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## **The fecal content of Veillonellaceae family bacteria correlates with cognitive parameters in young healthy human subjects**

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## ORIGINAL ARTICLE

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### **The fecal content of *Veillonellaceae* family bacteria correlates with cognitive parameters in young healthy human subjects**

Short title: Blanka Dwojaczny et al., *Veillonellaceae* bacteria and cognitive parameters

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#### **ABSTRACT**

**Introduction:** Several lines of evidence suggest that the composition of gut microbiota influences the central nervous system function. Previous studies demonstrated that gut microbiota composition can affect the mood, anxiety and cognitive performance. However, most of the research was focused on the animal models and older humans. Relatively limited number of reports examined the influence of gut microbiota on cognitive functions in young, healthy human subjects.

**Material and methods:** We examined the influence of gut microbiota composition on cognitive performance in 30 volunteers (24 females and 6 males; mean age  $22.53 \pm 1.97$  yr.). In order to evaluate the cognitive performance in our subject we used three standard tests:

Face/Name Association Test, Trial Making Test and Stroop Test. The composition of intestinal microbiota was determined in fecal samples using 16S rDNA V3-V4 regions analysis.

**Results:** The study demonstrated that the proportion of *Veillonellaceae* bacteria (phylum *Firmicutes*) in the subject's fecal matter positive correlated with the results of one of the cognitive tests — Trial Making Test.

**Conclusions:** Our results indicate that bacteria from *Veillonellaceae* family may influence the level of some cognitive functions (namely executive ones). Our results are consistent with previous studies describing the potential impact of intestinal bacterial on cognitive performance.

**Key words:** *Firmicutes*, *Veillonellaceae*, executive function, gut microbiota, gut-brain axis, Trial Making Test

## Introduction

Cognitive function refers to the mental processes such as learning, memory, perception, language skills, attention, reasoning and manipulation of information and decision making [1]. The level of cognitive function can depend on many factors. Following factors were found to be of particular importance: age [2, 3.], level of physical activity [4, 5], hormones [6, 7] and levels of adipose tissue [8, 9]. In recent years, various studies have demonstrated that composition of gut microbiota can influence functions of the central nervous system, such as cognition, mood and behavior [10–12].

The intestinal microbiota are all bacteria, viruses (including bacteriophages), and fungi residing in the gastrointestinal track. The majority of them are bacteria residing in the colon [13]. The bacterial species found in the intestines of adults belong above all to two types: *Firmicutes* (*Clostridium*, *Enterococcus*, *Lactobacillus*, *Faecalibacterium*, *Ruminococcus*) and *Bacteroidetes* (*Bacteroides* and *Prevotella*). In smaller numbers: *Actinobacteria* (mainly *Bifidobacteriu*), *Proteobacteria* (mainly *Escherichia*), *Verrucomicrobia* and *Euryarchaeate* [14, 15]. It is the composition of “healthy” gut microbiota which play role in pathogen protection, nutrition, host metabolism, immune modulation [16, 17]. It has been demonstrated that dysbiosis (loss of beneficial microbial organism or overgrowth of pathobionts) can have a negative effect on the host organism [18]. For example, dysbiosis may be implicated in the pathogenesis of obesity [19], diabetes [20], cardiovascular disease [21], irritable bowel syndrome [22] and colorectal cancer [23].

Several studies indicated the relationship between the composition of intestinal microbiota and the central nervous system. It was demonstrated that altered composition of

intestinal microbiota (elevated levels *Clostridium* species) occurs in an autism spectrum disorders [24], in patients with depressive and anxiety like disorder (elevated level of *Bacteroides*, reduced *Lachnospiraceae* and *Prevotellaceae*) [25]. Dementia has been associated with a reduction *Bacteroides* and increase *Firmicutes* [26]. Furthermore, amount of *Verrucomicrobia* was reduced, *Proteobacteria* and *Actinobacteria* elevated in Alzheimer disease patients [27].

Particularly interesting are the studies which show the influence of composition of gut microbiota on cognitive function. Results of previous studies indicate that bacteria living in the gut can modulate level of cognitive function. Bruce-Keleler et al. [28] reported the correlation between the size of *Verrucomicrobia* gut population and cognitive performance including attention, executive function, and memory. It was also demonstrated that higher proportions of *Bacteroides* is positively associated with learning and memory performance [29], whereas relative abundance of *Actinobacteria* phyla was related to cognitive performance such as speed attention and cognitive flexibility [30].

