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Exposure to the fungicide propamocarb causes gut microbiota dysbiosis and metabolic disorder in mice☆

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ABSTRACT

Propamocarb (PM) is a widely used fungicide with property of affecting fatty acid and phospholipid biosynthesis in funguses. In this study, we explored its effects on mice gut microbiota and metabolism by exposing mice to 3, 30, and 300 mg/L PM through drinking water for a duration of 28 days. We observed that the transcription of hepatic genes related to regulate lipid metabolism were perturbed by PM exposure. The microbiota in the cecal contents and feces changed during or after PM exposure at phylum or genus levels. 16S rRNA gene sequencing for the cecal content revealed shifted in overall microbial structure after PM exposure, and operational taxonomic unit (OTU) analysis indicated that 32.2% of OTUs changed by 300 mg/mL PM exposure for 28 days. In addition, based on ^{1}H NMR analysis,a total of 20 fecal metabolites mainly including succinate, short chain fatty acids, bile acids and trimethylamine were found to be significantly influenced by exposure to 300 mg/L PM.,. These metabolites were tightly correlated to host metabolism. Our findings indicated that high doses of PM exposure could disturb mice metabolism through, or partly through, altering the gut microbiota and microbial metabolites.

1. Introduction

A great number of microorganisms, including thousands of species of bacteria, fungi, protozoa and viruses, coexist with animals and humans, residing in their guts (Clemente et al., 2012). More than 3.3 million genes are encoded by the microbiota, which is about 150 times greater than the number of genes that are encoded in the human genome (Qin et al., 2010). The functions of the microbiota are critical to the host and include breaking down indigestible polysaccharides (fiber), synthesizing essential vitamins and amino acids, promoting neurodevelopment, and modulating the immune system (Clemente et al., 2012; Round and Mazmanian, 2010; Spanogiannopoulos et al., 2016). One of the most important functions of the gut microbiota is that, through their metabolites (such as short chain fatty acids and bile acids) and their ability to modulate the immune system (such as regulation of the TLR4 pathway) (Sonnenburg and Backhed, 2016; Wang et al., 2016), they can regulate the hosts' energy metabolism. Recent studies have revealed a relationship between the gut microbiota and metabolic diseases; they have implicated the gut microbiota as a trigger for metabolic diseases including obesity, glucose response disorders, and decreasing insulin sensitivity (Cho et al., 2012; Cox and Blaser, 2013). In support of this theory, it has been shown that the transplantation of an unhealthy guy microbiota can also transfer these phenotypes to the recipient host (Wang et al., 2016).

The gut microbiota is influenced by environmental factors present in our daily lives, such as diet, medicine and even temperature. For example, a change in diet resulting in an increase in both fat and cholesterol was shown to significantly alter the gut microbiota after just 24 h (David et al., 2014). Recently, a few environmental chemicals were also shown to be important factors that had an influence on the gut microbiota (Jin et al., 2017b). In fact, because of their widespread use, pesticide (and fungicide) residues have become a serious pollution problem. As reported, a lot of pesticide (and fungicide) residues were found in food materials, water, and soil; this resulted in a series of health problems in human and animals (Jin et al., 2015a; Liu et al., 2017; Liu et al., 2016). Some of the pesticides (and fungicides) were reported to have the ability to alter overall gut microbial structure, and these changes in microbiota were usually linked to several negative phenotypes (Xu et al., 2014; Jin et al., 2016a, 2017a; Joly Condette et al., 2015).

Propamocarb (PM) is a systemic carbamated fungicide used to control diseases caused by Oomycetes in soil, roots, and leaves. PM

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could affect the biosynthesis of fatty acid and phospholipid, thus change the membrane in funguses (Burden et al., 1988; Griffiths et al., 2003; Papavizas et al., 1978). PM residues may accumulate on the fruits to high levels, and people may subsequently ingest it when consuming the fruit (Wu, 2016). Previous reports found that PM is slightly cytotoxic through its interference with glycolysis and the construction of fibrillary proteins (Aydemir and Bilaloglu, 2004; Schmuck and Mihail, 2004). According to European Food Safety Authority, PM could induce vacuolar alterations in multiple organs, and it's relevant NOAEL (No Observed Adverse Effect Level) for long term toxicity in rats (1 year-study) was considered to be 29 mg/kg bw/day (EFSA, 2006). However, little is known about its effect on the gut microbiota and its subsequent effects in animals. In this study, we exposed mice to PM through their drinking water, and as a result, we found that this fungicide could alter overall gut bacterial composition, and drastically disturb the homeostasis of lipid metabolism of the host. The results acquired in the present study provide new information on PM induced mammalian toxicity.

2. Materials and methods

2.1. Reagents

Propamocarb (propyl -3-[dimethyl-amino] propyl carbamate, PM) was purchased from Aladdin (Product code: P115119, Lot number: H1514031, CAS: 24579-73-5, purity: 99%).

