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Phenylalanine enhances innate immune response to clear ceftazidime-resistant *Vibrio alginolyticus* in *Danio rerio*

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ABSTRACT

Antibiotic-resistant bacteria becomes a major threat to the economy and food safety in aquaculture. Although the antibiotic-dependent strategy is still the mostly adopted option, the development of antibiotic-free approach is urgently needed to ameliorate the severe situation of the global antibiotic resistance. In the present study, we showed that modulating the metabolism of zebrafish, *Danio reiro*, would enhance *D. rerio* to clear ceftazidime-resistant *Vibrio alginoyticus* (Caz-R) *in vivo*. By generating Caz-R *in vitro*, we found Caz-R stays longer than ceftazidime-sensitive *V. alginoyticus* (Caz-S) in *D. rerio,* where Caz-R induced less potent immune response than that of Caz-S. The differential immune response was associated with different metabolism of the host. Through functional metabolomics, we identified a crucial biomarker, phenylalanine. The abundance of phenylalanine was increased in both of Caz-S and Caz-R infected hosts but the abundance was higher in Caz-S infected group. This specific difference indicated phenylalanine could be a metabolite required to clear Caz-R by the host. Exogenous phenylalanine would enhance the host's ability to remove Caz-R, which was through upregulated production of lysozyme and C3b. Thus, our study demonstrates a novel strategy to boost host's immune response to combat against antibiotic-resistant bacteria.

1. Introduction

The wide spread of antibiotic-resistant bacteria became a severe threat to human health, aquaculture and poultry industry. In aquaculture, the treatment of bacterial infections with antibiotics that is sensitive to the infection-causing agents is still the most favorable strategy. It actually complicates the current situation by bringing antibiotic-resistant bacteria with pan- and stronger-resistance, imposing higher degree of threat to human being and food safety. Vaccination of the aquaculture animals with live or inactivated bacteria represent another strategy to prevent infections. As compared to antibiotic-dependent approach, vaccination shows higher protection efficiency and environment-free. But pipeline for the development of effective vaccines is always time- and labor-consuming. More importantly, bacterial vaccines are very specific to certain serotypes [1], and the development of polyvalent vaccines is still lagged behind expectation [2–4]. Thus, the development of alternative strategy is highly demanded to control or prevent infections by antibiotic-resistant bacteria in aquaculture.

Boosting host's immune response to pathogens is an attractive strategy. But the host respond differently to antibiotic-sensitive and -resistant bacteria. Previous study has shown that macrophage less efficiently to kill rifampin- and streptomycin-double mutations in *E. coli* than susceptible strains [5,6]. More importantly, the gene expression pattern of macrophage to susceptible strain, single mutation strains and double mutations strains are different, indicating that antibiotic-resistant bacteria induced differential immune response to the susceptible strain, and implying that the host may have different strategies to cope with susceptible- and resistant-bacteria [6]. Thus, the elucidation of host immune response to antibiotic-resistant bacteria is of prior importance in developing novel strategies.

Recently, we developed a platform that combine the use of functional metablomics to identify crucial biomarkers and use the

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biomarkers for metabolic reprogramming, which we termed reprogramming metabolomics [7]. Through functional metabolomics, we identified glucose, alanine and fructose as the crucial biomarkers that distinguished the kanamycin-resistant and kanamycin–sensitive bacteria. Exogenous glucose/alanine to kanamycin-resistant bacteria reprogrammed the metabolome, which restored their sensitivity to kanamycin [8,9]. Similarly, crucial metabolites identified from hosts may also reprogram the hosts' metabolome to eliminate bacterial pathogens, though different bacteria require different metabolite [10–15]. In the present study, we specifically address the immune response of zebrafish, a model organism for bacterial infection study, to *Vibrio alginolyticus* (Caz-S), a severe pathogen that infects both human and fish, and its ceftazidime-resistant strain (Caz-R) [16]. These two strains differ in their physiology, virulence to the host, and induces differential immune response. We further demonstrate that the differential immune response is associated with different host metabolism. And identification of crucial metabolic biomarkers allows the enhancement of host to clear Caz-resistant bacteria in synergy with Caz. Thus, our study highlights the importance of utilizing metabolism to kill antibiotic-resistant bacteria *in vivo*.

2. Materials and methods

2.1. Bacterial strain

The bacterial strain used in this experiment is *V. alginolyticus* 12G01, a collection of our laboratory. To generate ceftazidime-resistant *V. alginolyticus*, *V. alginolyticus* was subcultured in LB medium with 1/2 minimum inhibitory concentration (MIC) of ceftazidime (0.1 μg/ml) for 25 passages, which led to the elevated 16 MICs and unchanged MIC, respectively, namely ceftazidime-resistant *V. alginolyticus* (Caz-R) and ceftazidime-sensitive bacteria (Caz-S). MIC was determined by antimicrobial susceptibility testing by allowing the bacteria to grow until it reached an optical density of 0.5 (OD600 nm) at 30 °C as described previously [16,17]. The log phase cells were diluted 1:1000 into each well of a 96-well microtiter polystyrene tray. The tray contained a series of 2-fold dilutions of antibiotics. After 24 h of incubation, the MIC was defined as the lowest concentration that inhibited visible growth. Three biological repeats were carried out for each sample.