Several hypotheses have been proposed to explain the potential mechanisms underlying the influence of intestinal microbiota on central nervous system. The influence of gut microbiota on cognitive function may be associated with induced expression of hippocampal BDNF (brain-derived neurotrophic factor). BDNF plays a very important role in the regulation of neurogenic processes, regulates neuronal survival, differentiation and activity-dependent synaptic plasticity [31]. It was shown that germ-free mice have lower BDNF level in the cortex and hippocampus, lower cognitive performance (compared to normal, healthy mice) and increased anxiety indicators [32].

Furthermore, gut microbiota may contribute to increase in the level of intestinal permeability [33] for pathogenic, immune-stimulating and neuroactive substances. In the circulation these substances may activate a proinflammatory immune response and compromise the integrity of the blood brain barrier [34]. In this way microbiota may contribute to heightened microglial activation and production of pro-inflammatory cytokines in the brain [35]. It is known that chronic inflammation can contribute to cognitive decline [36]. In addition, metabolites of gut fermentation of dietary fibers such as acetic, butyrate, succinate, and propionic acids (short-chain fatty acid, SCFA) can also influence some functions of central nervous system. It was demonstrated that SCFA affect neurotransmitters production [37] and modulate neurotrophic factors (BDNF, NGF) [38]. Furthermore, SCFA can inhibit the production of proinflammatory cytokines and enhances expression IL-10 (anti-inflammatory cytokine) [39]. SCFAa has a beneficial and protective effect on blood-brain

barrier [40]. Decreased SCFA levels are observed in patients with neurodegenerative disorders [41]. It has been demonstrated that especially anaerobic bacteria (such as *Firmicutes*) significantly increase the levels of SCF's [42].

In the current study was investigated the relationship between composition of gut microbiota and the level of selected cognitive functions in young, healthy people.

## **Material and methods**

The study was conducted in accordance with the Declaration of Helsinki for Human Studies. The study protocol was approved by a local Ethics Committee.

Thirty volunteers (22 females and 6 males; aged  $22.53 \pm 1.97$ ) participated in this research. The volunteers were students from the Collegium Medicum in Bydgoszcz. Participants were qualified based on a questionnaire. Exclusion criteria included: history of antibiotic use in the past 30 days, history of alcohol or illicit drug dependence, history of taking medications and dietary supplements, history of sleep disorders. In addition, the volunteers assessed their mood, level of physical activity, eating habits and sleep quality. All persons underwent anthropometric measurements and have determined body fat content using body composition analyzer (type BC-418MA).

## **Cognitive test**

Three tests were used to assess the level of cognitive functions: Face/Name Association Test, Stroop Test and Trial Making Test. The Face/Name Association Test consists of two stages (acquisition and retrieval phase) separated by a 10-minute break. In the acquisition phase of the face/name association test, subjects were exposed to 100 faces associated with a single name on a computer screen (presented for 2 seconds). After 10 min from the end of acquisition phase the retrieval phase began. During this phase test subjects were presented with the same faces as in acquisition phase, but each face was associated with two names, one of which was the same name as in acquisition phase. The task of the subject was to indicate the name associated with the face during acquisition phase. No time limitations for retrieval phase were imposed by the protocol. The percent of correctly answered names, and the duration of the retrieval phase were monitored for each subject. Face/name test evaluating short-term declarative memory associated with hippocampal activity [43].

The Stroop test consisted of four pages. The first test page contained the names of colors written in two columns in black ink (20 words in each column). The task was to read the names of colors. The second page contained the rows of cross marks in two columns (20 rows in each column). The rows of cross marks were displayed in different colors. A color of each row was recognized and pronounced by each participant. The third and four pages contained the names of colors written in two 20-word columns. An ink color was different than the name of a color. The written name of color (third page) or the color of the ink (four page) were recognized and pronounced by each subject. For each page the time of reading duration was recorded. In the statistical analysis we used the reading time of the last page expressed as a percentage of the first page reading time. The Stroop test measures multiple cognitive processes such as executive control, selective attention and ability to inhibit habitual responses correct performance of the tasks in this test requires the ability to inhibit the automatic responses. These abilities are strongly associated with the activity of prefrontal and anterior cingulate cortical areas [44].

