Oral Probiotic Deficiency May Cause Common Allergies - Theory of Negative Trigger Marks the Interaction between Microbiota and Host Immune System

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Competing Interests statement: CSH is the owner of Knoze Jr Corp. that holds granted/pending patents covering the oral microbiota inducing method described here.

Abstract

This study seeks to identify causes for common allergies and to propose translational measures in the treatment of allergies. The research is based on the longitudinal/translational research of oral microbiota during the three-year observance of a subject with common allergies (allergic rhinitis). Through this observation, I was able to identify the cause of common allergies and develop a treatment resulting in lasting allergy relief. The results of the study indicate that the direct mechanism behind common allergies may be oral probiotic deficiency - the lack of probiotic bacteria such as Streptococcus and Veilonella in the oral/nasal cavities. These probiotic bacteria are mutualists and together produce metabolites that pacify the nearby immune system. Through the targeted promotion of those probiotics, the subject found long-term remission from common allergies. In addition, the study led to the development of the Theory of Negative Trigger (TNT). During the research, the study participant exhibited a moderate fever from an unrelated respiratory infection. The fever worked to clear the subject's oral biofilm, which in turn facilitated the development of a hot-water microbiota reseeding procedure. The clearing of the biofilm also suggests that fever triggers the release of the immune system that is normally restrained by metabolites from the microbiota. TNT advocates that oral probiotics are a negative trigger that controls the immune system to make peace between the host and commensal bacteria. Through clinical intervention, the interaction between microbiota and the immune system can be manipulative for the treatment of allergies, autoimmune diseases, infections, and potentially cancers.

List of abbreviations: TNT, Theory of Negative Trigger; OTU, operational taxonomic unit; QIIME, Quantitative Insights Into Microbial Ecology

Introduction

For 30 years, the hygiene hypothesis has been used to explain the increase of allergy diseases (Strachan, 1989). Many studies on personal and social hygiene practices suggest they have an association with the prevalence of allergy diseases (von Mutius, 2007). The hypothesis currently emphasizes the exposure of diverse environmental bacteria rather than infection (Ege et al., 2012; Jatzlauk et al., 2017). Allergic subjects are found to have microbiota dysbiosis (Chung, 2017). Indeed, certain bacteria species interact with the immune system through

metabolites or structural molecules produced by bacteria (Blacher et al., 2017; Gensollen et al., 2016). However, attempts at using probiotics to cure or prevent allergy diseases have had limited success (West et al., 2016). This study illustrates that the restructuring of oral microbiota may lead to lasting remissions of common allergies (allergic rhinitis).

Results

The subject (CSH, the author) began to exhibit symptoms of common allergies 18 years after immigrating to the US in the late nineties. Symptoms experienced include nasal congestion, rhinorrhea, red and itchy eyes, tearing, coughing, aches in the throat and a tight chest. This unusually longer lapse between immigration and the development of the symptoms comparing to published data indicate that the underlying trigger is likely to be personal rather than environmental (Lombardi et al., 2008).

Prior to the appearance of the first symptoms, the subject intensified his oral hygiene routine. In his first ten years in the US, he brushed his teeth only once a day. Slowly, he began introducing additional dental hygienic practices, such as dental cleaning, flossing, tongue scraping, and the use of mouthwash. In the winter before allergy symptoms first appeared, CSH was brushing and flossing his teeth once every day, tongue-scraping several times a week, and frequently using mouthwash. In addition, he took antibiotics for a week to treat a lasting cough, a variable which is consistent with previous observations regarding the association between allergy and antibiotic use (Foliaki et al., 2009).

He suspected that changes in his oral microbiota contributed to the development of his allergies and began to collect samples of his saliva and fecal matter periodically. Sample collection continued until spring 2017 (Table 1). Only data from saliva samples are presented here as no positive result was found from fecal samples.

Table 1. Samples collected for this study.

