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PCR-DGGE analysis of earthworm gut bacteria diversity in stress of *Escherichia coli* O157:H7*

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ABSTRACT

In order to test if the intestinal bacteria play an important role in antibacterial ability of earthworm, we chose *Escherichia coli* O157:H7, an anthropozoonosis pathogen, as a biological stressor and studied the change of intestinal bacteria community of earthworm by PCR-DGGE analysis. Results showed that the pathogen merely existed 1 - 3 days, then almost disappeared after through the earthworm's gut. In this period, the diversity and abundance index of intestinal bacteria increased first, then decreased, and finally kept stably after 7 days. The result demonstrated that the intestinal bacteria of earthworm had ability of adjust community structure to eliminate the pathogen *E. coli* O157:H7, and the amount of bacteria *Bacillus* increased significantly, which might be the positive antagonism to *E. coli* O157:H7.

Keywords: PCR-DGGE; Earthworm Intestinal Bacteria Diversity; *Escherichia coli* O157:H7 Stress

1. INTRODUCTION

The antibacterial ability of earthworm is not only depend on natural immunity, the intestinal microbes also play a important role on cooperative defense [1]. *Escherichia coli* O157:H7 is an anthropozoonosis pathogen with characters of acid-resisting, low temperature resistance, low infectious dose, strong pathogenicity, no specific medicine for clinical treatment etc. Therefore it can cause gastrointestinal disease with potentially fatal consequences as a result of systemic Shiga toxin activity [2]. The *Escherichia coli* O157:H7 could threaten human health through the food chain as the natural host of *Escherichia coli* O157: H7 (like cattle, sheep, dogs and chicken and other animals) is often animal origin food pollution source.

Because of the strong ability of digest, earthworm was used to dispose the wastes and livestock feces for a long time. However, we had hardly found they died of pathogens, even they only existed natural immunity (body wall, cellular immunity and humoral immunity), which is not enough to defend all pathogen, as the lower level in animals [3]. Kumar found the amount of *Escherichia coli* O157:H7 reduced through digestion of the earthworm [4]. Liu isolated 6 *Lactobacillus* strains from vermicompost of *Eisenia fetida*, which was stressed by *Escherichia coli* O157:H7 and each strain could restricted the pathogen obviously [5]. Base on these studies, we reasonably infer the microbe in gut may help earthworm to defense the pathogen *Escherichia coli* O157:H7.

In this study, we use PCR-DGGE technique to analysis the dynamics of *Eisenia fetida* intestinal bacterial community structure and diversity under the stress of pathogen *Escherichia coli* O157:H7. Furthermore, we try to explain the relationship between the disappearance of pathogen and the change of intestinal bacterial community by which to evaluate if the intestinal bacteria of earthworm play an important role to against pathogen in surrounding environments.

2. MATERIALS AND METHODS

2.1. The Experimental Organisms and Preparation of an Artificial Soil

The earthworm *Eisenia fetida*, which was bred in our lab, was used as the experimental organism. Adult earthworms were kept in test substrates for one week before experiment. Artificial soil was used as test substrate in the experiments and was prepared according to the improved OECD method, which comprised 10% dried cowdung instead of sphagnum peat, 20% kaolin, 70% sand, and Ca carbonate was used to adjust pH to 6.0 ± 0.05 [6]. Two days before experiments, the earthworms were transferred onto the filter paper, which was soaked with isotonic Lumbricus balanced salt solution, to cleaned the

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feces in gut [7].

Escherichia coli O157:H7 (the international reference strain EDL933) was purchased from Institute of Epidemiology and Microbiology, Chinese Academy of Preventive Medicine.