2.2. Motility test

The motility test was carried out as described previously [18]. Caz-S and Caz-R were inoculated into 5 mL of LB and incubated at 30 °C overnight, and then 5 μL of each sample was spotted onto the LB agar plates with 1.5% agar (Sangon Biotech, Shanghai, China) for 16 h of incubation. Swimming mobility was evaluated by measurement of the diameter of the halo. For every result, the value of mobility was determined over a minimum of three independent measurements.

2.3. Fish

Zebrafish (0.22 ± 0.03g body weight), were obtained from a zebrafish breeding Corporation (Guangzhou, P.R. China). These animals were free of *Vibrio species* infection through microbiological detection and reared in 25 L open circuit filtered water tanks at room temperature with aeration. They were maintained in our laboratory for two weeks before experimental manipulation and were fed twice daily with commercial blood worm on a 12 h/12 h rhythm of light and darkness photoperiod always.

2.4. Ethics statement

This study was conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and maintained according to the standard protocols (http://ZFIN.org). All experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University (Animal welfare Assurance Number:16).

2.5. Sample preparation for GC-MS analysis

Sample preparation was carried out as described previously [12–14]. *D. rerio,* around 60 days post-fertilization, 0.22 ± 0.04g in weight and 2.5 ± 0.2 cm in length, were euthanized on in ice slush (5 parts ice/1 part water, 0–4 °C) for at least 10 min following cessation of gill movement, or left in the ice water for at least 20 min after cessation of all movement to ensure death by hypoxia following the guidelines of NIH, which has also been approved by Institutional Animal Care and Use Committee of Sun Yat-sen University. *D. rerio* were rinsed with distilled water and then wiped thoroughly with sterilized gauze. Each individual fish was cut into five pieces on ice and then weighted. The appropriate volume of saline (100 μL/100 mg) was added according to the weight. After centrifugation at 3,000 g at 4 °C, 100 μL fluid was aliquoted for the following metabolomic analysis. Metabolites were extracted with 0.2 mL of cold methanol, containing 10 μL of 0.1 mg/mL ribitol, Sigma Aldrich, as an analytical internal standard. After centrifugation at 12,000g for 10 min, the supernatant was concentrated in a rotary vacuum centrifuge device, LABCONCO. The dried polar extracts were used for GC-MS analysis.

2.6. GC-MS analysis

GC-MS analysis was carried out with minor modification on the two stage techniques as described previously[7]. In brief, samples were derivatized in 40 μL of 20 mg/mL methoxyamine hydrochloride, Sigma Aldrich, in pyridine to protect carbonyl moieties through methoximation for 90 min at 37 °C, followed by adding 80 μL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) for derivatization of acidic protons, Sigma Aldrich, at 37 °C for another 30 min. The 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 in Agilent 7890A GC equipped with an Agilent 5975C VL MSD detector, Agilent Technologies. 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. Helium was used as carrier gas and flow was kept constantly at 1 mL min^{-1}. The MS was operated in a range of 50–600 *m/z*. For each sample, two technical replicates were prepared to confirm the reproducibility of the reported procedures.

2.7. Exogenous addition of phenylalanine and bacterial challenge

Sixty zebrafish were randomly divided into control and test groups and acclimatized for 14 days at 28 °C. after purchase from the fish corporation. *D. rerio* were anesthetized by immersing in 0.02% tricaine methanesulfonate solution, which was subsequently injected with 125 μg phenylalanine as test groups, and treated with the same volume of sterile saline as control. Both two groups were injected once daily for 3 days. After the last administration, these zebrafishes were challenged by intramuscular injection of 1.6×10^6 CFU of Caz-R cells/fish. These zebrafish were observed twice daily for 15 days for accumulative death.

2.8. Sample preparation of zebrafish in the presence or absence of exogenous phenylalanine

Bacterial were grown in 100 mL LB medium at 30 °C overnight and then diluted 1:100 into fresh LB medium until its absorbance value of OD600 equal to 0.2. Then the cells were washed by saline for three times and resuspended in saline. Zebrafish were divided to two groups with and without phenylalanine. The group with phenylalanine was injected with 125 μg phenylalanine as teste group and the group without phenylalanine was injected with the same volume of sterile