The Trial Making Test consisted of two pages. The first page contained numbers from 1 to 25 which are randomly arranged on a piece of paper. The task of the subject is to connect numbers of a continuous line (without revealing a papier pencil). The second page contained numbers (from 1 to 13) and letter (from A to L) which are randomly arranged on a papier. The task of the subject is to connect alternately numbers and letters (without revealing a papier pencil). The speed of task completion in part A mainly reflects visual-spatial abilities, while in part B additionally examines the ability to switch between two types of tasks (alternating numbers and letters). The result of the test is the time it took to complete part A and part B, respectively. TMT test measures prefrontal cortex-dependent attention and cognitive flexibility [45].

All tests were performed between 9:00 am and 15:00 pm.

### Gut microbiota analysis

Fecal samples were recruited to gut microbiota 16S rDNA V3-V4 regions analysis. All fecal samples were collected to a standard tube, stored in a freezer and within 24h sent to CM Medgen Laboratory. DNA was extracted using Qiagen QIAamp PowerFecal ® Pro DNA Kit. DNA quantity and integrity was checked through Nanodrop. V3-V4 DNA regions (2x250nt) were sequenced on MiSeq Illumina instrument. The primers 341F (CCTACGGGNGGCWGCAG) and 805r (GACTACHVGGGTATCTAATCC) were used.

Sequencing data were analyzed according to QIIME2 recommendations. Raw reads were demultiplexed with demux plugin and pairs were joined with vsearch join-pairs plugin, keeping the minimum length of 180nt. Contigs were filtered using the quality-filter q-score plugin (--p-min-length-fraction 0.8. --p-min-quality 8. --p-quality-window 6) and denoised with Deblur. Filtered contigs were classified against GreenGenes v13.5 database using Naive-Bayes classifier. Taxa other than bacteria were removed from the final dataset.

## Statistical analysis

Results are expressed as means  $\pm$  SD. The relationship between final taxa counts and metadata columns were measured by applying a generalized linear model of Negative Binomial regression. Multiple hypothesis testing on all columns of a data matrix were performed. FDR values lower than 0.05 were considered as significant.

## Results

Cognitive test results: the average score in Face/Name Association Test was  $68.1\% \pm 7.61$ . The mean retrieval phase duration equaled  $306 \pm 66$  seconds. In the Stroop test volunteers reached the mean score of  $194.72 \pm 33.80\%$ . In the Trial Making Test the average score was  $69.66 \pm 19.74$  seconds and  $85.78 \pm 19.53$ , respectively in part A and B.

Anthropometric analysis: the average value of the body mass index was  $22.50 \pm 3.57$ . In 5 volunteers BMI value was higher than norm ( $> 25$ ) and in 4 volunteers the BMI value was lower than norm ( $< 19$ ). The average fat content was  $24.78\% \pm 8.41$ . In 5 volunteers fat content was higher than norm and in 4 volunteers fat content was lower

Microbiological analysis: in 30 tested samples the presence of 3 main one's bacteria phyla was demonstrated: *Bacteroidetes*  $54.43\% \pm 0.6\%$ , *Firmicutes*  $37.78\% \pm 15.32\%$  and *Proteobacteria*  $7.73\% \pm 7.10\%$ . Bacteroidetes phyla dominated in 22 out of 30 persons, while in 8 persons dominance *Firmicutes* phyla was demonstrated (Fig. 1). The abundance of *Veillonellaceae* family was from 1.15% to 34.40% (Fig. 2).

Negative binomial regression analyses revealed negative association between that bacterial family *Veillonellaceae* (*Firmicutes* phyla) and results Trial Making Test part B ( $p < 0.05$ ; FDR  $< 0.05$ ) (Figure 3). We observed a statistically significant relationship ( $p < 0.005$ ; FDR  $< 0.05$ ) between other cognitive function (for example results of Trial Making Test part A) and taxa belong to Firmicutes (Table 1) but number of patients is smaller than number of

taxa detected in all samples what lowers the statistical power. Therefore, these results can only be regarded as preliminary. We did not observed association between other bacterial family and results Trial Making Test part A, Stroop Test and Face/Name Association Test (Figs. 4–6).

