



Effects of microcystin-LR on bacterial and fungal functional genes profile in rat gut



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ABSTRACT

The short-term exposure to microcystin-LR (MC-LR, one of the most common and toxic variants generated by toxigenic cyanobacteria) induced gut dysfunction such as generation of reactive oxygen species, cell erosion and deficient intestinal absorption of nutrients. However, till now, little is known about its impact on gut microbial community, which has been considered as necessary metabolic assistant and stresses resistant entities for the host. This study was designed to reveal the shift of microbial functional genes in the gut of rat orally gavaged with MC-LR. GeoChip detected a high diversity of bacterial and fungal genes involved in basic metabolic processes and stress resistance. The results showed that the composition of functional genes was significantly changed in rat gut after one week of exposure to MC-LR, and we found some relatively enriched genes that are involved in carbon degradation including chitin, starch and limonene metabolism, and these genes were mainly derived from fungal and bacterial pathogens. In addition, we found large amounts of significantly enriched genes relevant to degradation of the specific carbon compounds, aromatics. The dysbiosis of bacterial and fungal flora gave an implication of pathogens invasion. The enriched gene functions could be linked to acute gastroenteritis induced by MC-LR.

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1. Introduction

The development of cyanobacterial blooms has become a serious problem because many kinds of cyanobacterial metabolites have been reported to be toxic to organisms in the past decades (Carmichael, 1992; Codd et al., 2005). Special attention has been given to one of the members, microcystins (MC), because of its potent hepatotoxins with genotoxic and carcinogenic properties. In the previous studies, focus has been paid on necrosis or apoptosis of animal cells and tissues destructions caused by MC (Mackintosh et al., 1990; Runnegar et al., 1995). To our knowledge, there is little known about the transition of microbial community in the intestinal system after exposure to MC, where we should pay much more attention since it is the organ of humans or animals in direct

contacting with MC, furthermore, a substantial higher portion of MC was proven to be presented in intestine (about 89.5%) than in other organs including liver, kidney, spleen, gallbladder, gill, blood and muscles (Chen et al., 2007).

In natural environments, it is frequently appeared that surface aggregations of some cyanobacteria may accumulate to scum with toxin concentrations varying from traces up to 1800 µg/L or higher (Chorus and Bartram, 1999). Water uptake by humans with such high concentration of MC during a short term would result in acute gastroenteritis as that happened before (Velde, 1931) or trigger the release of the potent inflammatory cytokine TNF-α (Christen et al., 2013). Moreover, MC-LR has been shown to cause severe erosion of villi in the small intestine (Ito et al., 2000), generation of reactive oxygen species (ROS) (Amado and Monserrat, 2010; Ding et al., 2001), alteration of cellular membrane organization, membrane fluidity and modification in the activity of intestinal membrane enzymes (Moreno et al., 2003). These damages of intestinal physiology and function might lead to nutrient absorption

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deficiency of digestive system with elevated lipids and amino acids in ileum of rat, whereas decreased amino acids, lipids and choline in liver, which thereby disrupted some metabolic pathways in liver including choline metabolism, nucleotide de novo synthesis, glutathione synthesis and glutathione depletion (He et al., 2012).

Under such a situation, how the microflora accommodates itself to the gut and which genes are the prevalent ones of the shifted microflora, which especially attract our attention, since intestinal microflora is necessary to host as metabolic assistant and stresses resistant entities. For instance, gut microbes can supply short-chain fatty acids to host as energy and carbon source through catabolism of carbohydrates such as starch, cellulose, chitin, etc. (Blaut and Clavel, 2007). Meanwhile, gut microbes have potential ability to degrade MC as microbes did in nature, for example, species from *Bacillus*, *Bordetella*, *Brevibacterium*, *Rhodococcus* and *Pseudomonas* were reported to be capable of degrading MC (de la Cruz et al., 2011). To get an overview of the transition of microbial genes in rat gut after exposure to a high concentration of MC-LR during a short-time course, we used GeoChip to detect functional genes from bacterial and fungal species.