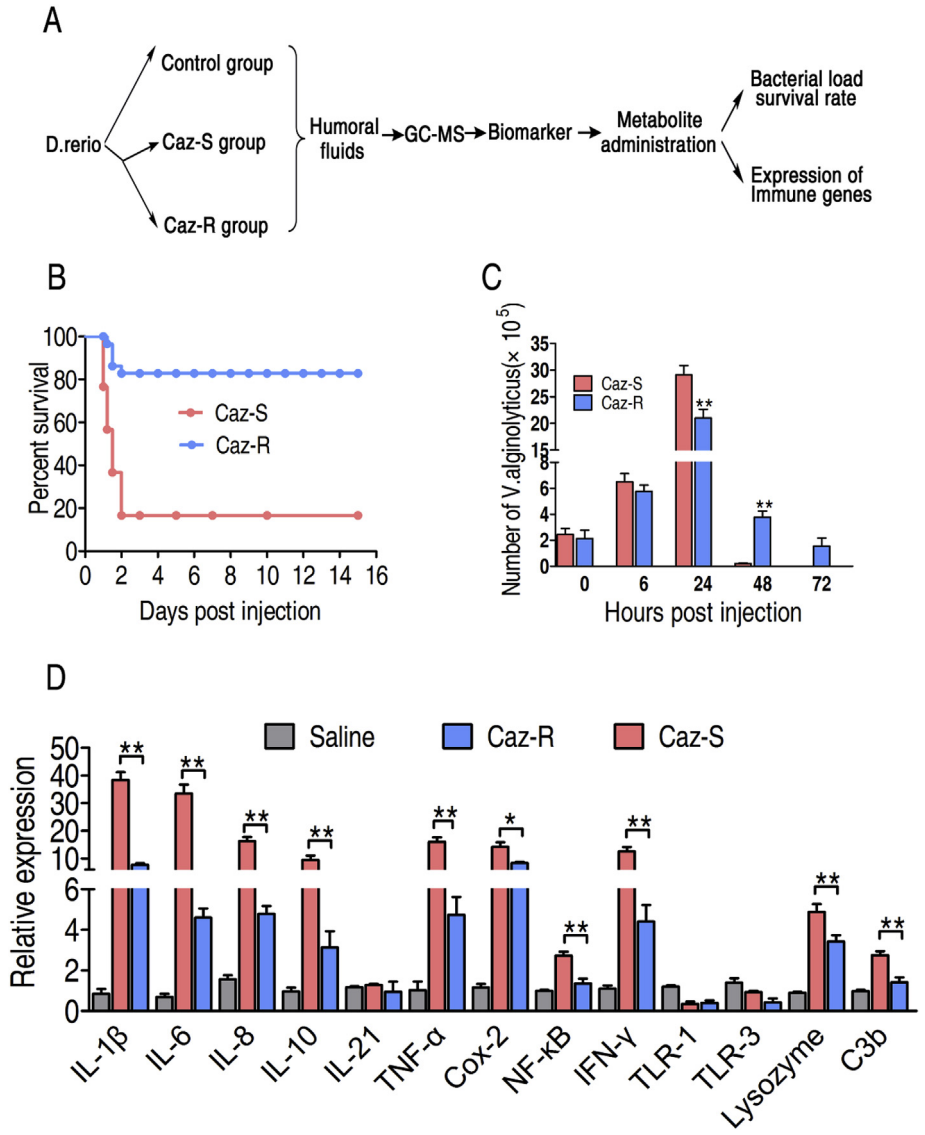

Fig. 1. Immune response of zebrafish to Caz-S and Caz-R. (A) Schematic representation of experimental flow. (B) Percent survival of *D. rerio* infected by Caz-S or Caz-R. Thirty zebrafish were infected with Caz-S or Caz-R at the dose of 1.2×10^6 bacteria The survival rates were injected with 30 were monitor for 16 days post-infection; (C) Persistence of Caz-S and Caz-R in *D. rerio* by calculating *gyrB* gene copy numbers; (D) QRT-PCR for quantifying transcriptional levels of innate immune genes in *D. rerio* infected by Caz-S or Caz-R.

saline as control group for three days. Samples were collected for measurement of lysozyme activity by commercial kit. Meanwhile, each of the other fish from the two groups was challenged 2×10^5 CFU (5 μL) of Caz-R by intramuscular injection. For qPCR detection of bacterial elimination, the fish at 0, 6, 24, 48, 72 h post injection were euthanized and stored at −80 °C until DNA extraction. Total DNA was extracted from the whole fish after removing the intestines using DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions with a minor modification. The samples were grounded with pestle in lysis buffer, and were incubated overnight. The purified genomic DNA was stored at −20 °C until qPCR amplification. The experiment was performed in six biological repetitions. For qRT-PCR detection of innate immune genes, zebrafish at 6 h post injection were anesthetized and spleens were pooled aseptically. All the spleens were stored at −80 °C until RNA extraction. Total RNA was isolated with Trizol (Invitrogen, USA), the RNA was then quantified by detecting the intensity of fluorescence. Reverse transcription-PCR was carried out on a Prime-Script™ RT reagent Kit with gDNA eraser (Takara, Japan) with 1 μg of total RNA according to manufacturer's instructions. The experiment was performed in six biological repetitions.