## Discussion

Previous studies have revealed that composition of the intestinal microbiota may affect cognitive function. It has been observed that in particular the presence of *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Verrucomicrobia* phyla is corelated with level of cognitive performance [26, 27, 46].

Our current study shows that abundance of bacteria from *Veillonellaceae* family (*Firmicutes* phylum) is associated with decreased cognitive function as determined by Trial Making Test. In the literature of the subject the results of Trial Making Test are considered indicative of the level of executive function (working memory, flexible thinking, self-control) [45].

Several studies revealed that bacteria that live in the intestines can influence cognitive functions, but the results are not always consistent. In 2019, Saji et al. reported that the ratio of *Firmicutes* to *Bacteroides* species is higher in demented human subjects [26]. Whereas Manderino et al. [47] observed that higher proportion of *Firmicutes* may have positive influence on cognitive function in elderly people (n = 43; aged  $64.08 \pm 6.49$ ). Results from other studies demonstrated decreased *Firmicutes* in depressed mice and humans [25, 46, 48] and in Alzheimer's disease patients [27]. Additionally, most studies described the effect of the bacterial phyla, not family or order. In our research only the presence of one bacterial family — *Veillonellaceae* correlated with cognitive performance. In research conducted by Liu et al. [27], it was also observed that the relative microbiome density of bacteria from *Clostridiaceae*, *Lachnospiraceae* and *Ruminococcaceae* families were decreased in Alzheimer's disease patients. *Veillonellaceae* family participation in microbiome composition was increased in mild cognitive impairment patients and was negatively associated with scores of Montreal Cognitive Assessment. Similarly, Bajaj et al. and Wright and Jalan observed association between increased levels of *Veillonellaceae* and developing inflammation, endotoxemia, and cognitive impairment in patients with hepatic encephalopathy [49. 50].

In our study we did not observe association between levels of *Bifidobacterium* and cognitive function. However, literature describes positive effect of *Bifidobacterium* on the cognitive performance and potential therapeutic effect of *Bifidobacterium breve* in preventing



cognitive impairment in a mouse model of Alzheimer's disease [51]. Also, studies in human subjects showed that administration of *Bifidobacterium breve* positively influences memory function in older adults [52]. Other studies reported increased levels of synapse-promoting genes and synaptic density in hippocampus of germ-free mice after the colonization with *Bifidobacterium* species [53]. We did not observe any association between levels of *Proteobacteria* and cognitive performance. In literature, however, the increase of *Proteobacteria* was reported in mouse model/human in Alzheimer's disease [41].

It is unclear what is the mechanism of association between *Veillonellaceae* bacteria levels and executive functions described in this report. Negative effects on cognitive function may have several pathways including neural, inflammatory and biochemical genesis. Previous studies have shown that cognitive impairment may be the result of neuronal cell apoptosis, brain mitochondrial dysfunction, elevated hippocampal oxidative stress, decreased hippocampal synaptic plasticity, decreased number of dendritic spine density at hippocampus or increased amyloid-beta deposition [54]. Moreover, negative changes in the brain are also associated with gut dysbiosis and inflammation.

While the number of subjects in the experimental group was relatively small, it should be noted that our group of volunteers was homogeneous. Association between *Veillonellaceae* and cognitive function was observed in young people before age-related cognitive decline. Volunteers declared similar levels of physical activity and no sleep irregularities.

The majority of studies examining the relationship between gut microbiota and cognitive functions are conducted in elderly subjects suffering from neurodegenerative diseases or emotional disorders. Our study demonstrates that composition of gut microbiota can have influence on cognitive performance in young, healthy humans.

## **Conclusion**

Composition of gut microbiota can have influence on cognitive performance. *Veillonellaceae* family is association with cognitive function determined by Trial Making Test. No associations between levels of *Bifidobacterium* and *Proteobacterium* phyla and cognitive tests outcomes.

