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Liver functional metabolomics discloses an action of L-leucine against *Streptococcus iniae* infection in tilapias

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ABSTRACT

Streptococcus iniae seriously affects the intensive farming of tilapias. Much work has been conducted on prevention and control of *S. iniae* infection, but little published information on the metabolic response is available in tilapias against the bacterial infection, and no metabolic modulation way may be adopted to control this disease. The present study used GC/MS based metabolomics to characterize the metabolic profiling of tilapias infected by a lethal dose (LD50) of *S. iniae* and determined two characteristic metabolomes separately responsible for the survival and dying fishes. A reversal changed metabolite, decreased and increased L-leucine in the dying and survival groups, respectively, was identified as a biomarker which featured the difference between the two metabolomes. More importantly, exogenous L-leucine could be used as a metabolic modulator to elevate survival ability of tilapias infected by *S. iniae*. These results indicate that tilapias mount metabolic strategies to deal with bacterial infection, which can be regulated by exogenous metabolites such as L-leucine. The present study establishes an alternative way, metabolic modulation, to cope with bacterial infections.

1. Introduction

Streptococcus iniae is a species of Gram-positive, sphere-shaped bacterium. Though originally isolated from Amazon freshwater dolphin, *S. iniae* has emerged as a leading fish pathogen in aquaculture operations worldwide with a broad host range of fresh and saltwater species such as tilapia, rainbow trout, salmon, barramundi, yellowtail, flounder, red drum and hybrid striped bass, resulting in great economic losses [1,2].

Streptococcal disease caused by *S. iniae* seriously affects the intensive farming of tilapias. *S. iniae* causes tilapia meningoencephalitis, with symptoms including lethargy, dorsal rigidity and erratic swimming behavior; death follows in a matter of days. *S. iniae* is highly lethal with 30–50 % mortality in outbreaks [2,3]. Much work has been conducted on prevention and control of *S. iniae* infection in farmed tilapias [4,5], but little or no published information is available on the metabolic response of tilapias to *S. iniae* infection, and no efficient way may be adopted to control this disease through metabolic modulation.

Recently developed metablomics gives a holistic view of the metabolism of the system studied and creates opportunities for studying metabolic pathways and metabolites [6]. Very recently, we have used gas chromatography-mass spectrometry (GC/MS)-based metabolomics approach to identify biomarkers differentiating survivals from death in crucian carps infected by *Edwardsiella tarda*. 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 [7]. Furthermore, we develop a functional metabolomics approach to demonstrate that interferon-α2b combats microbes through promoting biosynthesis of unsaturated fatty acids [8] and *N*-acetylglucosamine enhances survival ability of tilapias infected by *S. iniae* cultured at 22 °C [9]. Besides the functional metabolomics approach to modulate metabolism of hosts, we also establish a novel functional-metabolomics-based strategy to manage infection by antibiotic-resistant bacteria through metabolite-potentiating antibiotic efficacy [10]. These

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results indicate that metabolome is a crucial link between genotype and phenotype, reflecting a metabolic strategy against microbial infections. An understanding of this metabolic strategy will highlight an alternative way to cope with the microbial infections.

Here we showed varied metabolomics responsible for the dying or survival in tilapias infected by an LD50 of *S. iniae* at 30 °C in comparison with no bacterial infection. We characterized the two metabolomes using pattern recognition analyses and identified valine, leucine and isoleucine metabolism and L-leucine as the most key pathway and the most crucial metabolite, respectively, in differentiating the dying from survival. Finally, we demonstrated the action of exogenous L-leucine as a metabolic modulator.

2. Materials and methods

2.1. Bacterial strain and fish

S. iniae, which was isolated from dying tilapias, was kindly provided by Professor Anxin Li, School of Life Sciences. Tilapias were bought from a local fish farm of Panyu, Guangzhou, with 5 cm ± 0.5 cm length and 1.8 g ± 0.2 g body weight and acclimated in stock tanks (80 × 75 × 90 cm) for 1 week. The fishes were fed with commercial pelleted feed twice a day.

2.2. Bacterial culture, infection and fish symptoms

S. iniae was cultured in Brain Heart Infusion Broth (BHI) at 30 °C for 36 h. The cultures were centrifuged and diluted using sterile saline until 1.0 of OD_{600}. LD_{50} dose was determined by intraperitoneal injection into tilapias. In brief, 10 fishes for each dose were challenged with 5-fold dilution series of doses ranging from 2×10^4 to 5×10^5 CFU/fish. The LD_{50} was determined as 4×10^4 CFU/fish. Some of the fishes started to show infectious symptoms after 40 h and died within 60 h. The remaining infected fishes survived without visible symptoms. The fish were anesthetized before intramuscular injection as described previously [11]. The experimental fish infections in the project were approved by Sun Yat-sen University.