2.9. Quantitative PCR

Quantitative PCR (qPCR) was performed as described previously with modifications [19]. Gene *gyrB* was chosen as a target to quantify *V. alginolyticus* counts in this study. Primers of *gyrB* (Suppl. Table 1) were designed referring the sequence of *V. alginolyticus* published in GenBank. The specificity was tested primarily. Injected and non-injected fish were sampled. DNA extracted from each sample was used as templates for qPCR amplification. The resulting products were analyzed by agarose gel electrophoresis and were sequenced directly. All the samples were performed on LightCycle 480 system, Roche, Germany, according to the manufacturer's instructions. Reaction mixtures (10 μL) contained 5 μL SYBR® Select Master Mix, 1 μL DNA samples, 0.5 μL of each forward and reverse primers. The cycling parameters were listed as follows: 95 °C for 30 s to activate the polymerase; 40 cycles of 95 °C for 10 s; 60 °C for 30 s; Fluorescence measurements were performed at 70 °C for 1 s during each cycle. Cycling was terminated at 95 °C with a calefactive velocity of 5 °C per second and a melting curve was

obtained.

To establish the relationship between Ct values and *V. alginolyticus* counts, a ten-fold serial dilution series ranging from 10 to 10^7 numbers of cells of *V. alginolyticus* DNA were used to create the standard curve for quantification. The concentration of DNA was measured and converted to the initial template copy concentration using the following equation: DNA(copy) = 6.02×10^{23} (copies mol^{-1}) × DNA amount (g)/DNA length × 660 (g mol^{-1} bp^{-1}). Ct values were plotted against the logarithm of their copy concentration. Standard curve was generated by linear regression of the plotted points.

2.10. qRT-PCR

qRT-PCR was performed as described previously [20]. Primers for each gene were listed in Suppl. Table 1. Each primer pair was specific. The operation and reaction of qRT-PCR were referred to qPCR. The relative expression of each immune-related gene was determined by comparative threshold cycle method ($2^{-\Delta\Delta Ct}$ method) with β-actin as reference gene. To measure gene expression of immune genes, spleens from three *D. rerio* were pooled as one biological sample for RNA isolation, and six biological replicates were used for one gene.

2.11. Lysozyme assay

Lysozyme assay Kit was purchased from the Nanjing Jiancheng Bioengineering Institute, China. The activity of lysozyme in zebrafish body fluid and captured by Caz-S and Caz-R was determined according to the method based on the lysis of the lysozyme sensitive Gram-positive bacterium *Micrococcus ktsideukticus*. Shortly, 2 mL of *M. ktsideukticus* at a concentration of 0.2 mg/ml (w/v) in 50 mM phosphate buffer (pH = 6.2) and 100 μL body fluid or reaction mixture was added. The reduction in absorbance at 530 nm was measured after 15 min water bath at 37 °C. Results were expressed in units of lysozyme mL^{-1}. One unit in defined as the amount of sample causing a decrease in absorbance of 0.001 units per min.

3. Result

The experimental design to identify metabolite that could enhances *Danio rerio* to eliminate antibiotic-resistant bacteria is shown in Fig. 1A.

3.1. Ceftazidime-sensitive Vibrio alginolyticus (Caz-S) and ceftazidime-resistant V. alginolyticus (Caz-R) induces differential immune response in zebrafish Danio rerio

To explore the differential host response to antibiotic -sensitive and -resistant bacteria, we generated the ceftazidime-resistant *V. alginolyticus* (Caz-R), whose MIC is 16 times higher than the parental strain, Caz-S (Suppl. Fig. 1A). Although Caz-S and Caz-R has similar growth rate in rich medium (Suppl. Fig. 1B), they grew differently when grown in minimal medium supplemented with different type of carbohydrates including fructose, galactose, lactose, mannose, sucrose and glucose (Suppl. Fig. 1C). And the swarming ability of Caz-R is decreased about 20% (Suppl. Fig. 1D). These results suggested that Caz-R has altered physiology which might induces differential immune response to the host, *D. rerio*.

The pathogenesis of Caz-S and Caz-R was evaluated in zebrafish, *Danio rerio*, infection model. Thirty zebrafish was infected with either Caz-S or Caz-R at the same dose of 1.2×10^6 cells/fish. Caz-S kills more than 80% of the fish two days post-infection, but Caz-R kills only 20% even 15 days post-infection (Fig. 1B). To further monitor the persistence of bacteria during infection, we used *gyrB* gene copy number to quantitate the bacteria that are persistent in the host. Both of Caz-S and Caz-R reaches the climax at 24 h post-infection. Although the number of Caz-S is higher than that of Caz-R at 24 h, Caz-S is barely detected at 48 h. Whereas Caz-R is still detectable 72 h post-infection, implying *D. rerio* is less efficient to remove Caz-R than to remove Caz-S (Fig. 1C). Furthermore, the immune response of *D. rerio* to Caz-S and Caz-R are also different. We quantitatively examined 13 immune molecules including IL-1β, IL-6, IL-8, IL-10, IL-21, TNF-α, Cox-2, NF-κB, IFN-γ, TLR-1, TLR-3, lysozyme and C3b. The challenge of *D. rerio* with Caz-S and Caz-R induced strong immune response as demonstrated by the up-regulation of proinflammatory cytokines of IL-1β, IL-6, IL-8, IL-10, TNF-α, IFN-γ, and molecules of innate immunity, lysozyme and C3b (Fig. 1D). These data suggested that *D. rerio* mount differential immune response to cope with Caz-S and Caz-R.