2.2. Experimental Procedures

Escherichia coli O157:H7 was maintained in stock culture at 4˚C, and was shock cultivated in Luria-Bertani medium (pH 7.0, Tryptone 1%, Yeast extract 0.5%, NaCl 1%) at 37˚C, 200 rpm for 18 h. The cells of bacteria in the culture were collected by centrifugation at 3000 rpm for 30 min, washed, and resuspended in water. They were added into the artificial soil at final concentrations of approximately 10^7 CFU g^{-1} soil [8]. Then, the mixture was thoroughly mixed with water at least 30 min to achieve a homogenous distribution which was adjusted to 35% of the final water content. The soil samples were stored in the plastic containers at room temperature overnight. The control soil without bacterial addition was prepared similarly. Subsequently, the earthworms were transferred to the plastic containers. 300 earthworms were added into each container with 30 kg bacteria-soil mix as prepared in the above steps. The containers were placed in a room which maintained a constant environmental temperature under 23˚C; water was supplemented to maintain the moisture level of the soil [9]. The earthworms were fed with dried cow-dung, which was added onto the soil surface once a week after the dung was thoroughly wetted. The experiments lasted for 21 days.

2.3. The Extraction of the Total DNA of Bacteria in Earthworm Gut

On Day 0, 1, 3, 7, 14, and 21 of the feeding period, ten earthworms were random collected, and the intestinal content was gathered by anatomy. The total DNA of intestinal bacteria was extracted from 0.5 g intestinal content by E.Z.N.A. Soil DNA Kit (obtained from American OMEGA).

2.4. The Conditions of PCR and DGGE

The total DNA of bacteria in earthworm gut was used as a template (0.5 ng) in PCR reactions (2 × Easy taq Super mix were obtained from Beijing TransGen Biotech Co., Ltd.) with primers: GC-968F (5'-CgCCCgGggCgCgCCCCgggCggggCgggggCACgggggggAACgCgAAgAACCTTAC-3') and 1401R (5'-gCgTgTgTACAAgACCC-3'). PCR cycles were as follows: one cycle of 94˚C for 5 min, and 30 cycles of 94˚C, 30 s, 54˚C, 30 s and 72˚C, 30 s, followed by a final extension at 72˚C for 5 min.

DGGE condition was controlled as: 25 μL sample (DNA concentration more than 50 ng/μL at least) has been purified (PCR Cycle Pure Kit were obtained from American OMEGA Co.), then it was add into the gel with 40% - 65% denaturing gradient, ran 7 hour under 120 V.

2.5. Gel Extraction and Sequencing

Gel Extraction Mini kit (obtained from American OMEGA) was used to extract DNA fragments. After that, the products were linked with PMD 18-T vector to ensure the correct sequencing (the primers without GC clamp were used for sequencing).

3. RESULTS

3.1. Behavior of Earthworms under *Escherichia coli* O157:H7 Stress

At the beginning, few earthworms (about 70 - 80) stayed on the surface of artificial soil, without escaping. Then, all of them dip into the earth during 3 hours. The difference of weight per 100 earthworms between the control and stress groups was not significant (**Figure 1**).

3.2. The Change of Intestinal Bacteria Diversity

According to the cluster analysis of DGGE fingerprinting, the sample 1st and 3rd were classified to a group; the sample 7th and 14th were classified to the second; the last group contained sample 21st (**Figure 2**).

The diversity, abundance and dominance index of *Eisenia fetida* intestinal bacteria had a short increase in 24 h under *Escherichia coli* O157:H7 stress, but it reduced rapidly after 3 days, and finally kept stable during the last days (**Table 1**).

3.3. The Change of Intestinal Bacteria Community Structure

Band L, which means *Escherichia coli* O157:H7, merely existed in earthworm gut in the first day, and could not be found in the rest of days. With the pathogen disappeared, bond a, d and k, which existed in earthworm

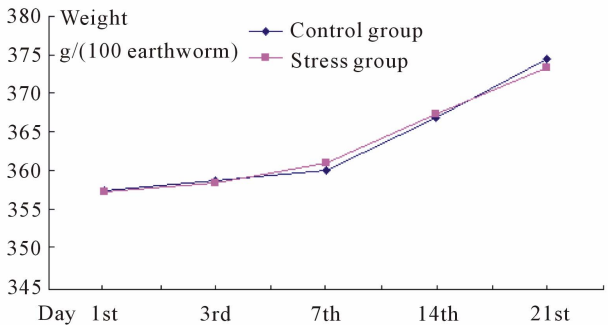

Figure 1. The increasing weight of two groups of earthworm. The growth rate of control group is 4.784%; and the number of stress group is 4.535%.

