Effects of Dachaihu Decoction and Its “Prescription Elements” on Intestinal Flora of Nonalcoholic Fatty Liver Disease Model Rats

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Abstract

Objective: The study objective was to observe the effect of Dachaihu decoction and its “prescription elements” on intestinal flora of model rats with nonalcoholic fatty liver disease (NAFLD). Materials and Methods: Healthy male Sprague-Dawley rats were randomly divided into the following seven groups (n = 105): normal, model, pioglitazone hydrochloride (PH), Shuganlidan, Jianpihuatan, Tongfuxiezhuo, and dachaihu decoction (DD). 16SrRNA high-throughput Illumina sequencing platform was used for sequencing, and bioinformatics analysis of rat intestinal flora was done. Results: Compared with the normal group, rats in the model, PH, Shuganlidan, Jianpihuatan, Tongfuxiezhuo, and DD groups showed significant differences in the intestinal microflora structure. The microorganisms that play an important role in the normal group are Ruminococcaceae, Oscillospira, Lactobacillales, Lactobacillaceae, Lactobacillus, bacilli, Ruminococcus, TM7, Spirochactes, Clostridium, Elusimicrobia, Elusimicrobiaceae, Elusimicrobiales, Lactobacillus helveticus, Lactobacillus reuteri, Elusimicrobiunum, RF39, and Christensenellaceae; in the model group are Ruminococcus gnavus; in the PH group are Prevotella, Paraprevotellaceae, and Blautia; in the Jianpihuatan group are Bacteroidetes, Bacteroida, Bacteroidales, Roseburia, Rikenellaceae, and Stridium methylpentosum; in the Tongfuxiezhuo group are Bacteroidaceae, Bacteroides, Porphyromonadaceae, Parabacteroidaceae, 4C0d_2, Cyanobacteria, YS2, Parabacteroides distasonis, Bacteroides uniformis, Verrucomicrobiaceae, Verrucomicrobiacia, Verrucomicrobiales, Akkermansia, Akkermansia muciniphila, Coprobacillus, Parabacteroides gordonii, Blautia producta, and Ruminococcus torques; and in the DD group are Erysipelotrichaceae, Erysipelotrichi, and Erysipelothrichaceae. Conclusion: The Tongfuxiezhuo prescription elements of the DD significantly improved the intestinal flora of rats with NAFLD, improving the relative abundance of beneficial bacteria in the intestinal flora of NAFLD rats such as Bacteroides, Sartre genus, Vibrio pseudostubutrate bacteria, and Akkermansia muciniphila. This subsequently improved the glucose and lipid metabolism in NAFLD rats, reducing fat deposition in the liver, inhibiting intestinal inflammatory reaction, and maintaining the integrity of intestinal mucosal barrier. The action target is the intestinal axis of “intestinal–hepatic axis.”

Keywords: Dachaihu decoction, intestinal flora, nonalcoholic fatty liver disease, prescription elements

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a clinicopathological syndrome characterized by overdeposition of fat in the hepatocytes, except for some definite liver damage factors. Intestinal flora in addition to genetic background, living environment, and dietary structure plays an important role in the pathogenesis and evolution of NAFLD.[1,2] Previous studies reported the changes in intestinal flora abundance[3] and microflora structure, and also the intestinal flora was disordered[4,5] in patients with NAFLD. Some researchers<br>
inoculated feces from mice with NAFLD into the intestines of wild mice, and then wild mice also had NAFLD.\textsuperscript{66} Moreover, several studies found that\textsuperscript{7–10} intestinal probiotic preparations have a significant effect on the treatment of NAFLD. These results suggested that intestinal flora plays an important role in the development and treatment of NAFLD.

The prescription element is a drug or a combination of drugs that is separated from the prescription, and is considered the simplest drug or drug group for the smallest pathogenesis unit, which emphasizes on the correspondence and targeting property, and to the syndrome elements. Dachaihu decoction (DD) is an effective prescription for the treatment of NAFLD. By analyzing the Traditional Chinese Medicine (TCM) pathogenesis of NAFLD and understanding the compatibility law of DD, three prescription elements in DD were analyzed, namely, Shuganlidan prescription elements (Bupleurum, Scutellaria root, and white peony root), Jianpihuatan prescription elements (prepared Pinellia tuber, fresh ginger, and Chinese date), and Tongfuxiezhuo prescription elements (rhubarb root and rhizome, with immature bitter orange).