2.3. Collection of tilapia liver samples

After seven days of acclimation, tilapias were randomly divided into control and two test groups, 20, 30 and 30 fish, respectively. While the control group was injected with sterile saline, the two test groups were intraperitoneal infected with 4×10^4 CFU/fish separately for preparation of GC/MS sample (test group 1) or investigation of survival rate (test group II) for validation of the LD50 dose of bacterial cells used. Fishes with and without infectious symptoms were separately collected at 48 h in test group 1, designed as the dying and survival groups, ten for each group. Meanwhile, ten fishes were randomly collected from control. Livers were isolated from these animals and used for GC/MS analysis.

2.4. Sample preparation for GC–MS

Sample preparation was performed as described previously [9,12]. In brief, appropriate volume of cold methanol was added into the liver samples above according to the weight (800 μL/100 mg). After centrifugation at 120,000× *g*, 4 °C, 50 μL supernatant was collected. The supernatant containing 10 μL 0.2 mg/mL ribitol (Sigma) as an analytical internal standard was concentrated in a rotary vacuum centrifuge device (LABCONCO). The dried extracts were used for GC/MS analysis.

2.5. GC/MS analysis

GC–MS analysis was performed with a variation on the two-stage technique as described previously [9,12]. In brief, bacterial samples were derivatized and used to firstly protect carbonyl moieties through methoximation, through a 90 min, 37 °C reaction with 80 μL of 20 mg/mL methoxyamine hydrochloride (Sigma–Aldrich) in pyridine, followed by derivatization of acidic protons through a 30 min 37 °C reaction with the addition of 80 μL N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA, Sigma–Aldrich). The resulting derivatized sample of 1 μL was injected into a 30 m × 250 μm i.d. × 0.25 μm DBS-MS column using splitless injection and analysis was carried out by Trace DSQ II (Thermo Scientific). The initial temperature of the GC oven was held at 85 °C for 5 min followed by an increase to 270 °C at a rate of 15 °C min^{-1} then held for 5 min. Electron impact ionization (EI) mode was selected and ionization energy was 70 eV. Helium was used as carrier gas and flow was kept constant at 1 mL min^{-1}. The MS was operated in a range of 50–600 *m/z*. Two technical replicates were prepared for each sample.

2.6. Data processing for GC/MS

2.6.1. Data processing

Data processing was carried out as described previously [9,12]. Spectral deconvolution and calibration were performed by using AMDIS and internal standards. A retention time (RT) correction was performed for all the samples, and the RT was used as reference against which the remaining spectra were queried. A file containing the abundance information for each metabolite in all the samples was assembled. Metabolites from the GC–MS spectra were identified by searching against National Institute of Standards and Technology (NIST 08) Mass Spectral Library. Among the detected peaks of all the chromatograms, 187 peaks were considered as endogenous metabolites excluded internal standard ribitol. The resulting data matrix was normalized by the concentrations of added internal standards and the total intensity. Normalized peak intensities form a single matrix with Rt-m/z pairs for each file in the dataset. This file was then used for subsequent statistical analyses.

2.6.2. Statistical analyses

Metabolites subtracted the median metabolites and were scaled by the quartile range in the sample. Z-score analysis scaled each metabolite according to a reference distribution [13], and was calculated based on the mean and standard deviation of reference. Hierarchical Clustering was completed in the R platform with the package gplots (http://cran.r-project.org/src/contrib/Descriptions/gplots.html) using the distance matrix.

2.6.3. Pattern recognition

Multivariate statistical analysis included principal component analysis (PCA) (SIMCA-P + 12.0.1), which was used to discriminate sample patterns, to identify the metabolites associated with infection and to minimize the inter-individual variation's influence. SPSS 13.0 and Prism v5.01 (GraphPad, La Jolla, CA, USA) were used to draw the histogram the scatter plot.

2.7. Exogenous addition of L-leucine and bacterial challenge

Fishes were randomly divided into control, test I, test II and test III groups, 20 fishes each group. Control fishes were intraperitoneally injected with 30 μL sterile saline. L-leucine group fishes in test I, test II and test III were injected with three concentrations of 0.15 mg, 0.3 mg and 0.6 mg L-leucine which was dissolved in 30 μL of sterile saline, once daily for 3 days. For oral administration, 120

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