Sample	Subject	Collecting time	Allergy status	Group*
CSA7	CSH	4/18/14	Yearlong	SAB
CSA8	CSH	4/23/14	Yearlong	SAB
CSA10	CSH	5/14/14	Yearlong	SAB
CSA11	CSH	6/4/14	Yearlong	SAB
CSA12	CSH	6/25/14	Yearlong	SAB
CSA13	CSH	7/1/14	Yearlong	SAB
CSA14	CSH	6/24/15	Yearlong	SAB
CSA15	CSH	7/1/15	Yearlong	SAB
CSA16	CSH	7/8/15	Yearlong	SAB
CSA17	CSH	10/28/15	Seasonal	SAA
CSA18	CSH	11/7/15	Seasonal	SAA
CSA19	CSH	1/23/16	Seasonal	SAA
CSA20	CSH	2/15/16	Seasonal	SAA
CSA21	CSH	2/20/16	Seasonal	SAA
CSA22	CSH	4/2/16	Seasonal	SAA
CSA23	CSH	10/2/16	Seasonal	SAA
BSA23	Family Member 1	10/2/16	Seasonal	SAF
SSA23	Family Member 2	10/2/16	None	SAF
NSA23	Family Member 3	10/2/16	None	SAF
BSA25	Family Member 1	4/11/17	Remission	SAC
CSA25	CSH	4/11/17	Remission	SAC
NSA25	Family Member 2	4/11/17	None	SAF

* SAB, samples collected during yearlong allergy; SAA, samples collected during seasonal allergy; SAC, samples collected after taking prebiotic mix; SAF, samples collected from family members.

The first allergic episode lasted for a year and a half and ended in the summer of 2015. It became seasonal afterward. During this time, CSH experimented with reducing his oral hygiene effort for several weeks. Local moderate periodontitis developed as well as bleeding and gum recession, and his allergy symptoms persisted. Resuming brushing and flossing for two weeks healed the inflammation, while the allergy symptoms continued. After that time, his only oral hygiene
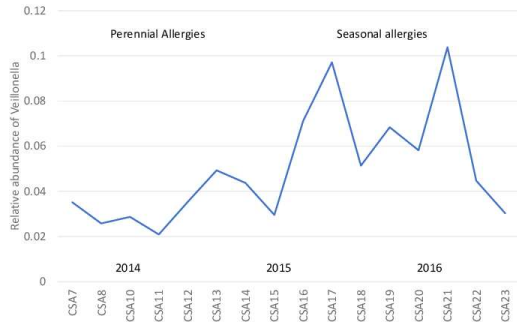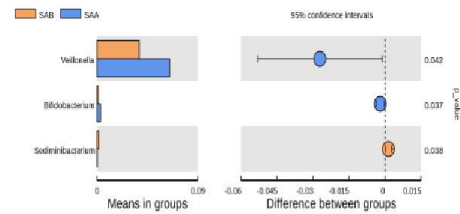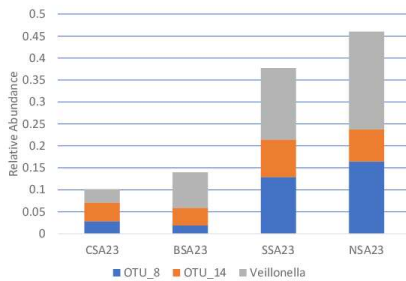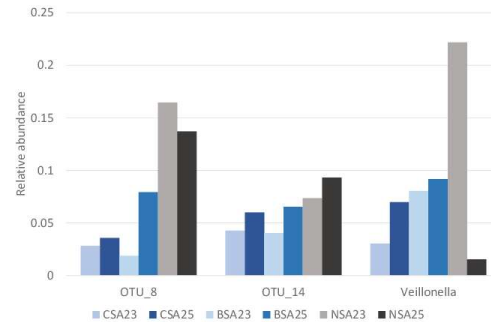
Figure 1. Consistent changes of the genera *Streptococcus* and *Veillonella* during the development of allergy and prebiotic intervention indicate their causal role in allergies. A. The relative abundance of *Veillonella* in samples before and after the change of yearlong allergies to seasonal allergies. B. Relative abundance of genera that are significantly changed after transition from yearlong allergy to seasonal allergy. Double sided t test was used in the analysis. C. Two OTUs of *Streptococcus* and *Veillonella* are less abundant in the saliva samples from subjects with allergies than those from people without allergies. D. The abundance of two OTUs belong to *Streptococcus* and that of genus *Veillonella* increased after prebiotic induction and remission of allergies.

practice was to brush his teeth once a day without toothpaste. His allergy symptoms alleviated during two 4-8 week trips abroad but never completely went away before the summer of 2015 (Timeline in Table 1 and Figure 1A).