The intestinal flora is closely related to the occurrence, development, and treatment of NAFLD, and DD is effective for the treatment of NAFLD. According to the principle of correspondence between formula–syndrome factors, the DD and each prescription element of it on the intestinal flora of rats with NAFLD were analyzed.

**Materials and Methods**

**Animals and grouping**

A total of 105 specific pathogen-free healthy male Sprague-Dawley (SD) rats, weighing 180 ± 20 g, were purchased from Speyer (Beijing) Biotechnology Co., Ltd. (license number SCXK (Beijing, China) 2016–0002). After 7 days of adaptive feeding, the animals were randomly divided into normal, model, pioglitazone hydrochloride (PH), PH tablets were selected as the experimental positive controls, and all the drugs used in the study were purchased from Beijing Tongrentang Pharmacy and were identified as authentic by the Research Center for Fundamental Theory and Key Technology of Traditional Chinese Medicine of the Beijing University of Chinese Medicine. The proportion of raw doses was referred to the “pharmacology of traditional Chinese medical formulae;”\textsuperscript{113} DD (15 g Bupleurum [Bupleurum chinense DC.], 9 g Scutellaria root [Scutellaria baicalensis Georgi], 9 g white peony root [Paeonia lactiflora Pall.], 9 g prepared Pinellia tuber [Pinellia ternata (Thunb.) Breit.], 15 g fresh ginger [Zingiber officinale (Willd.) ROSC.], and 12 g Chinese date [Ziziphus jujube Mill.], Rheum officinale Baill 6 g, and Fructus Aurantii Immaturus 9 g), according to the body surface area method,\textsuperscript{19} and the crude drug was 8.64 g/kg body weight. The composition of Shuganlidan medicine contained 15 g Bupleurum, 9 g Scutellaria root, and 9 g white peony root. According to the body surface area method, the crude drug yield per unit body weight was 3.39 g/kg. The composition of Jianpihuatan medicines included 9 g prepared Pinellia tuber, 15 g fresh ginger, and 12 g Chinese date. According to the body surface area method, the crude drug yield per unit body weight was expanded by ten times, which was 3.70 g/kg. The composition of Tongfuxiezhuo medicine included raw rhubarb/raw rheubarb powder 6 g and Citrus aurantium 9 g, according to the body surface area method, and the crude drug yield per unit body weight was 1.54 g/kg.

The herbs were soaked in distilled water for 30 min, heated under a strong flame until boiling, heated under a mild flame for a further 30 min, and then filtered using a gauze element. In addition, distilled water was added and the decoction process was repeated. After removing the drugs, the decoction was mixed, filtered through two layers of gauze, condensed in a boiling water bath, marked, and then stored in a refrigerator.

From weeks 13–16, the rats in PH, the Shuganlidan, the Jianpihuatan, the Tongfuxiezhuo, and the DD groups were fasted for 12 h before each administration. After 12 h, the last intragastric administration (with fasting water for 12 h) to rats in each group was done. The rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.3 mL/100 g) to dissect the large intestine, and then the feces were placed in a sterile cryopreservation
tube, which was previously autoclaved. After liquid nitrogen cryopreservation, it was then transferred to a refrigerator at −80°C for delivery to Shenzhen Huada Gene Technology Service Co., Ltd (ShenZhen, China). The 16SrRNA high-throughput Illumina sequencing platform was then used for sequencing, and bioinformatics analysis of rat intestinal flora was done. The entire operation process was carried out in strict accordance with the aseptic operation conditions to avoid sample contamination and repeated freezing and thawing. Shenzhen Huada Gene Technology Service Co., Ltd submitted seventy samples for library quality inspection, which were considered qualified for further experimental process.

The animal experimental process complies with the experimental animal care and the use of guiding principles advocated by the National Institutes of Health and the “3R” principle of animal experiment with reduction, replacement, and refinement as the core. This study was approved by the Medical and Experimental Animal Ethics Committee of Beijing University of Chinese Medicine (No. BUCM-I-2017051030-2030).

