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The effect of antibiotic exposure on eicosanoid generation from arachidonic acid and gene expression in a primitive chordate, *Branchiostoma belcheri*

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ABSTRACT

Chloramphenicol (Chl) is an effective antimicrobial agent widely used in veterinary medicine and commonly used in fish. Its use is restricted in the clinic because of adverse effects on the immune system and oxidative stress in mammals. However, the effects of Chl treatment on invertebrates remain unclear. Amphioxus, a basal chordate, is an ideal model to study the origin and evolution of the vertebrate immune system as it has a primary vertebrate-like arachidonic acid (AA) metabolic system. Here, we combined transcriptomic and lipidomic approaches to investigate the immune system and observe the oxygenated metabolites of AA to address the antibiotic effects on amphioxus. Tissue necrosis of the gill slits occurred in the Chl-treated amphioxus, but fewer epithelial cells were lost when treated with both Chl and ampicillin (Amp). The immune related pathways were dysregulated in both of the antibiotic treatment groups. The Chl alone treatment resulted in immunosuppression with down-regulation of the innate immune genes. In contrast, the Chl + Amp treatment resulted in immunostimulation to some extent, as shown by KEGG clustering. Furthermore, Chl induced a 3-fold reduction in the level of the eicosanoids, while the Chl + Amp treatment resulted in 1.7-fold increase of eicosanoid level. Thus in amphioxus, Amp might relieve the effects of the Chl-induced immune suppression and increase the level of eicosanoids from AA. Finally, the oxygenated metabolites from AA might be crucial to evaluate the effects of Chl treatment in animals.
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1. Introduction

Chloramphenicol (Chl) is a highly effective antimicrobial agent that has been used to treat typhoid fever, meningitis, and certain infections of the central nervous system since 1948 [1–3]. In the clinical context, a combination of Chl and ampicillin (Amp) treatment is used to treat severe purulent exacerbations of bronchitis and systemic haemophilus influenza disease [4–9]. Chl + Amp treatment is synergistic against gram-negative bacteria [10]. Furthermore, typhoid fever patients treated with Chl + Amp have a shorter febrile period compared to patients treated with Chl alone [11].

However, Chl is restricted in the clinic and banned in food-producing animals [12–15] because of its adverse effects. Chl treatment in mammals can induce complicated physiological and pathological effects. The most prominent feature of the adverse effects of Chl treatment is immune depression with irreversible aplastic anemia and gray baby syndrome [16–18]. Chl can also induce abnormal T cell differentiation and the inhibition of T cell apoptosis [19]. Chl-treated dogs show complex hematologic changes, which includes polychromasia, anisocytosis, vacuolation of lymphocytes, and basophilic granule formation in neutrophils [20]. Chl treatment could also significantly suppress antigen-induced lymphocyte blastogenesis [21]. Another remarkable feature associated with Chl treatment is the induction of the oxidative stress response and an increase in reactive oxygen species production [16–18].

Oxidative stress affects the oxidative metabolism of polyunsaturated fatty acids through enzymatic and free radical-mediated reactions [22]. Oxygenated metabolites from arachidonic acid

Abbreviations: Chl, chloramphenicol; AA, arachidonic acid; Amp, ampicillin; COX, cyclooxygenase; LOX, lipoxygenases; CYP, mono-oxygenases from cytochrome P450

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(AA) are produced by the catalytic reaction of two principal dioxygenase enzymes, namely cyclooxygenases (COX) and lipoxygenases (LOX), as well as mono-oxygenases (CYP) of the cytochrome P450 family. Eicosanoids from AA are a family of bioactive lipids, which are associated with a wide variety of physiological activities as well as pathological responses and often exhibit potent immune properties in mammals [23,24]. Thus, we hypothesize that the level of eicosanoids from AA may be crucial to evaluate the homeostasis of animals and might reflect the effects of Chl treatment on animals.

Chl is still widely used in veterinary medicine and commonly used in fish because it is a highly effective and well-tolerated broad-spectrum antibiotic [12,25]. However, the effects of Chl treatment on invertebrates remain unclear. Amphioxus is an example of a primitive chordate that has unique features, which make it an ideal model for investigating the origin and evolution of the vertebrate immune system [26,27]. Amphioxus has a complex mucosal immune system, which is involved in host defense after pathogen stimulation [28]. Furthermore, we previously identified an AA-metabolic system with COX, LOX, and CYP pathways in amphioxus [29]. Thus, considering the complicated consequences associated with Chl treatments in human and animal models, a comprehensive investigation will serve as a powerful approach to explore these complex effects in invertebrates. Thus, we combined transcriptomic and lipidomic approaches with KEGG clustering to address the impact of antibiotics on amphioxus. Understanding these effects on amphioxus will help to evaluate the efficiency of Chl usage in the aquatic invertebrate species.

2. Materials and methods

2.1. Animals and sample acquisition

Adult Chinese amphioxus *Branchiostoma belcheri* were obtained from the Beihai Bay, China. The location is 20°01′N, 107°42′E. The experimental use of amphioxus has been approved and supervised by the Animal Care and Ethics Committee (ACEC) of the School of Life Sciences (SOLS) at Sun Yat-sen University since 2010 (track: SYSU-SOLS-ACEC2010B0022). SOLS ACEC ensures that animal experiments abide by local laws and the *International Guiding Principles for Biomedical Research Involving Animals* as issued by the Council for the International Organizations of Medical Sciences.

Amphioxus were cultured in aerated seawater and fed microalgae daily for more than three months. They were sorted into two antibiotic treatment groups, Chl, and Chl plus Amp. A third group of amphioxus was cultured in fresh seawater as a control. For the control amphioxus group, day 0 was used as the reference basis for investigating the histological changes, lipid levels, and gene expression in both of the antibiotic treatments groups. The antibiotic-treated groups consisted of fifteen amphioxus that were cultured in 2 L of seawater with aeration and treated with either 62.5 mg/L Chl in seawater (Chl group) or 62.5 mg/L Chl plus 0.5 g/L Amp in seawater (Chl + Amp group). The dose of antibiotics was based on previous reports on the effects of Chl treatment [30–32]. The experimental ratio of Chl and Amp was based on the clinical usage and the bactericidal activity with Chl + Amp treatment [10,11,33]. For histological observation, the digestive tract tissues of adult amphioxus were collected after 0, 2, 4, 6, and 8 days of treatment. For transcriptomic and lipidomic analyses, the pooled tissues samples were collected from the pharyngeal gill slits to the intestine of the adult amphioxus digestive systems. Six female and male adult amphioxus were used for each sample in the histological, transcriptomic and lipidomic experiments, respectively.