For comparison, saliva samples of his family members were also collected (Table 1). Together the samples collected before 2017 were sequenced and analyzed using a commercial service (Supplemental material). No significant difference was found between the fecal samples before and after summer 2015 (data not shown).

Analyzing the results of CSH's saliva samples shows that the relative abundance of *Veillonella* increased (p=0.042) in saliva samples collected after summer 2015 (Figure 1A & 1B) and that an increase of *Streptococcus* was not significant. The initial analysis shows that the difference in abundance of the genus in CSH and his family members is not significant. However, the saliva of one family member, who also suffers from seasonal allergies, contained *Veillonella* at a level half of those of non-allergic family members. The average amount of *Veillonella* in the two non-allergic family members is 5-6 times as high as those in the two subjects with allergies. Also, two of the three OTUs (operational taxonomic units) in the genus of *Streptococcus* had similar distribution patterns in the family as the two OTUs in genus *Veillonella* (Figure 1C).

Numerous studies indicate that *Streptococcus* and *Veillonella* grow together in the oral cavity with a mutualistic relationship (Takei et al., 1968; van der Hoeven et al., 1978). *Streptococcus* attaches itself to surfaces, and *Veillonella* metabolizes the cavity-inducing lactic acid from the *Streptococcus* and converts it into short chain fatty acids (SCFA). Besides being less toxic, SCFA has been proven to benefit the human host in many other ways. For example, they interact with the immune system to reduce inflammation (Correa-Oliveira et al., 2016). Like the gut, the respiratory tract is surrounded by lymphoid tissue from the mouth/nose to the bronchi. A reasonable hypothesis is that allergy symptoms could be moderated or eliminated if prebiotics were given to promote the growth of these bacteria in the oral cavity.

Given these findings, a prebiotic mix (U.S.Pat. No. 9,795,579) was developed in a composition containing sugars and arginine to promote the growth of selected bacteria based previous studies of the fermentation profile of *Streptococcus* and *Veillonella*, both as individual species and as a community (Han, 2017; Kolderman et al., 2015; Willcox, 1996).

Subject CSH and the other family member with allergies took the prebiotic mix several times a day in the middle of March 2017 when their allergies were at their peak. The allergy symptoms were alleviated significantly. They did not have to take any other medication that might explain the change. However, the relief from their symptoms was temporary, forcing them to take the oral prebiotic mix several times a day to have continued success.

Five days after taking the prebiotic composition, CSH developed a fever likely due to an infection of the bronchi. It is possible that he contracted the infection from another family member who had become sick a week before. His temperature was between 38-39∘C, and the fever was accompanied by a phlegmy cough. CSH stopped using the compound once the fever began.