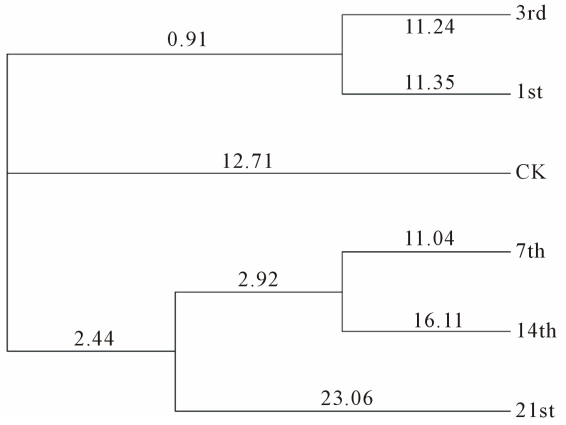

Figure 2. Cluster analysis of PCR-DGGE fingerprinting.

Table 1. Diversity of the intestinal bacteria in *Eisenia fetida.*

Sampling No.	Margalef index	Shannon index	Pielou index	1/Simpson index
CK	4.13	2.97	0.99	23.1
1st	4.56	3.07	0.99	26.27
3rd	3.91	2.90	0.98	20.441
7th	3.69	2.83	0.98	18.77
14th	3.69	2.83	0.98	18.63
21st	3.68	2.84	0.99	18.64

gut originally, also could not be found after the 7th day. Another bond, which need to be noticed is c. It occurred with *Escherichia coli* O157:H7, and had a high abundance during the culture period (the percentage in bacteria community of each sample was 3.24%, 3.87%, 5.31%, 5.28%, 5.29%) (**Figure 3**).

The phylotype, which band a, d, c and k represented, belong to *Anaerosporobacter*, *Aeromonas*, *Bacillus*, and *Rubritalea*, respectively. The band a (*Anaerosporobacter*), c (*Bacillus*), k (*Rubritalea*) had higher genetic relationship than band d (*Aeromonas*) with *Escherichia coli* O157:H7. The rest bacteria mainly belong to *Clostridiales*, *Aeromonadales*, *Chromatiales et al.*, which belong to phylum *Proteobacteria* and *Firmicutes* (**Figure 4**).

4. DISCUSSION

4.1. The Concentration of *Escherichia coli* O157:H7 in Artificial Soil

Hutchison found the concentration of *Escherichia coli* O157:H7 in livestock feces was 10^3 - 10^5 CFU/g, and Fukushima found the amount of the pathogens could increase to 10^8 CFU/g [8]. To ensure the stress effect, the concentration of *Escherichia coli* O157:H7 was maintained at 10^7 CFU/g. However, the growth of *Eisenia fetida* was not affected under these concentration (**Figure 1**).

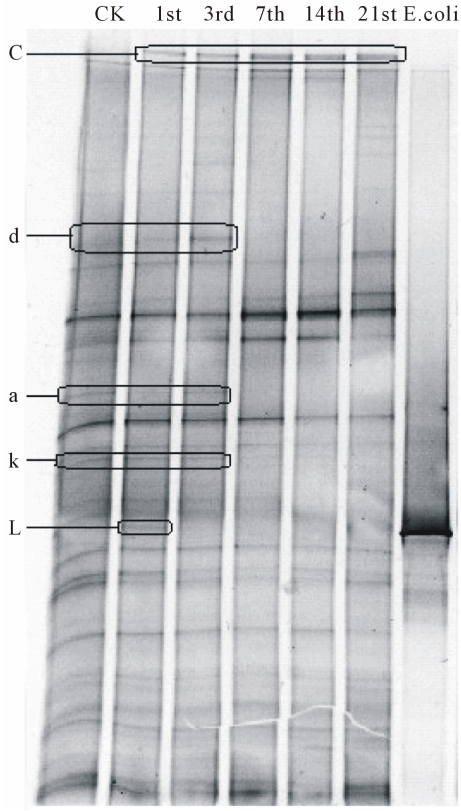

Figure 3. PCR-DGGE fingerprinting of intestinal bacteria in *Eisenia fetida* gut with *Escherichia coli* O157:H7.