2.2. Histological protocol

At 2, 4, 6, and 8 days, the adult amphioxus were killed and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) buffer at 4 °C for 12 h, dehydrated with graduated ethanol, and embedded in paraffin. The paraffin blocks were cut into 5 μm sections, stained with hematoxylin and eosin, and examined microscopically at 35×- and 630× with oil immersion.

2.3. Transcriptomic analysis of amphioxus

We collected tissue of the amphioxus digestive system from the pharyngeal gill slits to the intestine. Total RNA was extracted with TRIZOL (Invitrogen), and the cDNA libraries were prepared using the Truseq™ RNA Sample Preparation Kit (Illumina). The antibiotic-free, Chl1d, Chl + Amp1d, Chl4d, and Chl + Amp4d libraries were primer-end labeled with CGATGT, TGACCA, ACAGTG, CGATGT, and TGACCA, respectively. For PCR efficiency, the number of cycles was limited to 15 to reduce the effect of base mutations, redundant fragments, and guanine and cytosine (GC)% disparity. The PCR amplicon libraries were generated for each sample and detected with an Agilent Bioanalyzer 2100 (Agilent), quantified with a Qubit 1.0 spectrofluorometer (Invitrogen), and pyrosequenced by Genome Analyzer IIx (Illumina). The transcriptomic sequences have been deposited in the NCBI Short Read Archive database (accession No. SRP035372).

2.4. Transcriptome data analysis

Extracted RNA fragments of ~390 bp were subjected to sequencing (Table S1). The transcriptomic data was analyzed according to a previous report [29]. We identified 3530 differentially expressed genes (DEGs) (>1000 DEGs in each sample) that were significantly up- or down-regulated based on a *t*-test ($P < 0.05$) and false discovery rate (FDR < 0.05) (Fig. 3A). A GO function analysis (TermFinder: http://search.cpan.org/dist/GO-TermFinder/) (corrected $P < 0.05$) [34] and KEGG pathway analysis (DAVID: http://david.abcc.ncifcrf.gov/) ($P < 0.05$) of the DEGs was conducted [35]. A comprehensive comparison of a select set of KEGG pathways [36] in both of the antibiotics-treated amphioxus groups, on the basis of the time-course of the proportion of significantly regulated genes, is shown in Tables 1 and S3. The KEGG pathways were classified as the commonly and uniquely regulated pathways.

2.5. Expression patterns detected by real-time PCR

Total RNA was extracted from the samples using TRIZOL (Invitrogen) and treated with deoxyribonuclease (DNaseI). Double-stranded cDNA was synthesized using reverse transcription (TOYOBO) and quantified with the LightCycler 480 (Roche). Eighty-one immune and oxygenated-metabolic candidate genes were selected for qPCR to monitor their modulation of time in response to Chl (0–8 days) and Chl + Amp (0–8 days) treatments according to a previous report [28]. The values were considered to be significant at P < 0.05. Digital expression profiling with different antibiotic treatments were roughly consistent with the qPCR expression levels (Table S4), with a Pearson correlation coefficient of 0.82 and 0.80 on day 4 of Chl and Chl + Amp treatments, respectively ($P < 0.05$).

2.6. Section in situ hybridization

Male and female adult *B. belcheri* were cut into 1-cm sections and fixed in 4% paraformaldehyde in PBS buffer at 4 °C overnight, dehydrated with graduated ethanol, and embedded in wax. The tissue blocks were then cut into 8-mm sections. The 3′ end fragments

of the BbtCOX-c and BbtCOX-d cDNAs were amplified by PCR and cloned into the T Easy Vector (Promega) (Supplementary Table S1). DIG-labeled sense (synthesized by T7 RNA polymerase for BbtCOX-c and BbtCOX-d) and antisense (synthesized by SP6 RNA polymerase for BbtCOX-c and BbtCOX-d) probes were generated at the 3′ end sequences as a template with the DIG RNA Labeling Kit (Roche). The secting procedure for *in situ* hybridization was performed as previously described [29].

2.7. Eicosanoid measurement in the amphioxus digestive tract

Amphioxus tissues were snap-frozen in liquid nitrogen and stored at −80 °C until lipid extraction. For extraction, the tissue was pulverized and the frozen powder was immediately placed in 3 ml of ice-cold 100% ethanol and weighed. Ten microliter of the antioxidant cocktail (0.2 mg/ml butylated hydroxytoluene, 0.2 mg/ml EDTA, 2 mg/ml triphenylphosphine, and 2 mg/ml indomethacin in a solution of 2:1:1 methanol:ethanol:H_2O) was added to each sample, and all of the tubes were incubated on ice at −20 °C for 72 h. The samples were then centrifuged at 3500*g* for 30 min, and the ethanol supernatant was transferred to a new tube and dried under nitrogen gas. The samples were reconstituted with 2 ml of 10% MeOH and supplemented with 50 ng of the following deuterated eicosanoids (Cayman Chemical): (d4) 6-keto-prostaglandin F1α (6k-PGF$_1$α), (d4) thromboxane B2 (TXB$_2$), (d4) prostaglandin F2α (PGF$_2$α), (d4) prostaglandin E2 (PGE$_2$), (d4) prostaglandin D2 (PGD$_2$), (d4) 15-deoxy-prostaglandin J2 (15d-PGJ$_2$), (d4) leukotriene B4 (LTB$_4$), (d6) 20-hydroxyeicosatetraenoic acid (20-HETE), (d8) 5-hydroxyeicosatetraenoic acid (5-HETE), (d8) 15-hydroxyeicosatetraenoic acid (15-HETE), (d11) 8,9-epoxyeicosatrienoic acid (8,9-EET), and (d11) 11,12-epoxyeicosatrienoic acid (11,12-EET). Eicosanoid extraction and preparation for Ultra performance liquid chromatography and tandem mass spectrometry (UPLC/MS/MS) analysis was conducted according to a previous report [29]. Quantitative eicosanoid determination was performed by the stable isotope dilution method [29].

2.8. Fatty acid methylation and GC–MS analysis

Fresh visceral tissue from the amphioxus was homogenized and subjected to total lipid extraction. The fatty acids were then methylated for gas chromatograph and mass spectrometry (GC–MS) analyses [29]. Fatty acid methyl esters were measured using a Finnigan TRACEGC2000 gas chromatograph equipped with a TRACE-DSQ detector and fused-silica capillary column (DB-5, 30 m, 0.25 mm film thickness, 0.25 mm i.d.; Agilent, CA). The values are expressed as% total fatty acids, and the total fatty acid concentration (nmol/g viscera) was calculated by a comparison of the gas chromatography peak areas relative to that of the $C_{23:0}$ internal standard [29].