2. Materials and methods

2.1. MC-LR extraction and purification

MC-LR extraction and purification were performed according to a modified Ramanan method (Ramanan et al., 2000). Freeze-dried cyanobacterial blooms which were collected from Lake Dianchi in China were extracted with 60% MeOH at 220 rpm for 1 h at 4 °C (50 mL 60% MeOH for 1 g dry weight biomass of cyanobacterial blooms), after being sonicated at 32 kHz for 20 min and the suspension was centrifuged. The precipitant was extracted again. The total supernatant was evaporated to one-tenth of its initial volume and then isolated by HPLC with Waters C18 column. The purity of MC-LR was above 95% (the remainder was primarily comprised of pigment) and its identity was confirmed and determined by liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS, Thermo Electron, Waltham, MA) method described previously (He et al., 2012). MC-LR standard was obtained from Sigma–Aldrich Ltd (St. Louis, MO, USA, purity >95%).

2.2. Animal experiments and sample collection

Animal experiments were performed as He et al. described (He et al., 2012). Briefly, six male Sprague–Dawley rat (140–150 g) aged 6 weeks were obtained from National Resource Center for Rodent Laboratory Animal (Beijing, China), and then fed *ad libitum* at the animal facilities of Wuhan Institute of Virology, Chinese Academy of science under controlled conditions (temperature, 20–22 °C; relative humidity, 40–60%; day–night light cycle, 12–12 h). After one week of adaptation, the rats were randomly separated into two groups, kept one rat in one cage. The groups were orally gavaged with 0.9% saline (control: three replicates FC01, FC05 and FC07) or MC-LR suspended in 0.9% saline at dose of 1.0 mg/kg body weight/time (MC-LR treatment: three replicates FH01, FH05 and FH07), which was based on a prior acute experiment (Yoshida et al., 1997), for one week at 48 h intervals (totally four times), separately. On the second day after the last dose of MC-LR, fecal samples were collected immediately after defecation. Samples were stored at –80 °C before DNA extraction. All animal experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory animals (NIH Publication No.8023).

2.3. DNA extraction and microarray hybridization

GeoChip is a comprehensive microarray which contains highly specific probes from the genes with known biological functions, and available for studying functional activities of microbial communities (He et al., 2007). GeoChip 4.2 used in this study contained 83992 oligonucleotide probes (50 mer) targeting 410 functional groups of 152414 genes that are mainly assigned to two types of category: those required as basic metabolism for nutrient cycling (e.g., carbon cycling, nitrogen, sulfur, phosphorus and energy process), as well as those required for stress adaptation such as degradation of organic contaminants (e.g., aromatics), metal resistance and antibiotic resistance, etc. DNA extraction of all samples was performed according to a grinding method (Zhou et al., 1996), and purified using a Promega Wizard DNA clean-up system (Madison, WI). DNA samples were labeled with Cy-5 using random primers and purified (Yang et al., 2013). The hybridization was performed at 42 °C for 16 h on a MAUI hybridization station (BioMicro, Salt Lake City, UT, USA). After hybridization, the microarrays were scanned (NimbleGen MS 200, Madison, WI USA) at 100% laser power.

2.4. Data analysis

Microarray raw data were uploaded to the microarray data manager (<http://ieg.ou.edu/microarray>) and preprocessed using data analysis pipeline with following major steps: 1) remove microarray spots with Thermophile < 5%; 2) the signal intensity values for each spot were normalized by the sum of all spots' mean signals on the same slide and referred as mean ratio (MR) to represent the relative abundance of genes; 3) at least two spots out of the three replicate samples and the signal intensity > 1000 were required for a gene to be positive. The MR data was used for further data analysis. Functional gene diversity assessment including alpha diversity (Shannon–Wiener and Pielou) for individual sample and beta diversity (Bray–Cutis) between samples or groups and detrended correspondence analysis (DCA) were performed with R-based pipeline. A standard *t*-test was used to test the significance between MC-LR treatment and control. Cluster analysis of the significantly changed genes was performed using hierarchical clustering algorithm (Eisen et al., 1998) through CLUSTER (<http://rana.stanford.edu>) and then was visualized by TREEVIEW (<http://rana.stanford.edu/>).

