Fish & Shellfish Immunology 39 (2014) 215–222

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

GC/MS-based metabolomics approach to identify biomarkers differentiating survivals from death in crucian carps infected by *Edwardsiella tarda*

Chang Guo [a,1], Xiao-yan Huang [a,1], Man-jun Yang [a,b], Sheng Wang [a], Shi-tong Ren [a], Hui Li [a], Xuan-xian Peng [a,*]

[a] Center for Proteomics and Metabolomics, State Key Laboratory of Bio-Control, MOE Key Laboratory of Aquatic Food Safety, School of Life Sciences, Sun Yat-sen University, University City, Guangzhou 510006, People's Republic of China
[b] Tibet Vocational Technical College, Lhasha 850000, People's Republic of China

ARTICLE INFO

Article history:
Received 26 December 2013
Received in revised form
5 April 2014
Accepted 23 April 2014
Available online 14 May 2014

Keywords:
Palmitic acid
D-mannose
Metabolomics
Crucian carp
Biomarker

ABSTRACT

Microbial disease problems constitute the largest single cause of economic losses in aquaculture. An understanding of immune system in aquaculture animals how to function in defense against bacterial infections is especially important to control these diseases and improve food quality and safety. In the present study, we use a crucian carp model to explore which pathways and metabolites are crucial for the defense against infection caused by *Edwardsiella tarda* EIB202. We establish the metabolic profile of crucian carps and then compare the metabolic difference between survivals and dead fish by self-control. We identify elevating unsaturated fatty acid biosynthesis and decreasing fructose and mannose metabolism as the most key pathways and increasing palmitic acid and decreasing D-mannose as the most crucial metabolites differentiating survivals from death in these fish infected by *E. tarda*. Our findings highlight the importance of metabolic strategy against bacterial infections.

1. Introduction

Recently, aquaculture production has become one of the fastest-growing animal food- producing industries and supplied approximately one half of the fish and shellfish that is directly consumed by humans [1]. However, microbial disease problems constitute the largest single cause of economic losses in aquaculture [2]. Immunoprophylaxis has been an effective measure to maintain a sustainable aquaculture [3–5], but current vaccines do not cope with all of pathogens. Alternatively, using antibiotics to prevent and control bacterial infections may result in a hazardous effect on human health and the environment, which has become a global problem and affected food quality and safety [6]. In these regards, an understanding of immune system in aquaculture animals how to function in defense against bacterial infections is especially important to control these diseases and improve food quality and safety [7,8].

Genomics, transcriptomics and proteomics are now expected to facilitate the study of interactional response between bacterial pathogens and fish hosts. For example, microarray-based studies have renewed the knowledge of fish pattern recognition receptors (PRRs) [9]. RNA SEQ has enriched the present collection of expressed sequence tag sequences including rare transcripts like leukocyte immune-type receptors, cullin, or supervillin and showed the efficacy of oral vaccination against bacterial infection [10]. Proteomics have characterized host–microbe interaction, hosts and pathogens to environmental stresses [11,12]. Very recently, metabolomics as a mutually complementary technique with genomics, transcriptomics and proteomics has been used to investigate fish metabolic response to heavy oil, anoxia and hypoxia [13–15]. However, fish metabolic strategy for bacterial infections is largely unknown.

Metabolomics characterizes small-molecule metabolite profiles in a biological cell, tissue, organ or organism and describes chemical processes involved these metabolites [16]. While transcriptomics and proteomics data do not tell the whole story of what

* Corresponding author. Fax: +86 20 8403 6215.
E-mail addresses: pxuanx@sysu.edu.cn, wangpeng@xmu.edu.cn (X.-x. Peng).
[1] The first two authors contributed equally.

http://dx.doi.org/10.1016/j.fsi.2014.04.017
1050-4648/

might be happening in a cell, metabolic profiling can give an instantaneous snapshot of the physiology of that cell [17]. Gas chromatography/mass spectrometry (GC–MS), liquid chromatography-mass spectrometry (LC–MS) and nuclear magnetic resonance (NMR) are three most used analytical technologies in metabolomics investigation [18]. Each of these technologies has its own unique advantages and disadvantages. Out of them, GC–MS is ideally suited for the analyses of volatile compounds and thus is widely used [19,20].

In the present study, GC–MS based metabolomics carrying the entire metabolite repertoire from plasma of crucian carps has been developed to analyze changes in metabolites induced upon infection with *Edwardsiella tarda* EIB202. We wanted to exploit the metabolome data to identify, among the differential metabolites, crucial metabolites and key pathways as biomarkers capable of differentiating survivals from death in the crucian carps infected with the bacterial pathogen. To aim at this, we put forward to the hypothesis that half animals survive and the others die might be related to differential metabolomes between them when these animals are infected by median lethal dose (LD50) of bacterial cells. The most key metabolic pathway and the crucial metabolite in the pathway may be identified from the differential metabolomes. For guaranteeing the accuracy of our results, our identification of the most key metabolic pathway and crucial metabolite as the biomarkers is obtained by self-control. Our findings highlight the importance of metabolism against bacterial infections.