3. Results and discussion

3.1. Morphological and pathological characteristics of Chl and Chl + Amp-treated amphioxus

Amphioxus treated with Chl alone continuously swam erratically and displayed a weakened ability to move. Redness of the pharynx and gill area was observed in 80% of animals after 4 days of Chl treatment (Fig. 1A). This regional lesion slowly progressed to involve the entire body (Fig. 1A). However, amphioxus treated with Chl + Amp had no visible lesion by day 8 of the trial (Fig. 1A). Histological sections of Chl-treated amphioxus showed damage to gill cells of the parallel-sided gill slits at day 4 and necrosis and loss of gill cells from the primordial bar at day 8. The damage to the epithelial cells of the gill slits increased with time. In contrast, only a slight decrease of epithelial cells was found in the Chl + Amp4d sample, and the loss of gill cells from the primordial bar in Chl + Amp8d sample was significantly less than Chl8d sample (Fig. 1B). Thus, the combination of Chl and Amp might relieve the adverse effects of Chl treatment alone in amphioxus.

3.2. Differential digestive tract gene expression by antibiotic treatments

To understand how antibiotics affect amphioxus homeostasis, transcriptomic data was obtained to analyze the DEGs after antibiotic treatment at day 1 and day 4 according to the morphological and pathological alterations (Fig. 1). The expression profiles of the samples were remarkably different. Approximately 75% of the DEGs were down-regulated in the Chl1d sample; approximately 50% were down-regulated in the Chl4d and Chl + Amp1d samples; and about 25% down-regulation was observed in the Chl + Amp4d sample (Fig. 2B). Thus, Chl treatment induces the suppression of gene expression in amphioxus, especially in the Chl1d sample. Among the 3530 DEGs, approximately 30% were host immune genes (Fig. 2B and Table S6). The Chl1d sample had the threefold increased in the number of down-regulated immune genes compared to up-regulation, but the Chl + Amp treatment maintained a similar ratio of up-regulated to down-regulated genes (Fig. 2B). Thus, Chl treatment mostly likely induces a severe suppression of gene expression in the immune system of the amphioxus, especially in the Chl1d sample, but the status of the immune system in the Chl + Amp group needs further analysis.

3.3. Antibiotic treatments alter key regulated pathways at the transcriptomic level

The commonly regulated KEGG pathways between the antibiotic treatment groups include some pathways of infection and stress responses, such as the complement and coagulation cascades, *Staphylococcus aureus* infection, and autoimmune thyroid disease (Tables 1 and S3). In addition, pathways related to metabolism, protein digestion and absorption, and cytokine–cytokine receptor interaction, were also observed as seen in Table 1. Thus, antibiotic treatment induces complicated physiological and pathological effects in amphioxus. The number of down-regulated genes in these commonly regulated pathways was higher than the number of up-regulated genes in both of the antibiotics treatment groups (Table 1). Although the ratio of the up-regulated genes to total regulated genes in these pathways did not differ significantly between both antibiotics-treated groups (Table 1), the genes within the regulated pathways were different and the magnitudes varied. These differences ultimately contributed to the different biological effects. Within the uniquely regulated pathways, the number of down-regulated genes associated the phagosome, antigen processing, and the antigen presentation pathways was higher than the number of up-regulated genes in the Chl group. On the contrary, the genes found into be dysregulated in the Chl + Amp were enriched for pathways related to the significant activation of the arrhythmogenic right ventricular cardiomyopathy (ARVC) and AA metabolism (Table 1). A previous report indicated that amphioxus does not possess a proper heart, but has a circulatory system and a putative hematopoietic tissue domain that is closed to the dorsal aorta anlages, which is partially linked to the excretory tissues [37]. Thus, it is difficult to link the activation of the ARVC pathway to physiological changes in the amphioxus because of the lack of a heart. However, further measurement of the oxygenated metabolites produced from AA might provide useful information for the investigation of the effects of oxidative stress following Chl and Chl + Amp treatments.