3. Results

3.1. Composition variation of microbial genes and the enriched functions in gut after MC-LR treatment

We profiled microbial genes in fecal samples with their functional categories and phylogenetic classification from GeoChip 4.2. A total of 621 gene families from 21,460 sequences were detected. Alpha diversity is used to indicate the genes' number and abundance of each sample and beta diversity emphasizes the difference between each two samples or groups. A similar alpha diversity (Shannon–Wiener and Pielou evenness) based on genes relative abundances existed between MC-LR treated samples and controls. Beta diversity between each two samples from different groups (MC-LR treatment group and controls) varied markedly, and a much slight diversity variation existed between each two samples from the same group, indicating these genes detected in the three different samples from the same group gives a much better replications. From a dissimilarity test (Jaccard and Bray–Cutis distances), microbial communities of fecal samples after MC-LR treatment were significantly different from that of controls ($P = 0.001$), which

also can be clearly shown by the detrended correspondence analysis (DCA) plot (Fig. 1). T-test between MC-LR treatment samples and the controls was performed to find out the significantly changed genes in fecal samples after MC-LR treatment. The results showed that these significantly enriched genes were mainly associated to the processes of carbon degradation and aromatics degradation (Fig. 2A), and the significantly decreased genes referred to a variety of microbial functions including ammonification, phosphate limitation, metal resistance, etc. (Fig. 2B).

3.2. Carbon degradation

A substantial number of genes involved in the degradation of complex carbon compounds such as starch, hemicellulose, cellulose, chitin and lignin were detected, and genes relevant to hydrolysis of chitin and starch significantly enriched after MC-LR treatment ($P < 0.05$) (Fig. 3). In detail, relative abundances of 23 in 111 gene sequences encoding acetylglucosaminidase for chitin degradation changed significantly, and the enriched ones mainly derived from *Aeromonas hydrophila*, *Bacteroides vulgates*, *Akkermansia muciniphila* ATCC BAA-835, etc. (Fig. 4A). Relative abundances of 6 in 27 gene sequences encoding exochitinase changed significantly, and the enriched ones derived from *Shewanella denitrificans* OS217, *Acidothermus cellulolyticus* 11B and *Salinispora tropica* CNB-440 (Fig. 4B). Besides the chitin degradation genes, relative abundances of 12 in 72 gene sequences related to chitin synthase varied significantly ($P < 0.05$), and the enriched ones derived from fungi *Aspergillus flavus* NRRL3357, *Cryptococcus neoformans* var. *neoformans* JEC21 and *Nectria haematococca* mpVI 77-13-4, etc. (Fig. 4C). For starch hydrolysis, relative abundances of gene *amyA* encoding alpha-amylase from fungi and *amyX* encoding pullulanase significantly increased (Fig. 2A).

Genes relevant to other carbon compounds degradation were also detected, and several of them showed significantly increased signals after MC-LR treatment (e.g., *AssA*/alkylsuccinate synthase, *limEH*/limonene epoxide hydrolase) (Fig. 3). Intriguingly, most of the detected sequences of *limEH* for limonene degradation showed a significantly ($P < 0.05$) higher abundances in MC-LR treated samples than in controls, and these sequences were mainly derived from pathogenic species such as *Mycobacterium vanbaalenii*, *M. marinum*, *M. ulcerans*, *Pseudomonas aeruginosa* and *Rhodococcus erythropolis* (Fig. 5).

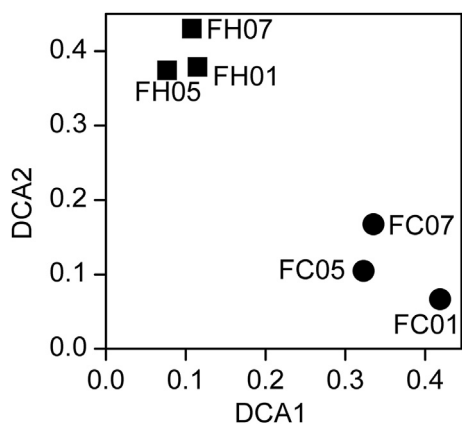


Fig. 1. DCA plot showed variation among samples according to the signal intensities of all detected genes.