## **Article information**

**Data availability statement:** *Data are available on request from the corresponding author.*

**Ethics statement:** *The study was reviewed and approved by Bioethics Committee of the Medical University in Bydgoszcz.*

**Author contributions:** *Conceptualization — BD; Methodology — BD, PZ, DM, DL; Collection Data — BD; Writing — BD, MB, PZ.*

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**Conflict of interest:** *None.*

**Supplementary material:** *None.*

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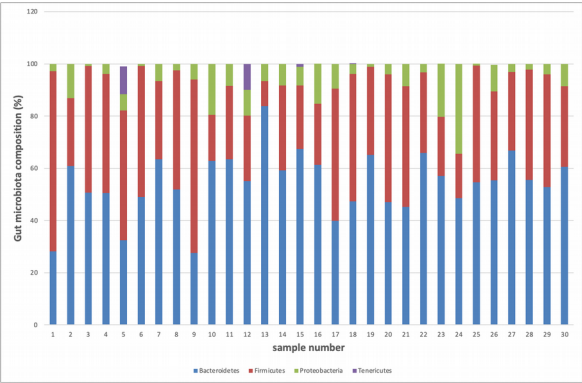
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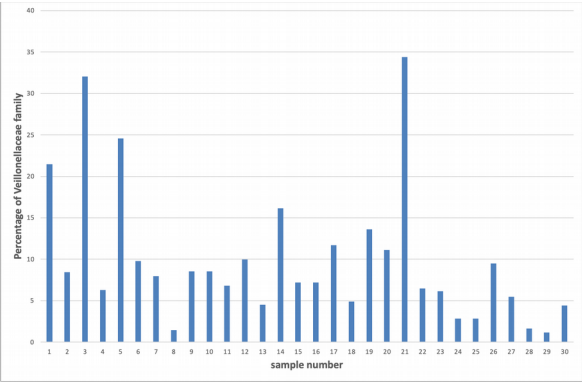
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**Figure 1.** Phylum-level gut microbiota composition in the fecal samples of 30 volunteers. Percentage content in the tested sample. Blue plot: *Bacteroides*, red plot: *Firmicutes*, green plot: *Proteobacteriae* and violet: *Tenericutes*

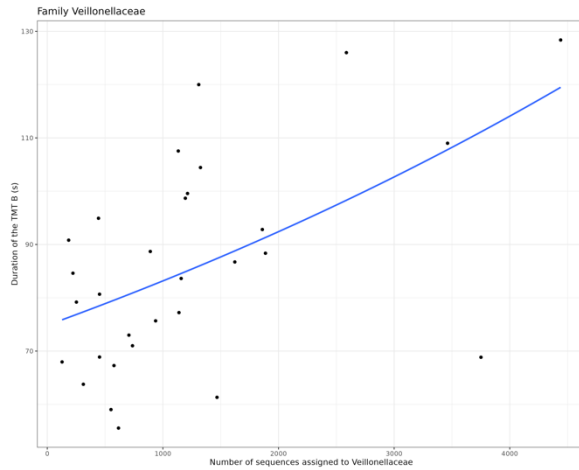


**Figure 2.** Family-level gut microbiota composition. Percentage content *Veillonellaceae* in the fecal samples of 30 volunteers

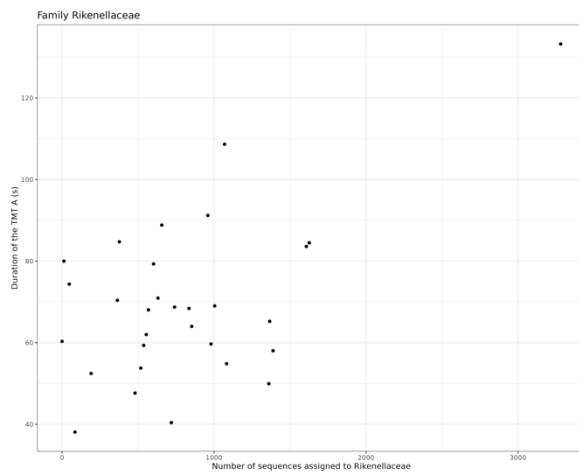


**Figure 3.** Scatter plot between results of Trial Making Test (part B) and *Veillonellaceae* family. X-axis is the number of reads/contigs (region sequences V3-V4 region) found in a sample and classified as of the given taxon. Y-axis is the result of Trial Making Test. FDR = 0.04