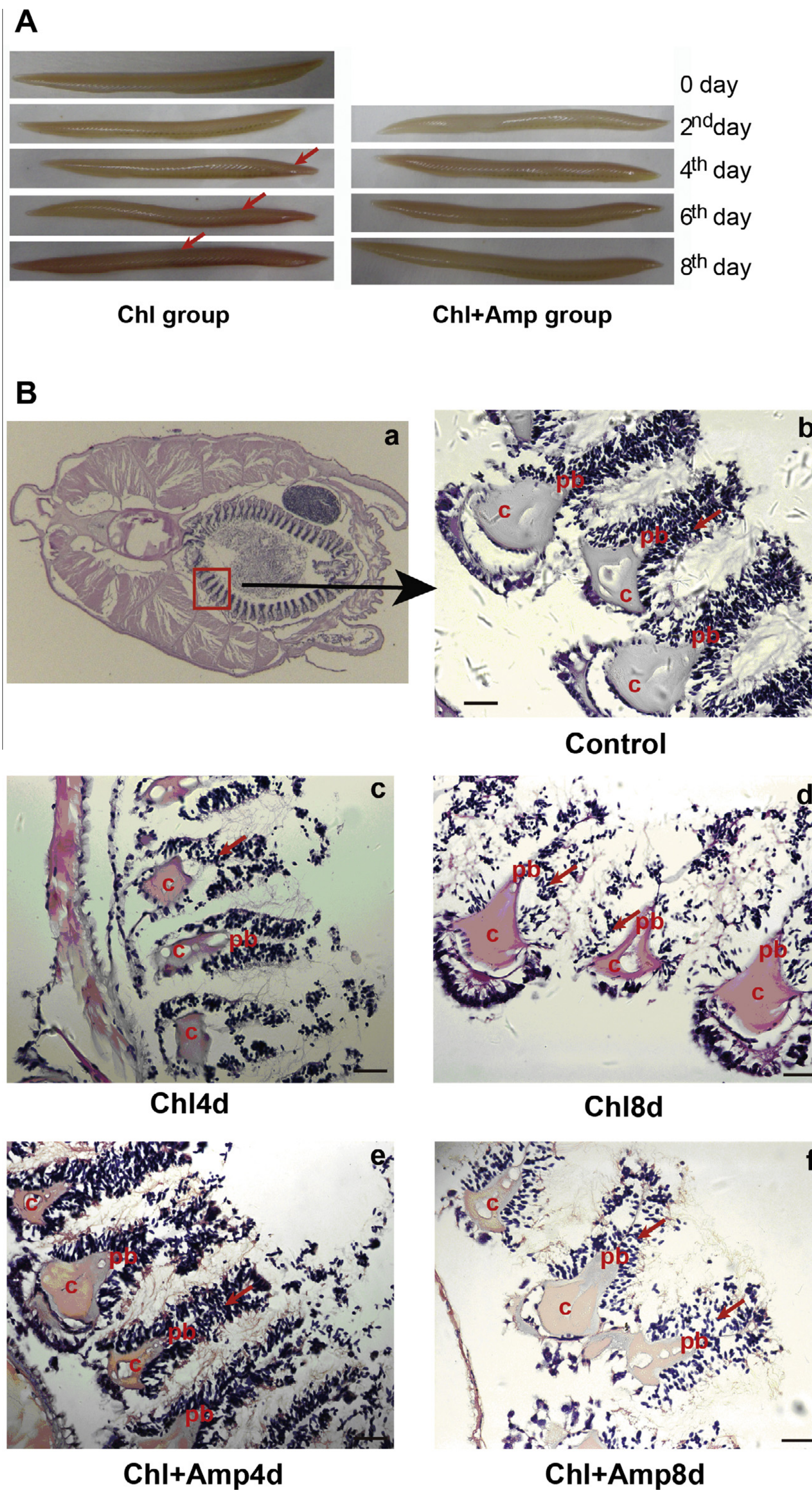

Fig. 1. The morphological and pathological observation of the antibiotic effects in amphioxus. (A) The morphological changes of the antibiotic treated amphioxus; (B) the pathological changes of the pharynx and gill of the antibiotic treated amphioxus. (a and b) Amphioxus not treated with antibiotic under 35×- and 630× oil. The red square indicates the parallel-sided gill slits of amphioxus. The deep blue is the gill cell, which is orderly and surrounded at the primordial bar. The arrow indicates the gill cell. (c and d) Amphioxus exposed to Chl for 4 and 8 days. Scale bar, 50 μm. (e and f) Amphioxus exposed to Chl + Amp for 4 and 8 days. Scale bar, 50 μm. The arrows were used to compare the tissue damage of the gill slit of the pharynx in the control, Chl-treatment, and Chl + Amp-treatment groups. c: coelom, pb: primordial bar.

Next, we examined the ten genes with the highest expression in both antibiotic-treatment groups. The genes up-regulated in Chl group but not in Chl + Amp treatment group were HSP68, a stress response protein gene, and RTase. The ten genes with the highest expression found in the Chl + Amp group but not in Chl treatment were the genes involved in the pathogen recognition and signal pathways, along with the stress protein of hematopoietic prostaglandin D synthase (HPGDS), which is involved in prostaglandin (PGs) production (Tables 2 and S6). Thus, although the immune-related pathways were to be dysregulated in both of the antibiotic treatment groups, the genes expression pattern between the treatment groups was different.

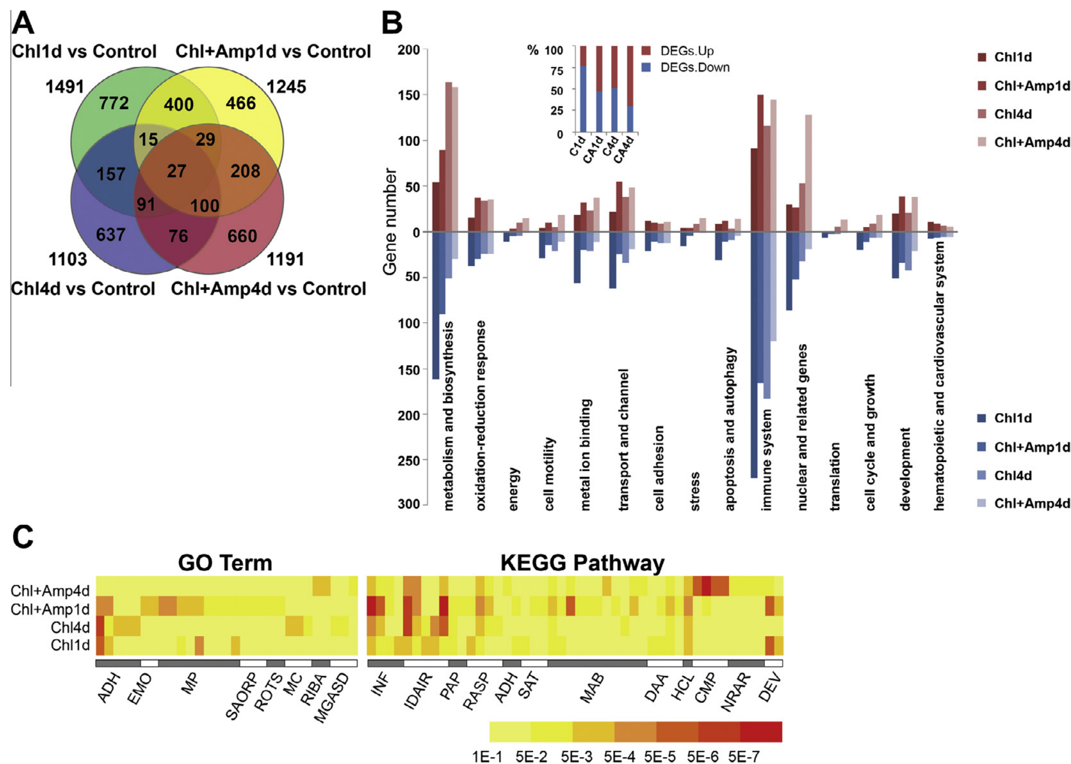

Fig. 2. Distribution, function, and expression pattern of DEGs in the libraries. (A) Distribution of DEGs in the Chl1d, Chl + Amp1d, Chl4d, and Chl + Amp4d libraries from the transcriptomic data. (B) The percent and number of up- and down-regulated genes of the 3530 DEGs in Chl1d, Chl + Amp1d, Chl4d, and Chl + Amp4d libraries. The red bar indicates the up-regulated DEGs, and the blue bar indicates the down-regulated DEGs. (C) The enriched GO type of the biological process and the KEGG pathway of the DEGs appeared in the Chl1d, Chl + Amp1d, Chl4d, and Chl + Amp4d vs. control. ADH: adhesion; EMO: extracellular matrix organization; MP: metabolic process; SAORP: stress and oxidation–reduction process; ROTS: regulation of inclusion body assembly; MC: molting cycle; RIBA: regulation of inclusion body assembly; MGASD: mammary gland and sex development; INF: infection; IDAIR: immune disease and immune response; PAP: phagosome and peroxisome; RASP: receptor and signal pathway; SAT: secretion and transport; MAB: metabolism and biosynthesis; DAA: digestion and absorption; HCl: hematopoietic cell lineage; CMP: cardiomyopathy; NRAR: nucleotide repair and recombination; DEV: development.