3.3. Fungal functions

We detected a diverse functional genes of fungal community and several of them showed significantly ($P < 0.05$) higher abundances in MC-LR treated gut, including genes that are related to carbon degradation (e.g., *amyA*/alpha-amylase, *AceB*/malate synthase ($P < 0.1$), genes encoding phospholipase C, chitin synthase and exoglucanase ($P < 0.1$)), organic remediation (*Dyp*), denitrification (*nirK*/nitrite reductase) and virulence (cyanide hydratase gene) (Fig. 2A).

3.4. Aromatics degradation

A list of 3738 common probes for 105 gene families that were relevant to aromatics degradation were detected, and abundances of 18 gene families have variation trends ($P < 0.1$), such as genes relevant to degradation of aromatic carboxylic acid (*GcdB*, *GCoADH*, *hmgB*, *nagK*, *ohbAB*, *pimF*, *POBMO* and *tphA*), BTEX and related aromatics (*tomA*), chlorinated aromatics (*cbdA*), heterocyclic aromatics (*arhA*), nitroaromatics (*nbz* and *pnbA*), polycyclic aromatics (*nahB* and *phdA*) and other aromatics (*AmiE*, *catechol_B* and *CDO*), in which abundances of 11 gene families changed significantly ($P < 0.05$) after MC-LR exposure (Fig. S1). For instance, the most abundant detected genes *pimF* and *GCoADH* enriched evidently (Fig. S2), which encodes enoyl-CoA hydratase and glutaryl-CoA dehydrogenase respectively that would be required for the β -oxidation of odd-chain dicarboxylic acids to crotonoyl-CoA (Carmona et al., 2009).

3.5. Nitrogen

Genes for nitrogen fixation (*nif*), ammonification (*ureC* and *gdh*), assimilatory nitrate/nitrite reduction (*nasA* and *NiR*), dissimilatory nitrate/nitrite reduction (*napA* and *nrfA*), nitrification (*amoA*, *amoB* and *hao*), and denitrification (*narG*, *nirS*, *nirK*, *nirK_fungi*, *narB*, *norB*, and *nosZ*) were detected by Geochip. There was a significantly ($P < 0.05$) increase of fungal *nirK* gene encoding nitrite reductase for nitrite reduction to nitric oxide, whereas, gene *ureC* encoding urease responsible for ammonification decreased significantly in relative abundance ($P < 0.05$) (Fig. 2).

4. Discussion

The significant variation of functional genes in fecal samples, together with our previous describing that nutrients such as amino acids and lipids were elevated in gut but deficient in liver (He et al., 2012), suggested that gut dysfunction happened when three doses of 1 mg MC-LR/kg bodyweight (48 h intervals) were given over one week. Generation of ROS including hydrogen peroxide (H_2O_2) induced by MC-LR as described previously (Amado and Monserrat, 2010; Ding et al., 2001) was confirmed by the significantly enriched gene *cat_bac* ($P < 0.05$) (Fig. 2A), which encodes catalase for protecting the cells against oxidative damage through decomposition of H_2O_2 . The significantly enriched genes detected in the present study were mainly those involved in carbon degradation and aromatics degradation.

Mammalian cells are generally limited in their capacity to hydrolyze and utilize a large quantity of undigested dietary carbohydrates such as polysaccharides from plant cell walls (e.g., cellulose, xylan and pectin) as well as chitin that are passing into the gastrointestinal tract. Fortunately, this deficiency can be compensated by microbes in gut, which are capable of degrading most of the dietary polysaccharides such as starch to their constituent monosaccharides. We detected much higher gene abundance involved in carbon degradation such as cellulose, chitin,