3.2. Metabolomic analysis of D. rerio humoral fluids associated with immune response to Caz-S and Caz-R

Metabolism plays pivotal role in regulating immune response [21]. To explore the metabolic mechanism in contributing differential immune response, GC-MS based metabolomics was applied. *D. rerio* were infected with Caz-S or Caz-R or saline only. Seventy-two hours post-infection, humoral fluid was collected from the survived *D. rerio*, and was prepared for subsequent GC-MS analysis. We prepared ten biological replicates for each group (saline, Caz-S infected group or Caz-R infected group), where each biological replicate had two technical replicates, thus yielding a total of 60 data sets for the three groups of samples. The metabolites that were differentially affected by bacterial infection were displayed in Fig. 2A. Compared to the saline group, both of the Caz-S-infected and Caz-R-infected groups had 60 metabolites with altered abundance, respectively. For a comparative study, Z-score plot based on the control showed that it spanned from −10 to 25 in the Caz-S group and from −15 to 300 in Caz-R group, respectively. Of the differential abundance of metabolites, the abundance of 32 metabolites were increased and 28 metabolites were decreased in the Caz-S group, and 35 metabolites were increased and 25 metabolites were decreased for the Caz-R group, which were listed in Fig. 2B. Principal component analysis (PCA) was carried out for the multivariate analysis (Fig. 2C), Obvious separations were detected between Caz-S and Caz-R as shown in the scores plot. No significant outlier was found in the score plot of all the samples. Component t [1] differentiated the Caz-R-induced metabolome from the control without bacterial infection, while component t [2] identified Caz-S group from the other two. Discriminating variables were present with S-plot when cut-off values were set as greater or equal to 0.05 and 0.5 for absolute value of covariance *p* and correlation p(corr), respectively (Suppl. Fig. 2A and 2B). Crucial biomarkers, including phosphoric acid, hypoxanthine,myo-inositol, leucine, taurine, glycine, inosine, glucose, valine, glycerol, phenylalanine, cadaverine, phenylalanine, pyroglutamic acid, lactose, mannose, malic acid, isoleucine, proline, tyrosine, tryptophan were screened by component p [1] and component p [2]. It is clear that the elevated phenylalanine was selected because it elevated in both component p [1] and component p [2], which is consistent with our purpose that elevated biomarkers differentiate from not only Caz-R group from Caz-S group.

In addition, the differential metabolites in abundance between Caz-S and Caz-R were further analyzed with pathway enrichment analysis. Thirteen metabolic pathways were enriched, where phenylalanine, tyrosine and tryptophan biosynthesis, valine, leucine and isoleucine biosynthesis and glycine, serine and threonine metabolism are the top three pathways that has the highest impacted pathway (Fig. 2D). Furthermore, the metabolites were put together and plotted on iPath, a versatile tool in visualizing enriched pathways (Suppl. Fig. 3). The pathway analysis indicated phenylalanine tyrosine the tryptophan analysis may play critical roles in removing Caz-R.

Indeed, higher phenylalanine was detected in Caz-S and Caz-R group than control, and also in Caz-S than Caz-R metabolome (Fig. 2E). These results taken together suggested that zebrafish mount differential metabolomic strategies to cope with the invading Caz-S and Caz-R, where phenylalanine is identified as the most crucial biomarker.