3.4. Effects of antibiotics treatment on the hematopoietic cell lineage pathway

A common pathway of the hematopoietic cell lineage was significantly down-regulated (FDR < 0.05) in both of the antibiotic treatment groups (Table S3). The main down-regulated cytokines included CD21, CD22, CD23, CD35, CD42, CD49, TPOs, and Mfhas1 (Fig. 3). The hematopoietic cell lineage can eventually differentiate into macrophage-like cells and lymphoid cells in mammals. In mouse, Chl induces the differentiation of activated T cells into lymphoblastic leukemia-like cells with expression of CD7, a marker for immature T cells and T-cell lymphocytic leukemia, thus phenotypically indicating differentiation toward leukemogenesis [19]. However, whether Chl treatment can inhibit the maturation of the hematopoietic cells in amphioxus remain elusive. Furthermore, angiopoietin-related protein like 1 (ANGPTL1) and angiopoietin-related protein 4 (ANGPT4) of the fibrinogen-related proteins were up-regulated in the Chl + Amp treatment group but down-regulated in Chl treatment (Table 2). This might mean that, to some extent, a different status of the immune system is induced in each of the antibiotic treatment groups.

3.5. Immunomodulation in the Chl alone and Chl + Amp treatment groups

Another common pathways dysregulated in both of the antibiotic treatment groups include the immune-related pathway of bacterial infections and the complement and coagulation cascades. The amphioxus immune system possesses a microorganism recognition system with expanded gene numbers and diverse domain architecture of PRR families, cytokines and signal transducers, and effectors. The microbe-associated molecular patterns (MAMPs) genes, including scavenger receptor cysteine-rich protein (SRCR), leucine-rich repeat-containing protein (LRR), and fibrinogen-related proteins, had a higher level of gene expression in the Chl + Amp group compared with the Chl group (Fig. 3). TLR2 was also increased in the Chl + Amp group, but down-regulated in Chl4d sample (Fig. 3). The potential Gram-positive bacteria receptors for the peptidoglycan recognition protein, chitin-binding protein, lysozyme, C-type lectin (CTL), and ficolin in the PRR families were down-regulated in both groups (Fig. 3). Further examination revealed that the expression of genes in the PRR families in the Chl + Amp treatment group was higher than Chl treatment group when assessing the ratio of Chl + Amp1d/Chl1d and Chl + Amp4d/Chl4d (Fig. 3). Thus, Chl + Amp treatment might relieve the effects of immune suppression in the PRR families in amphioxus treated with Chl alone.

As for signal transducers and effectors, MyD88 and IκB were up-regulated expression in both of the antibiotic treatment groups at day 4. TRAF3 and RIPK1 were significantly up-regulated in Chl + Amp4d sample. A majority of the NF-κB target genes, including NLR, TNFR, TNF, and various cytokines, were significantly induced in the Chl + Amp treatment group, but not with Chl treatment alone (Fig. S2). The expression dynamics analysis indicated that the lectin pathway and C1q-like pathway were activated in the mucosal immune responses of the digestive tract (Fig. S1). The expression of MACPF genes and the typical immune genes of the SOUL heme-binding protein (a regulatory factor in innate immune response) were increased in the Chl + Amp group but not in the Chl group (Fig. S1). The expression of the acute phase

Table 1
KEGG pathways enriched by gene regulation in Chl and Chl + Amp treated amphioxus. All selected pathways have a *P*-value < 0.05. The detailed digits refer to Table S3.

Path_name	Chl1d (Reg: 1491)				Chl4d (Reg: 1103)				Chl + Amp1d (Reg: 1245)				Chl + Amp4d (Reg: 1191)			
	Total	Reg	Up	EF	Total	Reg	Up	EF	Total	Reg	Up	EF	Total	Reg	Up	EF
Commonly regulated pathways																
Complement and coagulation cascades	269	26	3	4.4	264	28	7	6.1	277	41	3	7.2	266	24	3	5.0
Systemic lupus erythematosus	185	23	7	3.9	184	25	6	5.5	190	29	3	5.1	186	22	8	4.6
Staphylococcus aureus infection	185	23	4	3.9	177	21	1	4.6	190	33	4	5.8	181	20	1	4.2
Pertussis	194	20	2	3.4	195	20	3	4.4	203	28	4	5.0	198	16	4	3.3
Protein digestion and absorption	183	17	6	2.9	182	16	3	3.5	187	20	10	3.5	179	16	7	3.3
Tyrosine metabolism	168	17	5	2.9	166	14	3	3.1	171	19	8	3.4	171	16	7	3.3
Cytokine–cytokine receptor interaction	118	14	6	2.4	118	15	8	3.3	121	18	5	3.2	118	14	4	2.9
Hematopoietic cell lineage	96	14	5	2.4	95	13	2	2.8	96	16	1	2.8	96	13	2	2.7
Autoimmune thyroid disease	32	6	4	1.0	32	7	0	1.5	32	9	0	1.6	32	9	0	1.9
Uniquely regulated pathways																
Phagosome	368	33	10	5.6	352	24	6	5.2								
Rheumatoid arthritis	86	14	1	2.4	88	11	3	2.4								
Antigen processing and presentation	60	11	1	1.9	60	12	3	2.6								
Arachidonic acid metabolism									271	32	24	5.7	260	21	15	4.4
Arrhythmogenic right ventricular cardiomyopathy (ARVC)									226	20	14	3.5	228	31	30	6.5

Note: Total: total genes in the pathway; Reg: number of genes regulated in the pathway; EF: the enrichment factor of commonly regulated genes for a pathway is the ratio of the proportion of such genes in this pathway to its proportion in the 29,865 genes examined.

protein defensin was increased with both antibiotic treatments. A severe reduction of mucus was observed at day 4 in the Chl group but not in the Chl + Amp group (Table S6). In a more detailed comparison, the gene expression of the signal transducers and effectors in the Chl + Amp-treatment group was higher than the Chl-treatment group when the ratios of Chl + Amp1d/Chl1d and Chl + Amp4d/Chl4d were examined (Fig. 3). Thus, Chl treatment induces suppression of the PRR families and the signal transducers and effectors in amphioxus. In contrast, Chl + Amp treatment results in immune stimulation to some extent via KEGG clustering. These findings suggest that Amp might help to relieve immune suppression in amphioxus caused by Chl treatment.