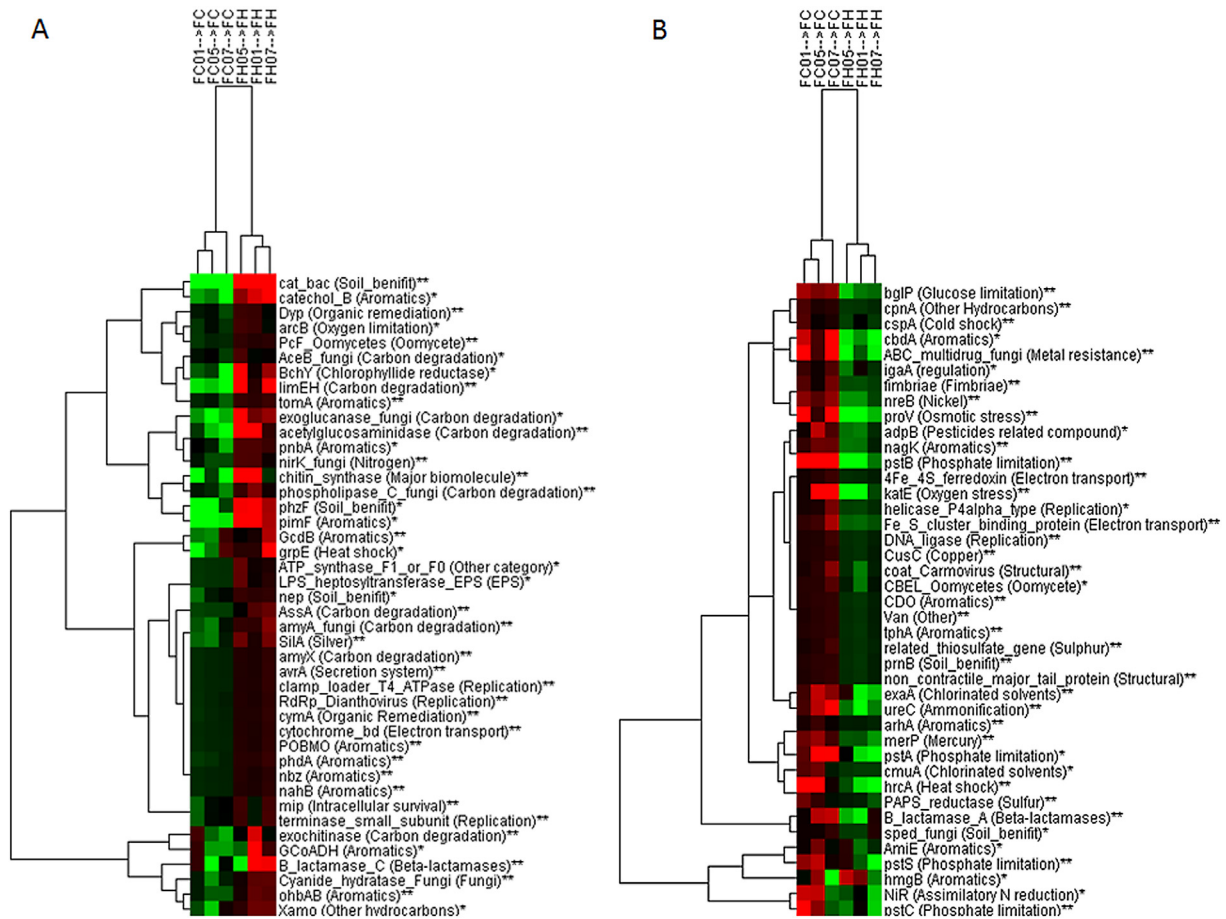


Fig. 2. Hierarchical cluster analysis of the evidently varied genes in fecal samples after MC-LR treatment. The relatively increased (A) and decreased genes (B) after MC-LR treatment. Results were generated in CLUSTER according to the relative abundance of genes' signal intensity and visualized using TREEVIEW. Green color indicated relatively lower signal intensities while red color represented relatively higher signal intensities. ** $p < 0.05$, * $p < 0.1$. FC, controls; FH, MC-LR treated samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