On the second day of the illness, he noticed that his tongue was completely red and absent of the natural biofilm. It was suspected that his fever/infection had cleared the micro eco-space in his oral cavity, which produced a unique opportunity for restructuring his oral microbiota.
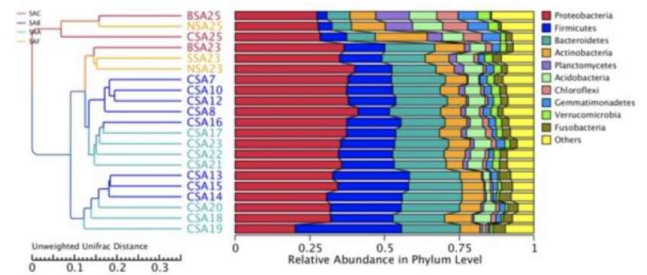
In response, CSH took two doses of the prebiotic mix as soon as his fever receded. The next day his allergic symptoms had been alleviated without additional medicines or doses of the prebiotic blend. To clear the oral micro eco-space of the other family member with allergies, he applied a local pyrotherapy (Supplementary Figure 1) for 10 minutes after brushing the teeth with water and scraping the tongue with a wet washcloth. The family member took some of the prebiotic mixes immediately after the pyrotherapy, and the allergy went into remission as well. Both CSH and this family member no longer required allergy medicines for the rest of the 2017 spring season.
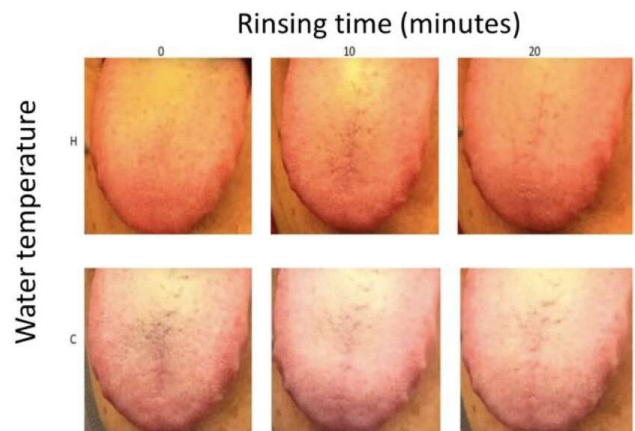
Supplementary Figure 1. Rinsing the mouth with hot water is more effective in removing biofilm than with rinsing with water with a lower temperature. H is achieved by rinsing the mouth with water with a temperature of 46-49∘C, and C with water at room temperature. 0 shows the tongue before rinsing; 10 depicts the tongue after 10 minutes continuous rinsing; and 20 is a picture of the tongue after 20 minutes continuous rinsing.

Approximately three weeks after their remission, the last batch of saliva samples was collected from three of the family members (two with allergies). They were sequenced for 16S rRNA gene profiling and analyzed together with the previous dataset using the same commercial service provider. The results showed that the relative abundance of *Streptococcus* and *Veillonella* increased in the final samples from both allergy subjects. The ratios of change varied between subjects and genera (Figure 1D). The differences between the NSA23 and NSA25 samples from the non-allergic family member were likely caused by a lasting respiratory infection flanking the time of NSA25 sample collection (Figure 1 D).

Figure 2. Non-metric multidimensional scaling shows that microbiota structures in the saliva are profoundly changed after prebiotic induction. CSA25, collected after induction, is much further away from the rest of the samples of the same subject.

Cross-sample diversity analysis, done with QIIME (Quantitative Insights Into Microbial Ecology) package (Kuczynski et al., 2011), confirms that the combination of pyrotherapy and prebiotic induction likely caused structural changes in oral microbiota community. Nonmetric multidimensional scaling shows that sample CSA25 is significantly different from the samples collected before the prebiotic treatment (Figure 2). The Unweighted Pair Group Method with the Arithmetic tree at phyla level clusters showed the samples after the treatment

was closer to ones from the non-allergic family members (Supplementary figure 2).
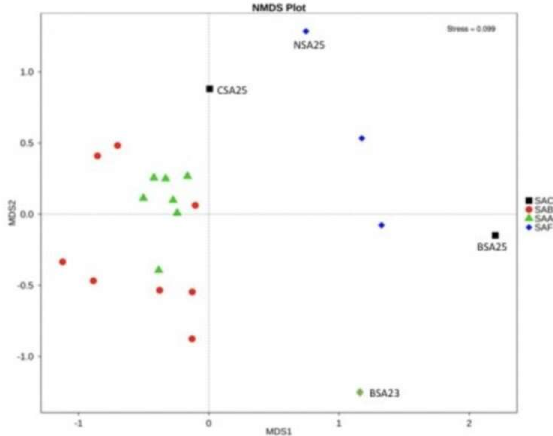

Supplementary Figure 2. Microbiota structures in the saliva are profoundly changed after prebiotic induction. A sample cluster analysis with Unifrac distance indicates that CSA25 and BSA25 are closer to samples from non-allergic subjects.