3.6. Antibiotic treatment results in altered eicosanoid production

A uniquely regulated AA metabolism pathway was activated in Chl + Amp group but not in Chl group, indicating one of the marked features of both of the antibiotic treatments (Table 1). As for the gene expression of the enzymes in AA metabolism, amphioxus LOX-1 mRNA was strongly expressed in the pharynx and gill, hepatic cecum, gonad, and intestine [29]. Amphioxus has two homologous COXs, named COX-c and COX-d [38]. Amphioxus COX-c/d mRNA was strongly expressed in the pharynx and gill, hepatic cecum, mature gonad, and intestine as shown by *in situ* hybridization (Fig. 4). The metabolites and mediators from AA through the COX, LOX, and CYP pathways in the amphioxus muscle and digestive tract were quantified. The eicosanoid concentration in muscle was found to be only about 5% of that in the digestive tract. Moreover, the levels of PGE_2, 12-HHTrE, and 9-HETE in muscle were lower than the detection limit (Table S7). Thus, the pharynx gill slits and the intestine are the major locations for gene expression of amphioxus COX and LOX-1, and eicosanoids production from AA.

The genes of phospholipase A2 (PLA_2), HPGDS, and CYP2J2, which are involved in the eicosanoids production, were significantly up-regulated in the Chl + Amp group (Fig. S2 and Table S6). The Chl + Amp1d sample showed the highest level of eicosanoids production from AA through the COX, LOX, and CYP pathways, whereas the Chl + Amp4d sample returned to the control levels (Fig. 5). The percentage of eicosanoids from AA through the COX, LOX, and CYP pathways in the amphioxus digestive tract reached 3%, 80%, and 17%, respectively. In detail, the high increased level of eicosanoids in Chl + Amp1d sample was from 8-, 12-HETEs from the LOX pathway and 14(15)-EET from the CYP pathway, while PGD_2 and PGE_2 from the COX pathway showed a relatively low increase (Fig. 5 and Table S8). In the Chl + Amp1d sample, PGD_2 and PGE_2 from the COX pathway had a 1.4-fold increase compared to control; and 8-, 12-HETEs from the LOX pathway and 14(15)-EET from the CYP pathway had a 1.6-fold and 2.1-fold increase, respectively. The lipid mediators from the COX and LOX pathways returned to the control levels in the Chl + Amp4d sample; but the lipid mediators from the CYP pathway showed a 1.3-fold decrease compared to the control level (Fig. 5 and Table S8). Eicosanoids from the COX, LOX, and CYP pathways showed a 2.3-, 2.2-, and 1.7-fold decrease in the Chl1d sample and a 1.8-, 3.2-, and 2.4-fold decrease in the Chl4d sample, respectively (Fig. 5 and Table S8). The substrate of AA was significantly increased in the Chl-treatment groups and decreased in the Chl + Amp-treatment group (Fig. 5D). Thus, the dynamic changes of AA and eicosanoid levels indicated that the inhibition of AA metabolism resulted in a decrease of eicosanoids from AA and AA accumulation in the Chl-treatment group; the activation of AA metabolism induced an increase of eicosanoids from AA and AA consumption in the Chl-treatment group.

Oxygenated metabolites of AA play a central role in immune responses through the recruitment and activation of neutrophil

