Clostridium* and *Bacteroides* that had correlation with BHB were indirectly related to *Enterobactericeae*. *Enterobacter* seemed to suppress *Fermicutes*, so butyrate producing bacteria would be suppressed in the colon. Possibility of metabolism by intestinal microbiota from butyrate to BHB in the body seemed to be independent.

In addition, there are many bacteria that can synthesize poly(3-hydroxy butyrate-co-3 hydroxyvalerate) oly-beta hydroxyl butyrate.[47-50] These bacteria are capable of using a broad range of carbon sources for their growth and for the production of polyhydroxyalkanoates (PHAs). They can use monosaccharides (glucose and fructose), disaccharides (sucrose), pentoses (xylose and arabinose), various organic acids (acetic acid, propionic acid and octanoic acid) for growth and the production of polyhydroxyalkanoates (PHAs). PHAs and BHB could be hydrolyzed inside and outside of the bacilli.

One case who did not show the direct or indirect correlation with Enterobacteriaceae could be related to such unknown bacteria.

## 4 . Conclusion

1. Wellness fasting caused metabolic and physiological changes , and induced hyperketonemia which showed correlation with prefasting microbiota profile.
2. High BHB level showed association with family *Enterobacteriaceae* directly or indirectly.
3. The dominance of *Enterobacteriaceae* seemed to suppress butyrate producing bacteria. So, the BHB production seemed to be independent from butyrate pathway in the gut.
4. The rout of BHB synthesis would be multiple by syntrophic and/or competitive growth of bacteria.

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