Discussions

The induced microbiota changes and immediate and lasting clinical responses to the intervention, though from a limited sample size, suggest a new hypothesis for etiology of allergy rhinitis — the Theory of Negative Trigger (TNT), in which probiotics are the negative triggers that control the power of the immune system.

TNT theory recognizes that common allergies is strongly influenced by local lymph tissues next to the respiratory tract. Metabolites such as SCFA produced by commensal bacteria likely act as a continuous messenger/pacifier to the immune system. The triggers can be removed/diminished by local pyrotherapy, elevated body temperature, or modern life events such as extreme oral hygiene or antibiotic usage (Figure 3).

Under healthy conditions, responsible probiotic bacteria produce enough pacifying metabolites that the immune system does not react to commensal bacteria and environmental allergens that do not cause harm (Figure 3A).

Figure 3A

Probiotics

37°C

Temperature

Immune System

Allergens

Figure 3B

Probiotics

37°C

Temperature

Immune System

Allergens

Figure 3C

Probiotics

39°C

Temperature

Immune System

Pathogens

Figure 3D

Probiotics

37°C

Temperature

Immune System

Commensal bacterial
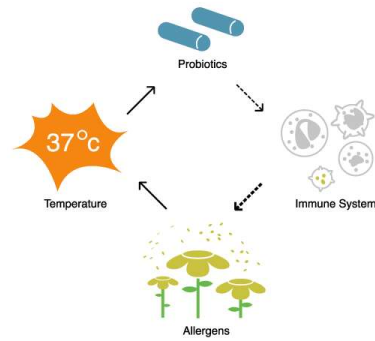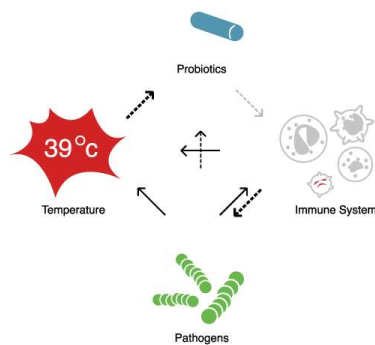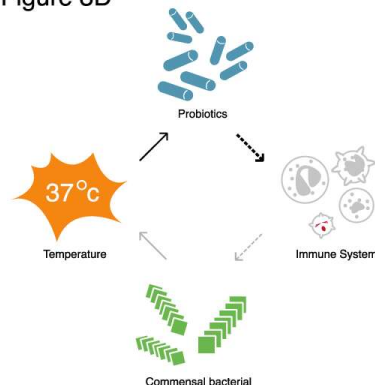
Figure 3. The Theory of Negative Trigger for interaction between oral probiotic bacteria that produce immune-pacifying metabolites and the immune system. A. Normal state, a proper dose of pacifying metabolites from probiotics calms the immune system so that it does not overreact to commensal microbiota and allergens. B. Allergic state, oral probiotics are suppressed, and an insufficient dose of pacifying metabolites results in an agitated immune system that overreacts to allergens. C. Acute infection leads to diminished pacifying metabolites and results in a strengthened immune system with more power to fight pathogens. D. An over-pacified immune system can lead to a vulnerability to infections. A solid line represents positive interaction; a dashed line represents negative interaction; greyed line indicates minimal cooperation; the thickness of the line shows relative scale of interaction.

Figure 3B demonstrate how common allergies develops. Intensive oral hygiene causes the biofilm to become vulnerable and collapse under the assault of antibiotics (Abeles et al., 2016). This process suppress oral probiotics. Species of bacteria which are less sensitive to antibiotics take over the ecospace of the previous probiotics. The immune system, without enough pacifying messengers from probiotics, then becomes hostile to allergens. Therefore, I propose a new name, **oral probiotic deficiency**, for the condition that oral probiotics reduce to a level where they cannot produce enough SCFA, the peaceful message to the immune system. The consequences of oral probiotic

deficiency may include allergy and autoimmune conditions.

The hypersensitive immune system may cause other inflammations in the airway such as periodontitis, rhinosinusitis, and tonsillitis by attacking commensal bacteria (Figure 3B). Therefore, this theory can explain previously observed association between oral "infectious" diseases and common allergies (Arbes and Matsui, 2011). In fact, taking the prebiotic composition relieved the allergic family member's inflammation when they had a sore throat and enlarged tonsils.