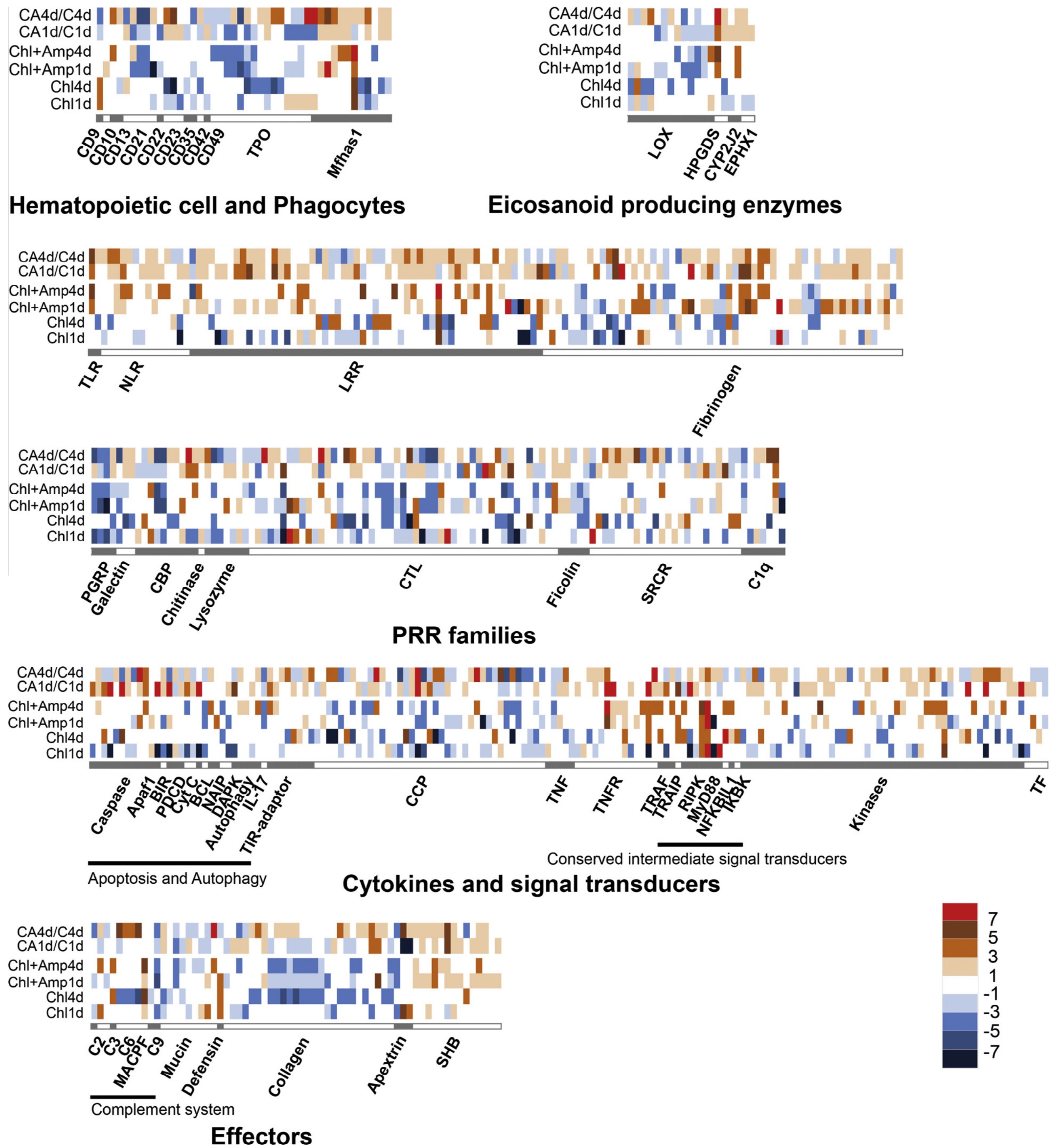

Fig. 3. Expression patterns of DEGs in amphioxus after Chl and Chl + Amp treatments. TPO: thrombopoietin; Mfhas1: malignant fibrous histiocytoma amplified sequence 1; LOX: lipoxygenase; HPGDS: hematopoietic prostaglandin D synthase; CYP2J2: cytochrome P450 2J2; EPHX1: epoxide hydrolase 1; TLR: toll-like receptor; NLR: NOD-like receptor; LRR: leucine-rich repeat-containing protein; PGRP: peptidoglycan recognition protein; CBP: chitin binding protein; CTL: C-type lectin; SRCR: scavenger receptor cysteine-rich protein; C1q: complement C1q protein; Apaf1: apoptotic peptidase activating factor 1; BIR: baculoviral IAP repeat-containing protein; PDCD: programmed cell death; CytC: cytochrome C; BCL: B-cell lymphoma; NAIP: neuronal apoptosis inhibitory protein; DAPK: death-associated protein kinase; IL-17: Interleukin-17; TIR-adaptor: Toll-interleukin receptor adaptor; CCP: complement control protein; TNF: tumor necrosis factor; TNFR: tumor necrosis factor receptor; TRAF: TNF receptor-associated factor; TRAIP: TRAF-interacting protein; RIPK: receptor (TNFRSF)-interacting serine–threonine kinase; MyD88: myeloid differentiation primary response protein; NFKBIL1: NF-kappa-B inhibitor-like protein 1; IKBK: I-kappaB kinase; TF: transcription factor; C2: complement component 2; MACPF: membrane attack complex component/perforin; SHB: SOUL haem binding protein. The detailed gene expression of the putative complement and coagulation cascade and TLR signaling pathway is shown in Figs. S1 and S2. The detailed digits are shown in Table S6.

granulocytes and T cells to inflammation sites [39]. An imbalance of bioactive lipid level can induce a series of diseases, such as chronic inflammation, diabetes, cancer, and cardiovascular disease [40]. In invertebrates, eicosanoids also play important roles at the cellular immune defenses and mediate certain host–parasite and predator–prey interactions [41–43]. Some enzymes for eicosanoid production may be involved in the regulation of the dinoflagellate symbiosis with corals [44]. The high level of PGs might be regarded as chemical defense in the struggle between predators and prey [45–47]. Furthermore, eicosanoids are mainly distributed in the amphioxus digestive tract, which is also the main locus of the distribution of phagocytes and lymphocyte-like cells and the occurrence of immune responses [28,48]. However, whether eicosanoids participate in the amphioxus immune response and

Table 2
Genes were highly up-regulated in the digestive tract of amphioxus with Chl/Chl + Amp treatment. Time course fold-change of the differential expression of the 10 most frequent genes (FDA < 0.05). Color-scale: −7 (dark-blue) – −1 (white) 1–7 (dark-red) [linear].

		Chl		Chl+Amp				Chl		Chl+Amp	
Gene symbol	Gene_ID	1d	4d	1d	4d	Gene symbol	Gene_ID	1d	4d	1d	4d
E1BRZ8	192120F	4.33	1.15	0.58	-0.31	TLR2	074010R	-0.51	0.43	3.35	5.59
US22	237850R	4.25	1.43	<-7	<-7	NLRC4	056340R	1.43	-1.66	2.58	2.99
CFDP2	302480R	3.95	5.01	-0.03	<-7	NLRC4	316280F	-0.85	1.01	2.94	3.37
CD9b	330990R	3.89	4.56	2.76	1.26	TNFRSF12A	128740F	<-7	3.60	6.81	7.12
FIBCD1	104570R	3.83	2.42	-0.02	0.80	TNFRSF12A	128750F	<-7	2.44	2.60	2.89
HSP68	187540F	3.30	5.40	-1.19	-0.47	ANGPTL1	230230R	<-7	<-7	6.85	4.40
HSP68	248920F	1.89	4.70	-0.80	-2.46	ANGPT4	170830F	-1.18	-1.03	5.14	3.67
SNS	137860R	2.35	4.89	<-7	<-7	ANGPT4	052410F	-1.89	0.43	3.32	3.26
IFI44	185680R	1.56	3.18	1.08	1.57	OAS1K	045050F	<-7	<-7	3.87	5.42
RTase	022360F	3.75	4.57	1.34	-0.99	HPGDS	220770F	<-7	<-7	3.74	6.49
Q08525	012450F	2.91	8.84	1.47	1.51	SLC27A2	186880R	0.15	-3.22	3.67	2.69