2.2. Animals

Five week-old male Institute of Cancer Research (ICR) mice (n = 32) were purchased from the China National Laboratory Animal Resource Center (Shanghai, China) and individually housed at 22 ± 1 °C on a 12 h: 12 h light:dark cycle (200 lux at cage level) for 1week prior to the onset of treatments. The mice were randomly divided into 4 groups (n = 8), and each mouse was harbored in one cage. Three groups of mice were orally administered 3 mg/L (PM 3), 30 mg/L (PM 30) or 300 mg/L (PM 300) PM in their drinking water for a duration of 4 weeks, each mouse in 4 groups consumed an average of 0, 0.5, 5, 50 mg/kg bw/day PM, respectively. In this study, the dosages were set according to the highest residue from the EU-MRLs (http://ec.europa.eu/food/plant/pesticides/) and the NOAEL (20 mg/kg bw/day) for long term toxicity (EFSA, 2006). The control group (PM 0) was treated with deionized water. Water and food were available *ad libitum* during the experiment. The feces from each mouse during PM exposure were regularly collected and stored at −80 °C until further use.

At the end of the experiment, all mice were fasted for at least 8 h and anesthetized by ether before being sacrificed. The serum, liver, colon, cecal content were collected and immediately stored at −80 °C. All experiments were performed in accordance with the Guiding Principles in the Use of Animals in Zhejiang University of Technology, and every effort was made to minimize animal suffering.

2.3. Histopathological analysis of the liver and colon

The liver and colon of three mice from each group were randomly selected for histopathological analysis (Ruifrok and Johnston, 2001). Briefly, the liver and colon were cut into 5 mm-thick sections, embedded in paraffin, fixed in 10% (v/v) formaldehyde, and then stained with hematoxylin & eosin (H&E).

2.4. Determination of serum, hepatic and colonic parameters

The hepatic TG, bile acids (BAs) and pyruvate levels were quantified using kits purchased from the Nanjing Jiancheng Institute of Biotechnology (Product code: A110-1, E003-2, A081, respectively, Nanjing, China) according to the manufacturer's instructions.

For hepatic TG, the TG were extracted by first homogenizing the liver in three times its volume of methanol, then adding six volumes of chloroform and incubating for 16 h at room temperature. The chloroform layer was collected after separation by centrifugation at 3000 rpm for 10 min.

For hepatic BA, pyruvate and colonic lipoprotein lipase (LPL), the liver and colon were homogenized in nine times their volume of PBS, centrifuged at 2000 rpm for 10 min, and then the supernatant was collected and used in the assay.

Colonic LPL levels were determined by ELISA (Product code: ML001947, MLBIO, Shanghai) following the manufacturer's instructions.

2.5. RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from the liver and the colon using the TRIzol reagent (Lot number: AKA2701, CAS: 108-95-2, Takara Biochemicals, China) cDNA was synthesized using a reverse transcriptase kit (Product code: FSQ-101, Toyobo, Japan) according to the manufacturers' instructions. RT-qPCR was performed using the SYBR Green system (Product code: QPK-201, Toyobo, Japan) in an Eppendorf MasterCycler ep RealPlex^2 (Wesseling-Berzdorf, Germany), and gene transcription was normalized to the level of 18S rRNA transcript in each sample. Primer sequences are shown in Table S1 (Jin et al., 2015a). The following PCR protocol was used: denaturation for 1 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The PCR and quantification of relative gene expression were performed as $2^{-\triangle\triangle}$ (Jin et al., 2015b; Livak and Schmittgen, 2001).

2.6. Microbial DNA extraction and structural analysis

Microbial genomic DNA (gDNA) was extracted from freeze-dried cecal contents and fecal samples using a commercial magnetic bead DNA isolation kit provided by Hangzhou Foreal Nanotechnology (Hangzhou, China), following the manufacturer's instructions, and then quantified using UV spectroscopy. The V3-V4 region of the 16S rRNA gene was PCR-amplified from microbial genome DNA using the following primers (forward primer: 5′-ACTCCTACGGGAGGCAGCAG-3'; reverse primer: 5′-GGACTACHVGGGTWTCTAAT-3′). The amplicons were normalized, pooled and sequenced on the Illumina Miseq deskstop sequencer (2 × 300 bp pairedend run). Sequence reads processing was performed using QIIME (version 1.9.0) and included additional quality trimming, demultiplexing (Caporaso et al., 2012). Operational taxonomic unit(OTU) picking using Vsearch v1.11.1, included dereplication, cluster, detectection of chimeras. Taxonomic assignment of individual datasets using the Greengene 13.8. followed by QIIME (vision 1.6.0) bioinformatic analysis. Alpha diversity was calculated with qiime, including index of observed species, chao1, shannnon, simpson, PD_whole_tree. Beta diversity was performed using Qiime with the matrix of Bray-Curtis distances.

In addition, a portion of the microbial genome DNA was amplified by a RT-qPCR using bacterial phyla specific primers (Table S1) under the following cycling conditions: 50 °C for 2 min; 95 °C for 10 min; 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 1 min, repeated for 40 cycles; and 72 °C for 10 min (Engevik et al., 2013).

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