**Illumina MiSeq sequencing of intestinal flora**

Genomic DNA was extracted from the intestinal flora according to the TIANamp Stool DNA Kit (TIANJEN, China) instructions. According to the designated sequencing region, polymerase chain reaction (PCR) amplification test was carried out by the TransStart FastPfu DNA Polymerase instructions. The PCR product was then recovered using the AxyPrep DNA gel recovery kit (Axygen Inc, production lot number: AP-GX-50 HangZhou, China), and a 2% agarose gel electrophoresis was performed for preliminary quantification. The PCR products were detected by Quantifluor-ST blue fluorescence quantitative system (Promega Inc, The Westin, America). The Illumina platform library was constructed, and the sequence of the template DNA fragment was obtained by Illumina platform sequencing. After that, the homologous sequence whose similarity was more than 97% was assembled into an operation taxonomic unit (OTU) for bioinformatics analysis. According to the results of Shannon–Wiener diversity index in different sequencing depths of microflora samples of rats in each group, a curve was constructed to reflect the alpha diversity of microflora in each sample, and then the Shannon index was used to analyze the diversity of the microflora. The relative abundance of OTU in the intestinal flora of rats in each group was analyzed by principal component analysis (PCA) and then the differences in the community structure were studied. The community difference analysis (LEfSe) was used to determine the groups with significant relative abundance differences of genus or higher taxonomic level between the groups of rat flora samples by linear discriminant analysis.

**Statistical analysis**

During the filtering of data process, the raw sequencing data were processed by using an internally written program to obtain the Clean Data. The specific steps were as follows:

1. Adopt the method of removing low quality by window
2. Remove the reads that are contaminated by joints
3. Eliminate the reads that contain N
4. Clear away the complexity reads (the length of successive occurrence of a base in default reads is ≥10).

Using Fast Length Adjustment of SHort reads, v1.2.11, software (http://www.cbcb.umd.edu/software/flash), and overlap relation, the pairs of reads sequenced by double ends were assembled into a sequence, and Tags with hypervariable region was obtained. The stitching conditions were as follows:

1. The minimum matching length is 15 bp
2. The allowable mismatch rate of overlapping region is 0.1.

The reads with no overlap relationship were removed.

The paired end reads were spliced into Tags by overlap relationship between reads, and a total of 2,189,006 Tags were obtained from all the samples, with an average of 31,271 Tags per sample, an Sprague Dawley (SD) value of 201, with an average length of Tag of 252 bp, and an SD value of 0 bp.

The clean tags treated above were clustered by OTU, and then the species classification of OTU was completed by OTU annotation.

The USEARCH (v7.0.1090) software (https://drive5.com/) was used to cluster the spliced tags into OTU. The main processes were as follows:

1. The UPARSE (https://drive5.com/) was used for clustering under 97% similarity, and the representative sequence of OTU was obtained
2. The UCHIME (v4.2.40) (https://drive5.com/) was used to remove the chimera produced by PCR amplification from the OTU representative sequence. The 16S uses a method to remove chimerism by comparing it with the existing chimeric databases. The 16S chimeric database was considered as the gold standard database (v20110519)
3. The USEARCH-global method was used to align all the Tags back to the OTU representative sequence, and a statistical table of abundance of each sample in each OTU was obtained.

After the OTU representative sequences were obtained, the OTU representative sequence was then compared with the database for species annotation by RDP classifier (v2.2), and the confidence threshold was set to 0.6.

**Comparison database**

The 16S (including bacteria and archaea) Greengene (default): V201305; RDP: Release9 201203.

Filter the annotation results as follows:

1. Delete the OTU without comments
2. Take away the species in the annotation results that do not belong to the analysis project. For example, if the sample is a bacterial 16s and if the OTU annotates the ancient bacteria, then it will be removed
3. The use of residual OTU for postanalysis.
**Experiment Results**

**Operation taxonomic unit statistics**

The species richness of the sample was preliminarily explained by clustering it into OTU, and the OTU for species classification was under 97% similarity. A total of 1128 OTUs were produced from seventy samples. The OTU number of each sample is shown in Figure 1, and the sample OTU number of rats’ feces in each group is shown in Figure 2.