Figure 3C illustrates how the immune system regains its strength under pathogenic infection. First, the invading pathogen can directly out-compete probiotics that produce pacifying metabolites. Second, severe infection induces pyrogens that raise body temperature to inhibit or kill many common, temperature-sensitive bacterial species in the oral microbiota, including oral species such as *Veillonella (Carlier, 2015)*. Suppression of probiotics will likely cease or significantly reduce the production of responsible metabolites and remove restraints on, or brake of, the immune system. Third, the pathogen cause damages to normal tissue and release toxins that stimulate the immune system directly, which like accelerator to the immune system. The immune system, with release of the brake pedal and pressing down of gasoline pedal, will then regain its full strength to fight pathogens. This mechanism could explain how fever increases immunity and helps the body fight diseases, even cancers (Atkinson, 1979).

Theoretically, too many immune pacifying metabolites from overgrowing probiotics may dampen the local immune system too much to fend off even the normal commensal bacteria and lead to chronic or acute infection which would reshape the whole community structure (Figure 3D). Study on people under normal life conditions will help to verify that theory, though. The TNT hypothesis would benefit from further testing from many different perspectives: infection, allergy, and autoimmunity.

TNT has the following propositions. First, the triggers can be removed and reapplied relatively easily and quickly. The epidemic of common allergies, the disappearance of oral biofilm under moderate fever, and the quick allergy-relieving effect of the prebiotic compound support this proposition.

Second, the gut has a less significant role in common allergies. Results in this study indicate the interactions between probiotics and the immune system come primarily at the local level. Long distance interaction or circulation of immune cells likely plays a secondary role.

Third, immune system programming is less important in this situation. Theoretically, the effects of the negative trigger should not last long after removing it as SCFA can be absorbed and metabolized by many different cells in human body. Otherwise, the power of the immune system cannot be released in time to protect the host. Practically, this study showed that allergies started before two years of age can be reversed with the prebiotic mix. The concept of a critical time for immune system development should be re-evaluated to determine what is programmed and what is impacted if critical time is missed.

This is all good news for many who have allergies, autoimmune diseases, inflammations, and other conditions (including cancer) and who would benefit from releasing the power of the immune system or suppressing it. Developing a method to fine-tune the strength of immune system temporally and spatially under these conditions would improve people's health significantly. For example, one may make the immune system more active by suppressing probiotics in the airway and gut with

physical and chemical (include antibiotics) means and by adding immune stimulants (such as vaccine). This may help to control chronic infection and cancers.

The theory also suggest the first **critical component of a microbiota** associated with a host should be the ones that can communicate with host to make a peace agreement. Bacteria in this role should first not cause immediate harm to the host and second are able to send pacifying signal to the host immune system. In human, this communication is likely achieved through bacteria producing short chain fatty acid. They are Streptococcus and Veillonella in the mouth and airway, fiber digesting bacteria in the gut, C. Akney on the skin. The second component of healthy microbiota should be enough diversity to occupy all nutrient space with members not harmful or even better benefit to the host. The last components are guest members dropped in accidentally.

This hypothesis suggests possible solutions to many practical issues. It explains why it is beneficial for parents to transfer their microbiota to their children by confinement of mother and newborn at the beginning of life, and fever should be kept if it is not too high. This theory also suggests what comprises healthy microbiota and whether it is possible to reshape microbiota for optimal immune status in other situations.

Declarations

Ethics approval and consent to participate: All Participates consented to participate.

Consent for publication: N/A

Availability of data and material: The sequence data is available upon request.

Competing interests: CSH is the owner of Knoze Jr Corp. that holds granted/pending patents covering the oral microbiota inducing method described here.

Materials and Methods

Sequencing

1 Sample collection

Family members provided fully informed consent to participate the study by providing samples and related information. Saliva samples were collected by directly expectorating into 15 ml tube and immediately frozen for storage.

2 Extraction of genome DNA

Total genome DNA from samples was extracted using Nucleo spin DNA stool kit (cat No. 740472.50) according to manufacturer's instructions. DNA concentration and purity was monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1ng/µL using sterile water.