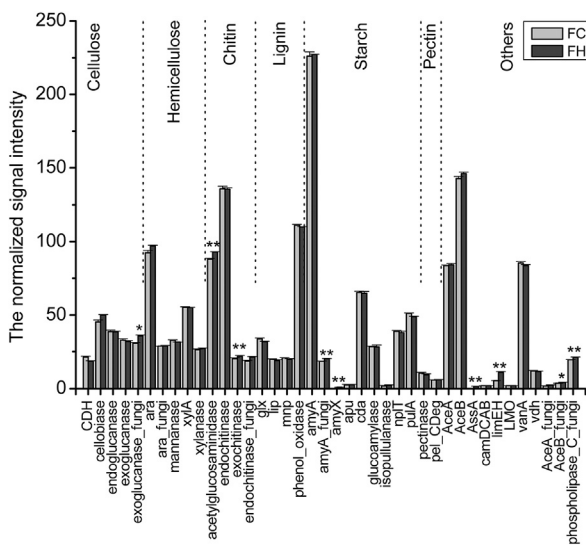


Fig. 3. The variations of the detected key genes involved in carbon degradation after MC-LR treatment. All data are presented as mean \pm SE with p value of t -test (** $p < 0.05$, * $p < 0.1$). Most of the genes involved in carbon degradation have litter variation after MC-LR treatment except that encoding chitinolytic enzymes (acetylglucosaminidase and exochitinase), genes involved in starch degradation (*amyA*_fungi/alpha-amylase and *amyX*/pullulanase), and genes for other carbon compounds degradation (i.e., *AssA*/alkylsuccinate synthase, *limEH*/limonene epoxide hydrolase, *phospholipase_C*_fungi).

lignin and starch, and genes encoding chitinolytic enzymes and amylase showed significant enrichments after MC-LR exposure. Chitin, a polymer of N-acetylglucosamine (GlcNAc), is widely found in cell walls of bacteria, fungi and exoskeleton of crustaceans (Lee et al., 2011). In our study, chitin- or chitin-like structures were synthesized through the significantly increased chitin synthase that was mainly derived from fungal pathogens as discussed below. The bacterial chitinolytic enzymes including exochitinase and N-acetylglucosaminidase would efficiently hydrolyze these chitin-like structures of fungi to produce GlcNAc (Patil et al., 2000), which together with glucose from starch hydrolysis that was catabolized by amylase, could supply a large percent of glucose to microbes or host cells. But as an alternative carbon source, the relative abundance of *bgIP* gene (aryl-beta-glucoside-specific enzyme II) relevant to utilization of aryl- β -glucosides decreased (Fig. 2B), which was in agreement with the previous studies that the expression of *bgIP* was repressed by glucose (Krüger and Hecker, 1995). Glucose plays important roles in microbes or host cells as carbon and energy source. For instance, glucose was fermented to the intermediates such as pyruvate, acetyl-CoA, malate, oxaloacetate and succinate by microbes in glycolysis and pentose phosphate pathway (Macfarlane and Macfarlane, 2003), and the intermediates were finally catabolized to short-chain fatty acids that are utilized by host cells as energy source (Macfarlane and Macfarlane, 2003). Also, much higher proportion of glucose could be fermented to lactate by intestinal epithelial cells through the up-regulated glycolysis after

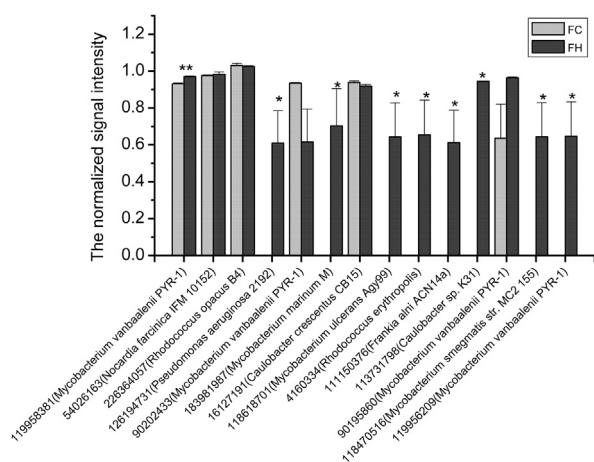


Fig. 5. The normalized signal intensity of the *limEH* genes encoding limonene epoxide hydrolase for limonene degradation. The signal intensity for each function gene was the average of the total signal intensity from all the replicates. Gene number is the protein ID number for each gene from GenBank database. All data are presented as mean \pm SE, ** $p < 0.05$, * only detected positive signals in FC or FH group.

crotonyl-CoA. An evident enrichment of *PimF* and *GCoADH* indicated that enriched microbes can ferment aromatics to acetyl-CoA through the anaerobic benzoyl-CoA pathway after MC-LR treatment, and the relatively increased processes of nitrite reduction (*nirK*) supported the anaerobic degradation of aromatics for supplying electron acceptors (Altenschmidt and Fuchs, 1991; Rudolph et al., 1991). The results together indicated that microbes could strengthen the usage of aromatics such as aromatic amino acids that were accumulated and much available to microbes in gut.