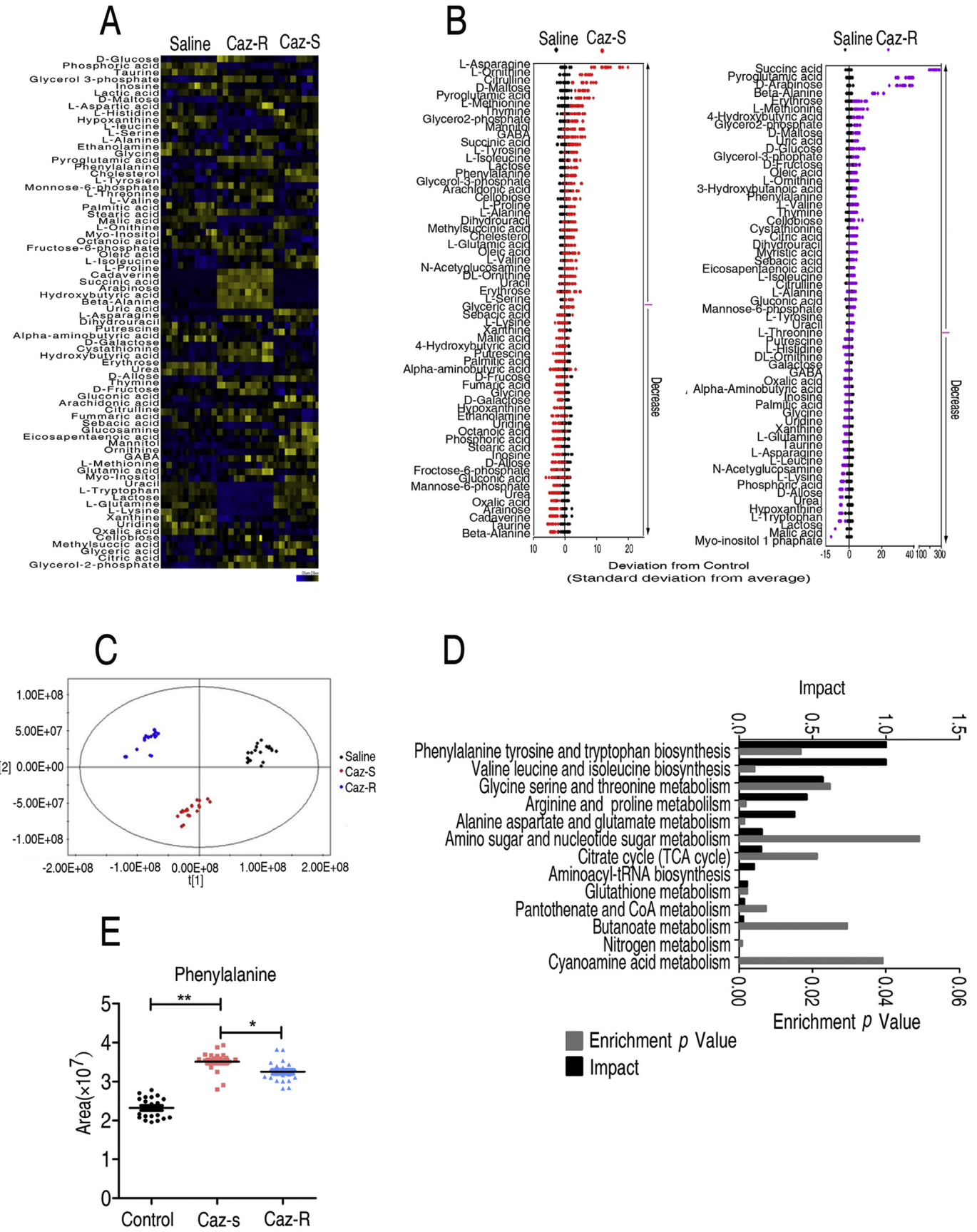

Fig. 2. Metabolomic analysis of humoral fluids of *D. rerio* infected by Caz-S or Caz-R. (A) Heat-map showing the abundance of metabolites (Wilcoxon, $p < 0.01$) in Caz-S and Caz-R as compared to saline control. Scales are shown at the right bottom. (B) Z scores (standard deviation from average) of Caz-S to saline control (left panel) and Caz-R to saline control (right panel), which are corresponding to the data shown in (A). Each point represents one technical repeat of metabolite. (C) Independent component analysis (ICA) of saline control, Caz-S and Caz-R. Each dot represents one technical replicate. (D) The abundance of phenylalanine in Caz-R, Caz-S and saline control. (A) Enriched metabolic pathways of Caz-R; (E) iPath analysis of altered abundance of metabolites.

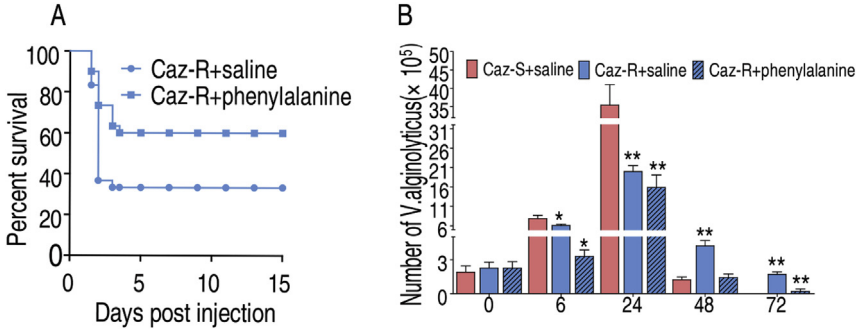

Fig. 3. Exogenous phenylalanine potentiates zebrafish to kill Caz-R *V. alginolyticus*. (A) Percent survival of *D. rerio* infected with Caz-R or Caz-R plus exogenous phenyalanine; (B) Persistence of Caz-R in the presence of exogenous phenylalanine; (C) Clearance of Caz-R *V. alginolyticus* in the presence of phenylalanine.

3.3. Exogenous phenylalanine potentiates D. rerio to kill Caz-R V. alginolyticus

Phenylalanine is the crucial biomarker that distinguishes Caz-S from Caz-R. Thus, reprogramming metabolomics by phenylalanine may be a strategy to help the host clear Caz-R. When thirty zebrafish were infected either with Caz-R (1.6×10^6 cells/fish) or Caz-R plus phenylalanine, their consequences were different. Phenylalanine greatly increase the survival rate of Caz-R-infected group for more than 20% (Fig. 3A). The dynamic analysis of the Caz-R that are persistent in the host further demonstrated that phenylalanine enhances the host clearance of Caz-R, which is barely detected at 72 h (Fig. 3B). Therefore, phenylalanine is a crucial biomarker that could restore *D. reiro* capability to clear Caz-R.