2. Materials and methods

2.1. Animals and treatments

Single colony of *E. tarda* EIB202 was propagated in tryptic soy-broth (TSB) medium at 30 °C for 24 h. The culture was separately diluted into 1:100 using fresh TSB medium and grown at 30 °C. The bacterial cells were harvested at 1.0 of OD_{600} by centrifugation at 6000 g for 5 min at 4 °C. The resulting cells were washed with saline solution for three times and resuspended in saline solution. Crucian carps (*Carassius auratus*) weighting 180 ± 10 g were purchased from the local market, Guangzhou, People's Republic of China and were free of *E. tarda* infection through microbiological detection. They were kept in the aquaria with a water recirculation system and were acclimated to the animal facility. The tank was stocked with one fish per tank and each tank was coded. These fish were fed a commercial dry pellet diet twice daily at about 1% of body weight each time. Followed by the acclimation of seven days, blood of 0.1 mL was drawn from tail venous sinuses each animal before the first feed and mixed with 1% sodium heparin. The resulting plasma was isolated by centrifugation at 2000 g and 4 °C for 10 min as pre-infection samples. Then fishes were infected with 400 μL 5×10^6 CFU/mL (LD50 dose) by intraperitoneal injection. Blood of 0.1 mL was drawn from tail venous sinuses each animal at 24 h post the infection and treated as the same as described above. The resulting samples were used as in-infection group. The in-infection group was further divided into dead and survival subgroups according to the result whether these fish were survival or dead after twelve days observation following the infection.

2.2. Sample preparation for GC–MS

Sample preparation was performed as described previously [21,22]. In brief, 50 μL plasma was quenched using 50 μL cold methanol and collected by centrifugation at 6000 g for 3 min. This step was repeated two times. 3 μL ribitol (3 mg/mL) was added as an analytical internal standard. The supernatant was transferred into a new 1.5 mL centrifuge tube and then dried in a rotary vacuum centrifuge device (LABCONCO) for 4 h. The resulting samples were used for GC/MS analysis.

2.3. GC–MS analysis

GC–MS analysis was carried out with a variation on the two-stage steps as described previously [23]. In brief, firstly protect carbonyl moieties of samples were carried out through a 2 h 37 °C reaction with 80 μL of 120 mg/mL methoxyamine hydrochloride in pyridine, followed by derivatization of acidic protons through a 1 h 37 °C reaction with 80 μL N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA). The samples were centrifuged for 7 min at 8000 g at 4 °C. Chemical analysis of samples was carried out by DSQ II Single Quadrupole GC/MS (Thermo Scientific). The injection port was maintained at 270 °C. The derivatized sample of 0.5 μL was injected into a DBS (Dodecyl Benzene Sulfonic Acid) column (30 m length × 250 μm i.d. × 0.25 μm thickness) using splitless model and interface temperature is 270 °C. The MS source temperature was maintained at 230 °C and the MS quadrupole temperature was held constant at 150 °C. Electron impact ionization (EI) mode was selected and ionization energy was 70 eV. For full-scan and selected ion recording (SIR) experiments, the initial temperature of the GC oven was programmed at 70 °C for 5 min followed by an increase to 270 °C at a rate of 2 °C/min and then held for 5 min. Helium was used as carrier gas. The flow was kept constant at 1 mL/min. The MS was operated in a range of 60–600 m/z.

2.4. GC–MS data processing and normalization

Peaks of total ion chromatograms (TIC) were identified by searching in National Institute of Standards and Technology (NIST) library (2005 edition) used the NIST MS search 2.0. Then, search for structural candidates and their associated match factors in databases [24]. Rank and match factor (score) were used to evaluate the hits. Scores ranged from 0 to 999. 0 showed spectra with no peaks, while 999 indicated a perfect match. Scores between 999 and 600 were acceptable [25]. Corrected hits were ranked first with a score bigger than a predefined threshold (600). We extracted the area of each compound peak from the TIC with XCalibur software (Thermo fisher, version 2.1). Signal intensity or relative abundance of peaks which indicated the number of ion has no unit [26]. Single ion was used to deconvolute overlapping peaks [27]. The compounds in each sample were matched with retention time and mass spectrum. Silylation reagent was removed from the resulting data matrix. Total 83 compound peaks with the same metabolite name were merged, which resulted in identification of 67 metabolites. Finally, the data matrix was normalized using internal control and the total peak area. Normalization factor for internal control was ratio between average ribitol intensity and individual ribitol intensity. The ion peak of ribitol was removed. In total area normalization, total peak area in a spectrum is scaled to average peak area of all spectra tested. Data normalization was completed in the excel 2010.

2.5. Statistical analysis and multivariate data analysis

Statistical analysis was performed in the SPSS 13.0. The difference among metabolites was conducted with Mann–Whitney test (Wilcoxon rank sum test) and Kruskal–Wallis (KW) test. A 2-sided P value <0.05 was considered statistically significant. Both tests were non-parametric test which assumed the data was not normal distribution. Mann–Whitney U test judged the significance by the way of comparing the two samples' rank. False discovery rates (FDR) which indicated the proportion of true null hypotheses in the research were determined from q-value. The quantity π was