We have discussed genes functions of fungi together with that from bacteria, and the dysbiosis of fungal flora as well as bacterial flora also should be paid much more attention since it has been associated to some diseases of humans (Iliev et al., 2012), as considerable potential for use in clinical studies of these organisms. Of the 72 detected sequences for fungal chitin synthase, 18 were statistically different between the control and MC-LR exposed communities, and sequences from *Schizophyllum commune* H4-8, *Sordaria macrospora*, *Alternaria carotiincultae*, *A. flavus* NRRL3357, *Moniliophthora perniciosa*, *Ajellomyces dermatitidis* SLH14081, *Nectria haematococca* mpVI 77-13-4 and *C. neoformans* var. *neoformans* JEC21 showed higher abundances in MC-LR treated samples compared with controls (Fig. 4C). The enriched fungal species are always pathogenic or/and frequently appeared in patients with inflammatory diseases, for instance, *A. flavus* produces the most toxic and potent hepatocarcinogenic compound aflatoxins and the associated clinical syndromes include chronic granulomatous sinusitis, keratitis and osteomyelitis (Hedayati et al., 2007). *N. haematococca* represent pathogens associated with opportunistic fungal infections and keratitis in humans (Godoy et al., 2004). *C. neoformans* was proven to be prevalent in mucosal inflammation in crohn's disease (Li et al., 2014). Besides, the significantly increased sequences encoding acetylglucosaminidase (Fig. 4A) and limonene epoxide hydrolase (*limEH*, Fig. 5) derived from the bacteria that were also involved in human inflammatory diseases, which gave further evidences of pathogens invasion. For instance, *A. hydrophila* was clinically associated to diarrhea (Agger et al., 1985), *B. vulgates*, *A. muciniphila* and *R. erythropolis* are commensal gut species and a high abundance of them were related to inflammatory bowel disease (Derrien et al., 2004; Lepage et al., 2011), the prevalence of *P. aeruginosa* was associated to gut-derived sepsis (Laughlin et al., 2000), and species in genus *Mycobacterium* (*M. marinum* and *M. ulcerans*) can cause diverse diseases in humans (Stamm and Brown,

2004). We do not know why these pathogenic fungi and bacteria were prevalent in gut after MC-LR treatment, but their antioxidant activities (Hamilton and Holdom, 1999; Lynch and Kuramitsu, 2000; Tally et al., 1977) such as catalase protecting themselves against ROS and the capabilities such as using ROS-generated products as electron acceptors for anaerobic respiration (Kamada et al., 2013) might be one of the reasons. Thus, it indicated that the enriched genes involved in carbon cycling including chitin, starch and limonene in MC-LR treated gut were mainly contributed by the pathogenic fungi and bacteria.

In conclusion, we characterized the significantly changed functional genes of the altered microbial community in rat guts exposed to MC-LR during a short time scale. These genes were not only contributed by bacteria but also fungi that indicated the dysbiosis of micro- and macro-flora. We hypothesized that the enriched bacteria and fungi in rat guts after MC-LR exposure could protect themselves against ROS and mainly use chitin, starch and aromatics as carbon and energy sources, meanwhile, the generated chitin and its derivatives have the potential to enhance the pathogen invasion. Our results gave an overview of the potential functions of enriched genes, however, to further understand the responses of host to these enriched microbial functions, much more work including the interaction between host and microbes should be taken.

Ethical statement

All animal experiments in this research were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory animals (NIH Publication No.8023) which is approved by the State Council of People's Republic of China. All authors have read the manuscript and agree to its publication in Toxicon and agree that it has followed the rules of ethics presented in the Elsevier's Ethical Guidelines for Journal Publication.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.toxicon.2015.01.011>.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.toxicon.2015.01.011>.

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