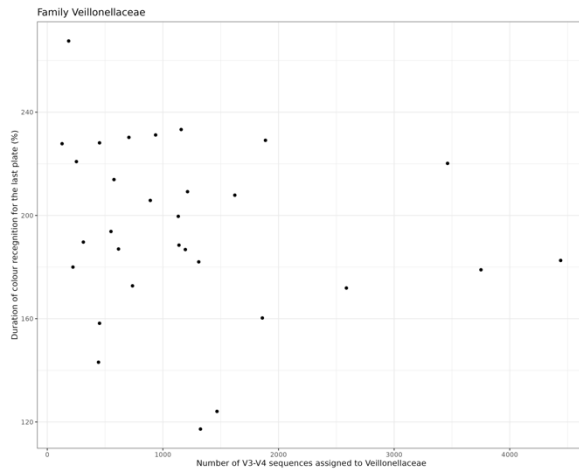


**Figure 4.** Scatter plot between results of Trial Making Test (part A) and *Rikenellaceae* family. X-axis is the number of reads/contigs (region sequences V3-V4 region) found in a sample and classified as of the given taxon. Y-axis is the result of Trial Making Test. FDR = 0.3

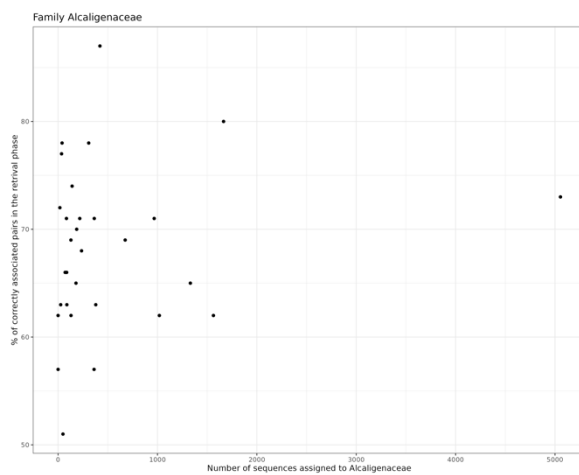


**Figure 5.** Scatter plot between results of Stroop Test and *Veillonellaceae* family. X-axis is the number of reads/contigs (region sequences V3-V4 region) found in a sample and classified as

of the given taxon. Y- axis is the result of Stroop Test. FDR = 1



**Figure 6.** Scatter plot between results of Face/Name Association Test and *Alcaligenaceae* family. X-axis is the number of reads/contigs (region sequences V3-V4 region) found in a sample and classified as of the given taxon. Y-axis is the result of Face/ Name Association Test. FDR = 1



**Table 1.** Negative Binomial GLM results with FDR correction. Positions with FDR lower than 0.05 indicates significant relationship between examined feature (results of cognitive test) and sequences count (interpreted as the amount of given taxon in sample)

<b>Face- Name Association Test results</b>	<b>PValue</b>	<b>FDR</b>
Bacteroidetes;Bacteroidia;Bacteroidales;_	0,185303182	1
Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae	0,185515021	1
Firmicutes;Bacilli;Lactobacillales;Enterococcaceae	0,221087842	1
Proteobacteria;Betaproteobacteria;Burkholderiales;Alcaligenaceae	0,227109718	1
<b>Strooptest results</b>		
Proteobacteria;Betaproteobacteria;Burkholderiales;Alcaligenaceae	0,056344283	1
Firmicutes;Bacilli;Lactobacillales;Enterococcaceae	0,131430063	1
Firmicutes;Clostridia;Clostridiales;Veillonellaceae	0,229341238	1
Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae	0,331262268	1
<b>TMTA results</b>		
Firmicutes;Bacilli;Lactobacillales;Enterococcaceae	0,00020514	0,006564492
Tenericutes;RF3;ML615J-28;_	0,00305959	0,048953436
Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae	0,016630256	0,177389397
Bacteroidetes;Bacteroidia;Bacteroidales;Rikenellaceae	0,041861823	0,334894587
<b>TMTB results</b>		
Firmicutes;Clostridia;Clostridiales;Veillonellaceae	0,001426021	0,045632687
Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae	0,051591768	0,825468284
Bacteroidetes;Bacteroidia;Bacteroidales;S24-7	0,208123717	1
Verrucomicrobia;Opitutae;[Cerasiococcales];[Cerasiococcaceae]	0,256444616	1