According to the statistical results, the number of OTUs in the model group was significantly lower than that in the normal group, and the difference was statistically significant ($P<0.01$). There was no significant difference in the OTUs between the Western medicine group, the Shuganlidan group, the Jianpihuatan group, the Tongfuxiezhuo group, and the Dachaihutang group, when compared with the model group. Compared with the model group, the number of OTUs significantly decreased in the Tongfuxiezhuo group, and the difference was statistically significant ($P<0.01$).

**Operation taxonomic unit principal component analysis**

PCA analysis is used if two samples are closer and the composition of the two samples is similar. There were significant differences in the composition of intestinal flora between the seven groups in the dimensions of PC2 and PC1, and the results are presented in Figure 3.

The abscissa represents the first principal component, and the percentage in the parentheses indicates the contribution of the first principal component to the sample difference, which was 21.85%. The ordinate represents the second principal component, and the percentage in the parentheses indicates the contribution of the second principal component to the sample difference, which was 9.27%.

**Operation taxonomic unit rank curve**

The OTU rank curve is a form of graph that shows species diversity in the sample, and the curve shows the richness and uniformity of the species contained in the sample. The length of horizontal axis of the curve reflects the richness of species in the sample, and the wider the curve, the richer the species in the sample. The shape of the longitudinal axis of the curve reflects the uniformity of the species in the sample, and the flatter the curve is, the higher the uniformity of the species composition in the sample. In this study, the species richness of normal group remained the highest. In this study, the species richness of the normal group was the highest, and the OTU rank curve of the experimental sample is shown in Figure 4.

Abscissa is the OTU abundance ranking for sample (from high to low), and the ordinate is considered the OTU abundance.

**Diversity analysis of individual samples**

Alpha diversity involves analysis of species diversity in a single sample, including the Observed Species Index, Chao index, ACE index, and Simpson index. The larger the first three indexes, the richer the sample species, and the smaller the last index, the richer the sample species.
these three indexes also reflect whether the sample sequencing quantity is sufficient or not. If the curve tends to be flat or reaches the plateau stage, it can be considered that the depth of sequencing has basically covered all the species in the sample; otherwise, the diversity of the species in the sample was higher, and there are still more species that have not been detected by sequencing. In this study, these three exponential curves tended to be flat, indicating that the sequencing depth has basically covered all the species in the sample [Figure 5].

**Total operation taxonomic unit classification comparison**

According to the absolute taxonomic chart of sample number, the composition of bacteria in each sample at each taxonomic level was calculated. In this study, the highest abundance distribution of 12 phylum in seventy samples included *Bacteroidetes*, which was 54.08556%, followed by *Firmicutes* (38.19605%), *Proteobacteria* (5.926266%), and other phylums (including *Cyanobacteria* [0.800365%], *Tenericutes* [0.320534%], *Verrucomicrobia* [0.218843%], *Actinobacteria* [0.174874%], *Elusimicrobia* [0.130494%], *Deferribacteres* [0.103984%], *TM7* [0.001646%], *Lentisphaerae* [0.001352%], and *Spirochaetes* [0.000823%]), which were below 2%. From the level of phylum, the unclassification (other) of other phylums accounted for 0.039207% of the total. In the phylum of *Bacteroidetes*, 54.06793% was *Bacteroidia*, and the remaining percentage of other bacteria was 0.017634. In the phylum of *Firmicutes*, the total proportion of *Clostridia* was 35.27909%, and the percentage of *Erysipelotrichi* was 1.7548555%, that of Bacilli was 1.157932%, and that of others was 0.004173%. In the phylum of *Proteobacteria*, the total proportion of *Deltaproteobacteria* was 3.64238%, that of *Betaproteobacteria* was 1.75944%, that of *Epsilonproteobacteria* was 0.330821%, that of *Gammaproteobacteria* was 0.113507%, and that of *Alphaproteobacteria* was 0.080119%. The result is shown in Figure 6.

**Species annotation analysis**

The profiling area map and the histogram map of each sample species clearly showed the composition of different classes of fungi in each sample from six taxonomic levels of phylum, class, order, family, genus, and species. Figure 7 shows the profiling histogram of species in seventy sample phylum classification levels.