4.2. The Diversity and Community Structure Change of *Eisenia fetida* Intestinal Bacteria

The result of cluster analysis show that the dynamics of earthworm intestinal bacteria community have experienced 3 difference periods under the stress of *Escherichia coli* O157:H7, and it was in accordance with the results of diversity, abundance and dominance index (**Table 1**). Combined the results of PCR-DGGE fingerprinting and ecology index, we speculated that the appearance of band c lead a short increasing of diversity and abundance index in the first day. In second period, the disappearance of *Escherichia coli* O157:H7 result in the reducing of diversity index and abundance index, and might have some relationship to the disappearance of band a, d, k. The third period performed relative stable, the indexes and structure basically did not change, and the rebalance might be build in this period.

4.3. The Defense Effection to *Escherichia coli* O157:H7 of *Bacillus*

Bacillus is a kind of aerobic microbe, through take biological oxygen to reduce the REDOX, and further reduce the aerobium, so as to keep the intestinal microecological balance [10]. *Bacillus* could produce a variety of diges-

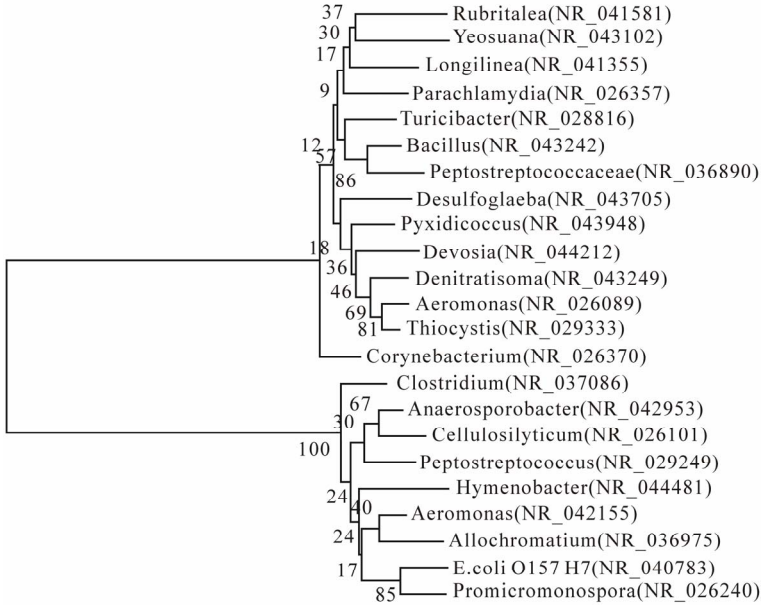

Figure 4. A neighbor-joining phylogenetic tree contain main intestinal bacteria of *Eisenia fetida* Bootstrap analyses were made with 1000 cycles.

tive enzyme and antibiotics (peptide antibiotics, lipopeptide antibiotics, phosphatide antibiotic, polyketide etc.) in intestinal [11]. Therefore, *Bacillus* is the source of many probiotic like some bacteria helpful for digestion and antimicrobial.

The bacteria *Bacillus*, which appeared with *Escherichia coli* O157:H7, might be a strain with antagonism to this pathogen. The *Bacillus* usually has a low level in earthworm gut. Therefore DGGE could not detect it. When the intestinal microbes were stressed by *Escherichia coli* O157:H7, the proliferation of *Bacilllus* made it to be found. The ability to defense *Escherichia coli* O157:H7 might depend on the secretion of antimicrobial compound [12]. The isolation of this strain and separation of antimicrobial compounds need to be promoted.

Other interesting bacteria are belong to *Anaerosporobacter sp.* and *Aeromonas sp.*, which also could lead diarrhea like *Escherichia coli* O157:H7. They were also found disappeared in 7 - 14 days combined with *Bacillus* appeared. Whether this *Bacillus* strain could influence these two pathogens, it needs to be confirmed and complemented with further studies.

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