3.4. Phenylalanine enhances innate immune response

To investigate the phenylalanine in promoting immune response to Caz-R, we measure the transcriptional levels of the pro-inflammation molecules. Surprisingly, phenylalanine significantly increased the expression of C3b, lysozyme and IL-6 (Fig. 4Aand B). C3b, a component of complement system, and lysozyme are important molecules in innate immunity. Both of the two molecules are targeting cell wall, forming membrane attacking complex or destructing cell wall synthesis. Thus, phenylalanie may promote the innate immunity to clear Caz-R through regulation of bacterial membrane, which may enhance the efficacy of antibiotics. To prove that, we treated Caz-R with lysozyme plus Caz or plus kanamycin. Lysozyme alone has very weak effect in killing Caz-R, however, the synergy of Caz or kanamycin with lysozyme could increase the killing efficacy of different types of antibiotics (Fig. 4Cand D). Thus, these data suggested that phenylalanine increased lysozyme production, which might alter bacterial membrane permeability becoming susceptible to antibiotics.

4. Discussion

Eliminating antibiotic-resistant bacteria is an emergent issue in aquaculture. Despite the lack of progress in the development of novel antibiotics, the non-antibiotic approach also meets limited success [22,23]. Thus, boosting host's immune response to combat against antibiotic-resistant bacteria is an alternative strategy.

In this study, we adopted reprogramming metabolomics that we proposed in our previous study to investigate the differential immune response of zebrafish to antibiotic-sensitive and -resistant bacteria, Caz-S and Caz-R, respectively. Caz-R was selected from Caz-S by serially cultured in sublethal dose of Caz, but Caz-S and Caz-R showed distinct cellular behavior like the use of carbon source and the swarming ability. These phenotypes may be resulted from the gene mutations that confer Caz resistance. Gaining antibiotic resistance shift bacterial metabolism that downregulate tricarboxylic acid cycle (TCA cycle) [9,24]. The reduced efficiency in using of carbon sources in Caz-R is thus not surprising since the promoted TCA cycle would sensitize antibiotic-resistant bacteria to antibiotics[18]. However, how gaining of antibiotic resistance would change the swarming ability is not yet reported. The mechanism still waits for further investigation. The change of these phenotypes may lead to different host-pathogen interaction. The pathogenicity of Caz-R is reduced as compared to Caz-S. Although fitness cost of bacteria with antibiotic resistance is known before [24], Caz-R persisting longer in the host than Caz-S was unknown. This observation suggested that antibiotic-resistant bacteria was not easy to be eradicated by the host, which may cause longer time of infection than the antibiotic-sensitive bacteria. This can be partially explained by the reduced antigenicity of Caz-R in triggering immune response. Thus, antibiotic-resistant bacteria suggested another severe consequence of the infection that last longer than the antibiotic-sensitive bacteria.

In searching for ways to boost immune response to clear antibiotic-resistant bacteria, we started from metabolism, which is recently recognized as an important regulator of immunity. We thus adopted functional metabolomics to identify crucial metabolic biomarkers, which could be used to revert kanamycin-resistant bacteria to kanamycin-sensitive bacteria as termed reprogramming metabolomics [7]. This strategy is based on the metabolite from organisms themselves, thus it has low antigenicity and less or even no toxicity to the organism. We previously showed that *Edwardsiella tarda*, a fish pathogen, kills the host if they have low levels of glucose, but this could be rescued by exogenous administration of glucose to the host, whose survival percentage was increased two folds [14,25], indicating the metabolic state determines the consequence of bacterial infection. Similarly, zebrafish mounts differential metabolic response towards Caz-S and Caz-R infections. The major differences between the two metabolomes located on amino acid metabolism and carbohydrates metabolism (Fig. 2G). And the increased amino acid metabolism is critical for zebrafish to clear Caz-S, thus suggesting the importance of promoting amino acid metabolism, where the crucial marker is phenylalanine.

The mechanism of how phenylalanine promoted the clearance of antibiotic-resistant bacteria is not yet explored. Phenylalanine is an essential amino acid that is the precursor of tyrosine. The role of phenylalanine in antimicrobial has not been explored yet. But phenylalanine and glutamine, cysteine, tryptophan and arginine are important for T-cell function and play critical roles of immune response to cancer [26]. The decreased level of phenylalanine would inhibit T-cell function but increased hydrogen peroxide production, which is modulated by an antigen presentation cell (APC)-expressing IL-4 induced gene 1 (IL4I1) [17,27]. This suggested phenylalanine a potential player in modulating the innate immunity and adaptive immunity. In addition, our studies showed that phenylalanine promotes the transcription of lysozyme and C3b, both of which are crucial innate immune molecules to clear pathogens. Lysozymes are enzymes produced by macrophages and polymorphonuclear neutrophils (PMN). Thus, whether phenylalanine increased the production of lysozyme in macrophages needs more