Figure 7 shows the distribution of bacterial abundance at the phylum classification level for each sample. The horizontal coordinate is the sample number, the vertical coordinate is the abundance of each phylum, and the sum of abundance was 1. Figure 7 shows that the intestinal flora structure of each sample (i.e. each rat) has its own specificity, but overall, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* are still considered the dominant flora of the intestinal tract of each group of rats, accounting for a high proportion of each sample. The proportion of a phylum in some samples was higher than that of other samples, for example, the proportion of *Verrucomicrobia* in F9 and F10 was slightly higher, i.e., as high as 8.603378% and 4.833206%, respectively. The proportion of *Deferribacteres* in F5 samples was slightly higher (3.681152%). The proportion of *Cyanobacteria* in F4, C5, F10, F3, E4, E6, and E7 was slightly higher, i.e., 6.658402%, 5.153419%, 3.292281%, 2.7457755%, 2.571671%, 2.374844%, and 2.308898%, respectively.

Using the above methods, the intestinal flora structure of each sample (each rat) can be displayed at different levels. With the gradual refinement of bacterial classification, the diversity of species and the individual differences among the samples are more obvious. Figure 8 shows the profiling histogram of seventy species at the taxonomic level of the sample genus.

As shown in Figure 8, in each sample, *Bacteroides*, *Prevotella*, *Oscillospira*, *Sutterella*, *Blautia*, and *Ruminococcus* abundance of elements is high. Observation and contrast can be seen, where the expression of *Bacteroides* in PH group, the Shuganlidan group, the Jianpihuatan group, the Tongfuxiezhuo group, and the DD group was higher than those in the normal group. However, the expression of *Ruminococcus* abundance was lower in different degrees than that in the normal group. *Lactobacillus* was expressed in the normal group, but almost showed no expression in other groups. The expression of *Prevotella* in the normal group was higher, but was significantly lower in the model group, the Shuganlidan group, the Tongfuxiezhuo group, and the Dachaihuatang group and higher in the PH and the Jianpihuatan groups. The expression of *Oscillospira* was higher in the normal group than in other groups, but higher in individual samples, such as G7, G10, and D4. *Sutterella* was rarely expressed in the normal group. However, the expression of Blautia was higher in other groups than that in normal group, but it was higher in other groups, especially in the PH group. The above is considered as the gross analysis of species abundance difference in each group, and the differences of specific bacteria in the intestinal tract of rats in each group require further analysis by informatics.

**LEiSe analysis**

The LEiSe software (https://huttenhower.sph.harvard.edu/galaxy/) was used to analyze the micropopulation that plays...
an important role in grouping, which was represented by the color. A color circle point represents a legend of biomarker in the upper right corner of the biomarker’s name. Yellow nodes represent microbial groups that do not play an important role in different groups. The concrete list diagram is shown in Figure 10.

As shown in Figure 10, the microbes that play an important role in the normal group are Ruminococcaceae, Oscillospira, Lactobacillales, Lactobacillaceae, Lactobacillus, bacilli, Ruminococcus, TM7, Spirochaetes, Clostridium, Elusimicrobia, Elusimicrobiaceae, Elusimicrobiales, Lactobacillus helveticus, Lactobacillus reuteri, Elusimicrobium, RF39, and Christensenellaceae. The model group microbes that played an important role were Ruminococcus gnavus. The XY group played an important role in the microorganisms of Prevotella, Paraprevotellaceae, and Blautia. The invigorating spleen and resolving phlegm groups played an important role in the microorganisms of Bacteroidetes, Bacteroidia, Bacteroidales, Roseburia, Rikenellaceae, and Stridium methylpentosum. The microbes that played an important role in the group of purging and turbidity of Fu-organs are Bacteroidaceae, Bacteroid, Porphyromonadaceae, Parabacteroid, 4COD-2,
Cyanobacteria, YS2, Parabacteroides distasonis, Bacteroides uniformis, Verrucomicrobiaceae, Verruco-microbiae, Verrucomicrobiales, Akkermansia, Akkermansia muciniphila, Coprobacillus, Parabacteroides gordonii, Blautia producta, and Ruminococcus torques. DD group played an important role in the microbes of Erysipelotrichales, Erysipelotrichi, and Erysipelotrichaceae.