3 Amplicon Generation

16S rRNA genes of 16SV4 region were amplified used specific primer(16S V4: 515F-806R) with the barcode. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs).

4 PCR Products quantification and qualification

Mix same volume of 1X loading buffer (contained SYB green) with PCR products and operate electrophoresis on 2% agarose gel for detection. Samples with bright main strip between 400-450bp were chosen for further experiments.

5 PCR Products Mixing and Purification

PCR products was mixed in equidensity ratios. Then, mixture PCR products was purified with Qiagen Gel Extraction Kit (Qiagen, Germany).

6 Library preparation and sequencing

Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina HiSeq 2500 platform (Illumina, USA) and 250 bp paired-end reads were generated.

Data analysis

1 Paired-end reads assembly and quality control

1.1 Data split: Paired-end reads was assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence.

1.2 Sequence assembly: Paired-end reads were merged using FLASH (V1.2.7) (Magoc and Salzberg, 2011), a very fast and accurate analysis tool, which was designed to merge paired-end reads when at least some of the reads overlap the read generated from the opposite end of the same DNA fragment, and the splicing sequences were called raw tags.

1.3 Data Filtration: Quality filtering on the raw tags were performed under specific filtering conditions to obtain the high-quality clean tags according to the Qiime (V1.7.0) (Kuczynski et al., 2011) quality controlled process.

1.4 Chimera removal: The tags were compared with the reference database(Gold database using UCHIME algorithm (Edgar et al., 2011) to detect chimera sequences, and then the chimera sequences were removed. Then the Effective Tags finally obtained.

2 OTU cluster and Species annotation

2.1 OTU Production: Sequences analysis were performed by Uparse software (Uparse v7.0.1001) (Edgar, 2013)Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation.

2.2 Species annotation: For each representative sequence, the GreenGene Database (McDonald et al., 2012) was used based on RDP classifier(Version 2.2) (Wang et al., 2007) algorithm to annotate taxonomic information.

2.3 Phylogenetic relationship Construction: In order to study phylogenetic relationship of different OTUs, and the difference of the dominant species in different samples(groups), multiple sequence alignment were conducted using the PyNAST software(Version 1.2) (Caporaso et al., 2010) against the "Core Set" dataset in the GreenGene database.

2.4 Data Normalization: OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed basing on this output normalized data.

3 Alpha Diversity

Alpha diversity is applied in analyzing complexity of species diversity for a sample through6 indices, including Observed-species, Chao1, Shannon, Simpson, ACE, Good-coverage. All this indices in our samples were calculated with QIIME(Version 1.7.0) (Kuczynski et al., 2011) and displayed with R software(Version 2.15.3).

Two indices were selected to identify Community richness:

Chao - the Chao1 estimator (Chao, 1984);

ACE - the ACE estimator (Chao et al., 1992) ;

Two indices were used to identify Community diversity:

Shannon - the Shannon index (Seaby R. M. & Henderson, 2006);

Simpson - the Simpson index (Harper, 1999);

One indices to characterized Sequencing depth:

Coverage - the Good's coverage (Seaby R. M. & Henderson, 2006)

4 Beta Diversity

Beta diversity analysis was used to evaluate differences of samples in species complexity, Beta diversity on both weighted and unweighted unifrac were calculated by QIIME software (Version 1.7.0) (Kuczynski et al., 2011).

Non-metric multidimensional scaling(NMDS) was performed to get principal coordinates and visualize from complex, multidimensional data. A distance matrix of weighted or unweighted unifrac among samples obtained before was transformed to a new set of orthogonal axes, by which the maximum variation factor is demonstrated by

first principal coordinate, and the second maximum one by the second principal coordinate, and so on. NMDS analysis was displayed by WGCNA package, stat packages and ggplot2 package in R software(Version 2.15.3).

Unweighted Pair-group Method with Arithmetic Means(UPGMA) Clustering was performed as a type of hierarchical clustering method to interpret the distance matrix using average linkage and was conducted by QIIME software (Version 1.7.0) (Kuczynski et al., 2011).

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