Note:
Color-scale: >7 5 3 1 -1 -3 -5 <-7
<-7: -1.79769e+308

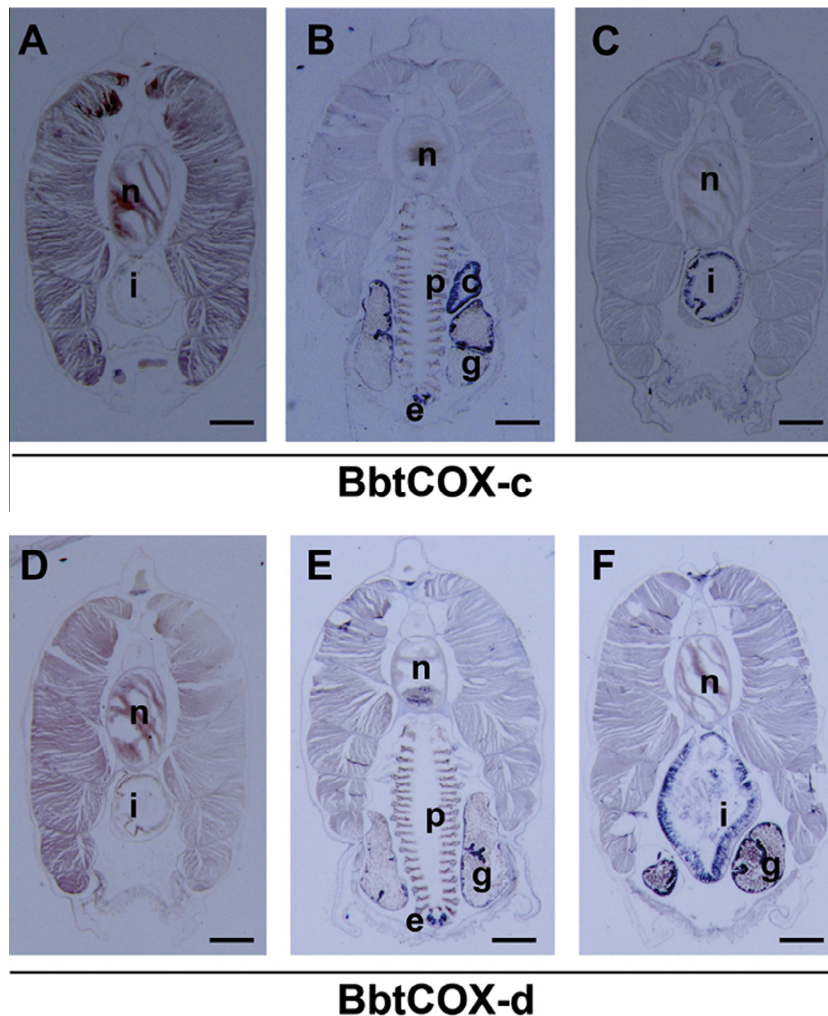

Fig. 4. Section *in situ* hybridization analysis of BbtCOX-c and BbtCOX-d. Positive hybridization signal is shown in deep blue. (A) Section *in situ* hybridization analysis of BbtCOX-c with sense probe; (B and C) BbtCOX-c with anti-sense probe; positive hybridization signal is strongly expressed in the pharynx and gill, cecum, gonad, and intestine. (D) Section *in situ* hybridization analysis of BbtCOX-d with sense probe; (E and F) BbtCOX-d with anti-sense probe; positive hybridization signal is strongly expressed in the pharynx and gill, cecum, gonad, and intestine. n = notochord, i = intestine, g = gonad, p = pharynx and gill, c = cecum, e = endostyle. Scale bar = 500 μm.

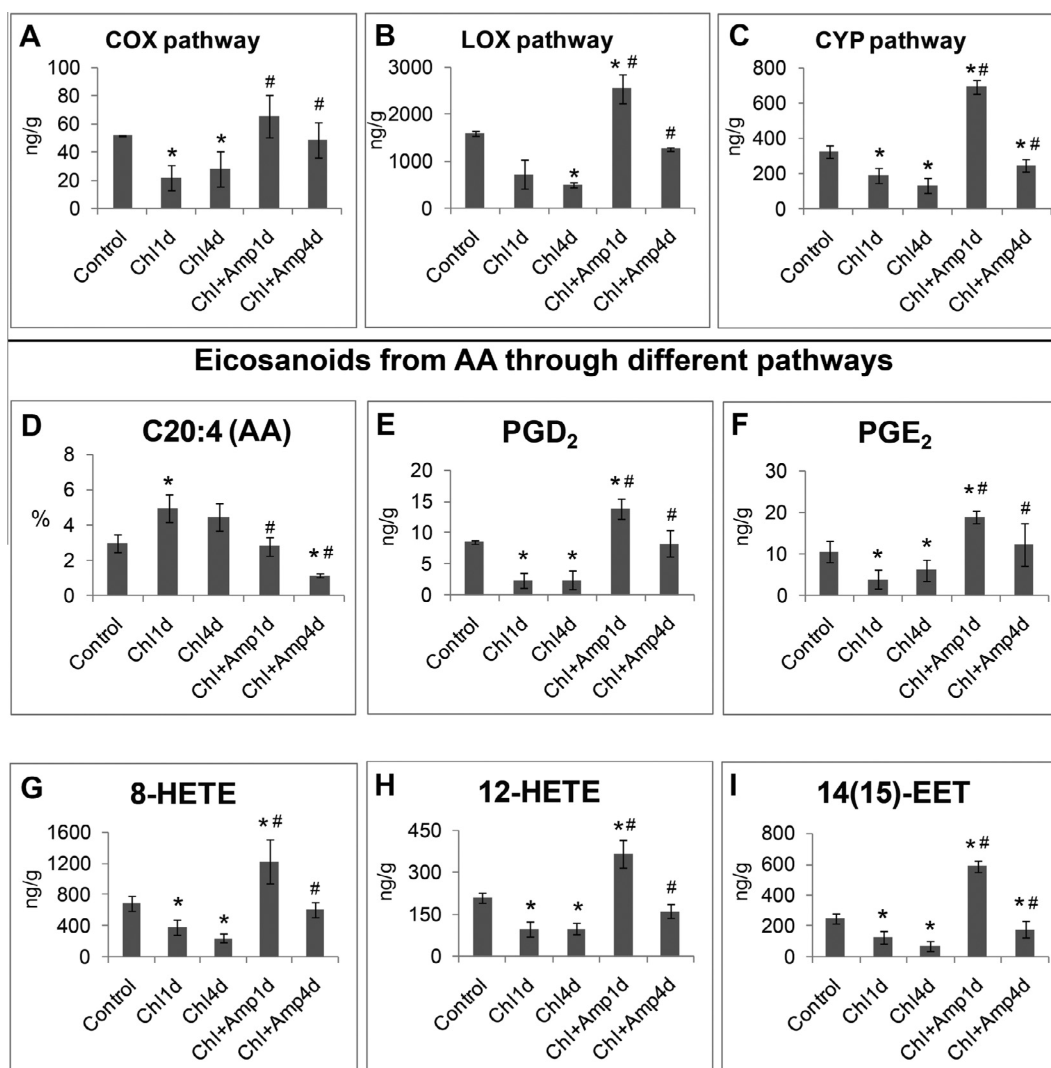

Fig. 5. Dynamic changes of the level of AA and eicosanoids from AA in amphioxus with antibiotics treatments. *Chl1d, Chl4d, Chl + Amp1d, Chl + Amp4d compared to control, respectively (t-test, $P < 0.05$); #Chl treatment compared to Chl + Amp treatment (ANOVA and post tests, $P < 0.05$).

whether the dynamic eicosanoid level reflects the immune status requires further investigation.

4. Conclusion

In summary, the present study indicates that Chl treatment induces immune suppression in amphioxus. The addition of Amp might relieve the suppression effects induced Chl in the amphioxus immune system. Furthermore, oxygenated metabolites of AA are the crucial signals that reflect the effect of Chl on amphioxus. These results exploring the effects of Chl and Chl + Amp treatment on AA metabolism in amphioxus describes a previously unknown pathogenesis that has been reported as an adversarial effect associated with Chl therapy in aquatic animals.

Competing interests

The authors declare that they have no competing interests. All of the authors read and approved the final manuscript.

Author contributions

D.Y. conceived the study, carried out most of the experiments and drafted the manuscript. M.P. extracted total RNA from amphioxus. Q.Z. performed the measurement of eicosanoid level. C.C. performed the qPCR assay. S.C. participated in the transcriptomic data analysis. A.X. co-designed the study, advised on the experiments and co-wrote the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2015.07.004.

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