**DISCUSSION**

The intestinal flora consisted of about 1014 kinds of microbes that participate in the metabolism and immunity of the host, and can respond to various adaptive responses caused by external stimuli. Liver and gastrointestinal tract are closely related in anatomy and function. In recent years, international studies showed that the occurrence and development of liver diseases are closely associated with the changes of intestinal microecology. Intestinal dysbacteriosis and endogenous endotoxemia play an important role in the pathogenesis of NAFLD.

In this study, NAFLD rat model was successfully established by high-fat and high-sugar diet, and then the intestinal flora of each group was analyzed. The results of flora abundance showed that the diversity of intestinal flora of normal rats was significantly higher than that of NAFLD rats. This was consistent with the results of foreign Le Chatelier et al. in their study. In their study, Le Chatelier et al. invited 123 nonobese and 169 obese Danish crowds to detect their intestinal flora abundance. The results showed that the intestinal flora with “low microbial abundance” carried more inflammatory bacteria, such as active rumen coccus. Similarly, individuals with low intestinal flora abundance tend to have characteristics associated with inflammation. Cotillard et al. found that people with low microbial richness had a higher risk of metabolic disease than those with higher intestinal microbial richness. Although the proportion of intestinal flora of each group was slightly different in terms of microflora structure, it still consisted of three phylums, namely Bacteroidetes, Firmicutes, and Proteobacteria. By analyzing the specific bacteria in the five medications’ administration, the rat intestinal-specific bacteria in the Tongfuxie group showed a more significant effect than those in the other treatment groups in NAFLD treatment. The specific flora in rats in the Shuganlidan group mainly consisted of Bacteroides, Vibrio pseudobutyrate, and Akkermansia muciniphila of the family Verrucomicrobia and the genus *Akkermann*. Bacteroid bacteria can decompose polysaccharides that cannot be
It also assists in the production and maintenance of gut microbiota. It has been reported that the enhancement of intestinal flora, and the expression of inflammatory factors in serum, increase insulin sensitivity, and reduce fat deposition in the liver. At the same time, some studies showed that capsaicin improves obesity in hyperlpidemic mice and is associated with increasing relative abundance of *A. muciniphila*, and metformin in the treatment of Type 2 diabetes is also associated with increasing the relative abundance of *A. muciniphila*. In general, many studies showed that several metabolic diseases are associated with the relative abundance of *A. muciniphila*, which can be used as an intervention or intermediate process to improve glucose, lipid metabolism, and anti-inflammatory effects; increase immunity; and maintain the integrity of intestinal mucosal barrier. And, hence, *A. muciniphila* is considered an important probiotic.

Chinese medicine believes that during the pathogenesis of NAFLD, phlegm, dampness, turbidity, poison, blood stasis, and even heat and other pathological products accumulate. The disease is located in the liver, and is closely related to spleen, stomach, and intestines. Therefore, one of the key points in NAFLD treatment includes reduction of various pathological products by using the method of Tongfuxiezhuo. The “Tongfuxiezhuo” prescription elements of the DD (rhubarb root and rhizome and rhubarb root and rhizome) utilized the function of intestinal conduction dross, detoxifying and releasing solid, making clear yang rising and turbid yin descending, recovering the function of spleen and stomach, and healing the disease.

**Conclusion**

Among the three prescription elements of the DD, the “Tongfuxiezhuo” prescription elements have a significant advantage in improving the intestinal flora of model rats with NAFLD. By enhancing the *Bacteroides* genus, *Sartre* genus, *Vibrio* pseudobutyrate bacteria, *A. muciniphila*, and other beneficial bacteria’s relative abundance in the intestinal flora of NAFLD rats, glucose lipid metabolism can be improved in NAFLD rats. Reduction of fat deposition in liver inhibited inflammatory reaction in the intestines and maintained the integrity of intestinal mucosal barrier. According to the theory of “entero-hepatic axis” put forwarded by Marshall in 1998 and the role of intestinal factors in the pathogenesis of NAFLD, the “Tongfuxiezhuo” prescription elements (rhubarb root and rhizome and rhubarb root and rhizome) in DD have a positive effect on the regulation of intestinal flora of NAFLD.

**Figure 10:** List of microbes that play an important role in each group.
Financial support and sponsorship
This study was supported by the National Natural Science Foundation of China (81673868).

Conflicts of interest
There are no conflicts of interest.

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