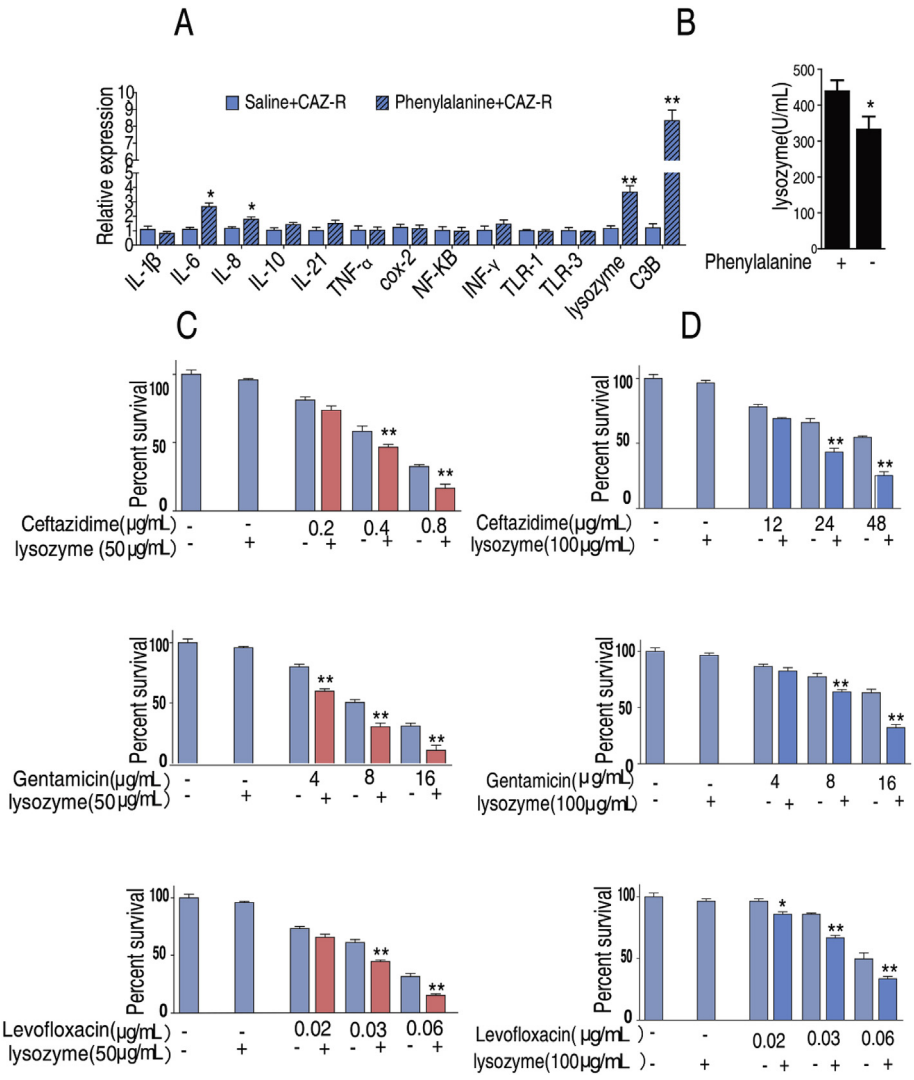

Fig. 4. Exogenous phenylalanine promotes pro-inflammatory cytokine production and lysozyme expression. (A) qRT-PCR for expression of innate immune genes in the presence of phenylalanine; (B) Quantitative analysis of lysozyme in the presence of phenylalanine; (C) & (D) Synergistic effects of lysozyme with antibiotics in killing Caz-S and Caz-R.

investigation. One interesting observation here is that lysozyme can degrade the bacterial cell wall but does not kill bacteria directly. The clearance of bacteria might be still dependent on other factors like C3b, which constitutes one of the major players in killing Gram-negative bacteria. But whether the increased production of lysozyme and C3b are in cooperation in killing pathogens requires further investigation. Therefore, enhanced innate immunity is required for the host to eradicate antibiotic-resistant bacteria *in vivo*.

Taken together, our results showed that bacteria have changed physiological state and interaction to the host after gaining antibiotic resistance. More importantly, the Caz resistance make the bacteria hard to be eradicated by the host. This phenomenon may put another line of evidence of the danger of the antibiotic-resistant bacteria. We proposed the use of reprogramming metabolomic to identify crucial biomarkers like phenylalanine in this study may shed light on the alternative strategy to combat against antibiotic-resistant bacteria infection in an antibiotic-independent approach.

Author contributions

BP conceptualized and designed the project. MJ, SSL and MYL performed experiments. MJ, QYG and JZ performed data analysis. BP, MJ, ZXC, ZGC and JZ interpreted the data. BP wrote the manuscript. All the authors reviewed the manuscript.

Conflicts of interest

All authors declare there is no conflict of interest.

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Appendix A. Supplementary data

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