Title:
Evaluation of immune response to OTC in red seabreams

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Aim:
Increasing seafood consumption results in intensive aquacultural system, which has been accompanied by an increased prevalence of the fish disease. The antibiotic oxytetracycline (OTC) is commonly used to prevent and treat bacterial diseases in fish. However, overuse of OTC has led to side effects, such as immune alteration. In view of this, the aim of present study is to reveal to immune effects by OTC administration in aquacultured red seabream (*Pagrus major*).

Procedure
Our previous study done the in vivo OTC administration to the red seabream juveniles. Three groups were established followed OTC administration concentration and sampling of immune organ spleen and liver were collected followed by OTC feeding-withdrawal days cycle.
Three experimental groups were established: control group (fed corn oil-supplemented food) and experimental groups (40 and 178 mg OTC per body kg feed, respectively). Each oral administration (total twice administration each for seven days), we collected spleen, and liver from each group of red seabream juveniles at the three-sampling time (at day 8, 43, and 70; after the first fed period, the first and second fed period, and after two-times fed and withdrawal period, respectively) (Figure 1). Using the liver and spleen from the OTC administrated red seabream juveniles, measured the expression level of immune-related genes by quantitative real-time PCR (qRT-PCR) and compare with the alteration of immune cells distributions by immunophenotyping.

① *In vivo* OTC administration to the red seabream juveniles was performed. Three groups were established followed OTC administration concentration (0, 40 and 178 $mg \cdot kg^{-1}$ diet), and three sampling times. Liver and spleen were collected followed by OTC feeding-withdrawal day cycle (Figure 1).

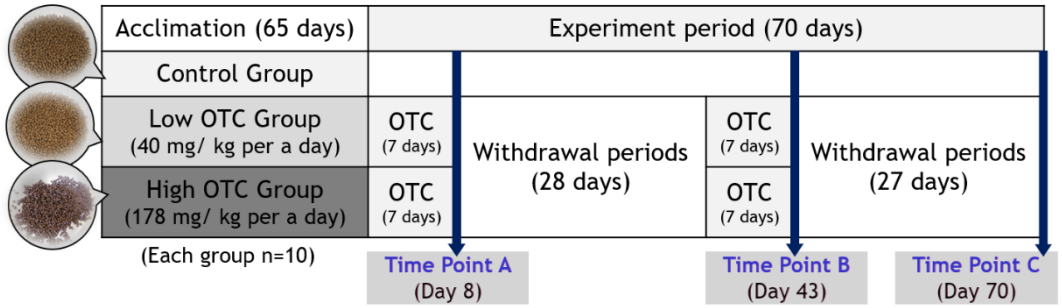

Figure. 1 Experimental groups and three-sampling schedules.

② Primer design (Table 1):

- Immunoglobulin M (IgM; membrane-bound IgM; mIgM and secreted IgM; sIgM)
- One reference gene: Elongation factor 1α (Ef-1α)

Table 1. Primers used for Real time qPCR

Gene name	Gene abbreviation	Gene Bank	Primer sequences (5'→3')
Elongation factor 1α	Ef-1α	AF184170	(F) CTGTCAAGGAAATCCGTCGT (R) TGACCTGAGCGTTGAAGTTG
Immunoglobulin M	mIgM	KX599199	(F) GCTATGGAGGCGGAGGAAGATAACA (R) GCAGAGTGATGAGGAAGAGAAGGATGAA
Interleukin-1β	sIgM	JQ811851	(F) ACCTCAGCGTCCTTCAGTGTTTATGATGCC (R) CAGCGTCGTCGTCAACAAGCCAAGC

③ Real-time qPCR: to evaluate the differences of immune related gene's expression level, measured the expression level of immune-related genes by qRT-PCR in liver and spleen. Normalized using Ef-1α gene expression level.

④ The immunophenotyping: Leukocytes were successfully isolated from blood and spleen from each red seabream samples. The used fish antibodies successfully detected the anti-CD4, anti-CD8a and anti-IgM expressed immune cells in red seabream. Spleen and blood lymphocytes were gated on FS & SS dot plot and lymphocytes were analyzed with antibody. The CD4-positive helper T cells, CD8α-positive cytotoxic T cells and IgM-positive B cells STAT3-positive T helper17 (Th17) cells and FOXP3-positive T regulatory (Treg) cells were detected and compare each group.

Result:

The immunophenotyping results shown that there are no significant differences between OTC treated and control groups due to the high individual differences in blood leukocytes and splenocytes (Figure 2 and 3). Overall, OTC treatment might have no effects on the immune cell populations after first OTC administered red seabream juveniles.

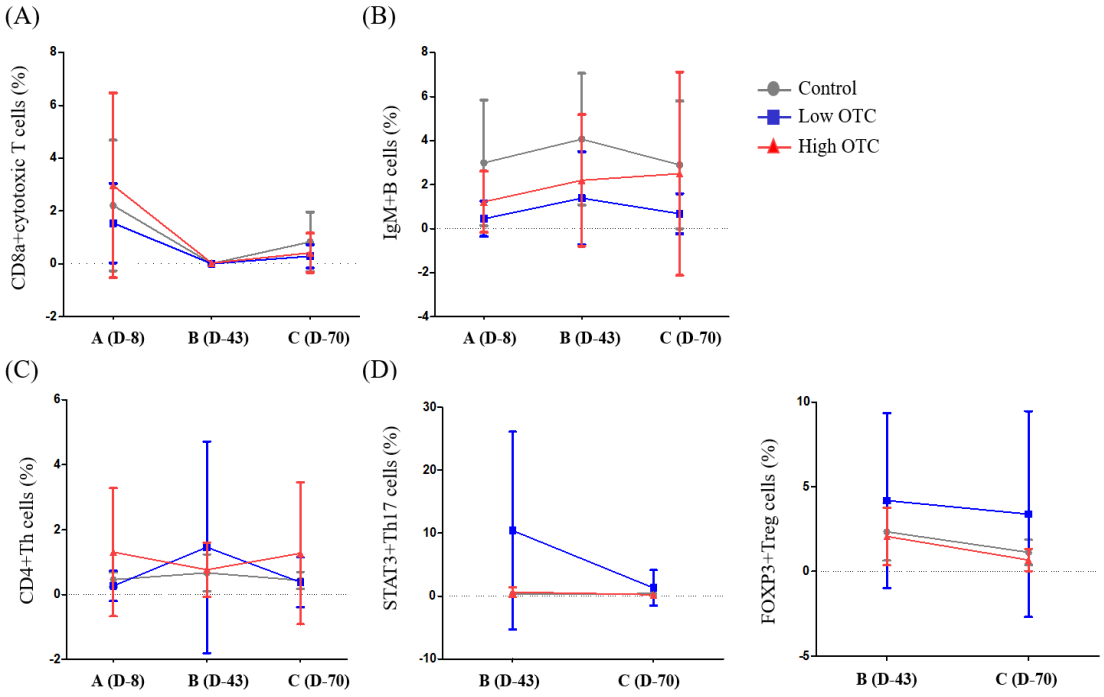

Figure 2. The immunophenotyping results in blood leukocytes. Summary graphs ($n = 6$ per group) of B and T cell frequencies (mean ± SD) are shown. The average percentages (%) of CD8α-positive T cells (A), IgM-positive B cells (B), CD4-positive T cells, STAT3-positive Th17 cells and FOXP3-positive Treg cells were represented with SD.

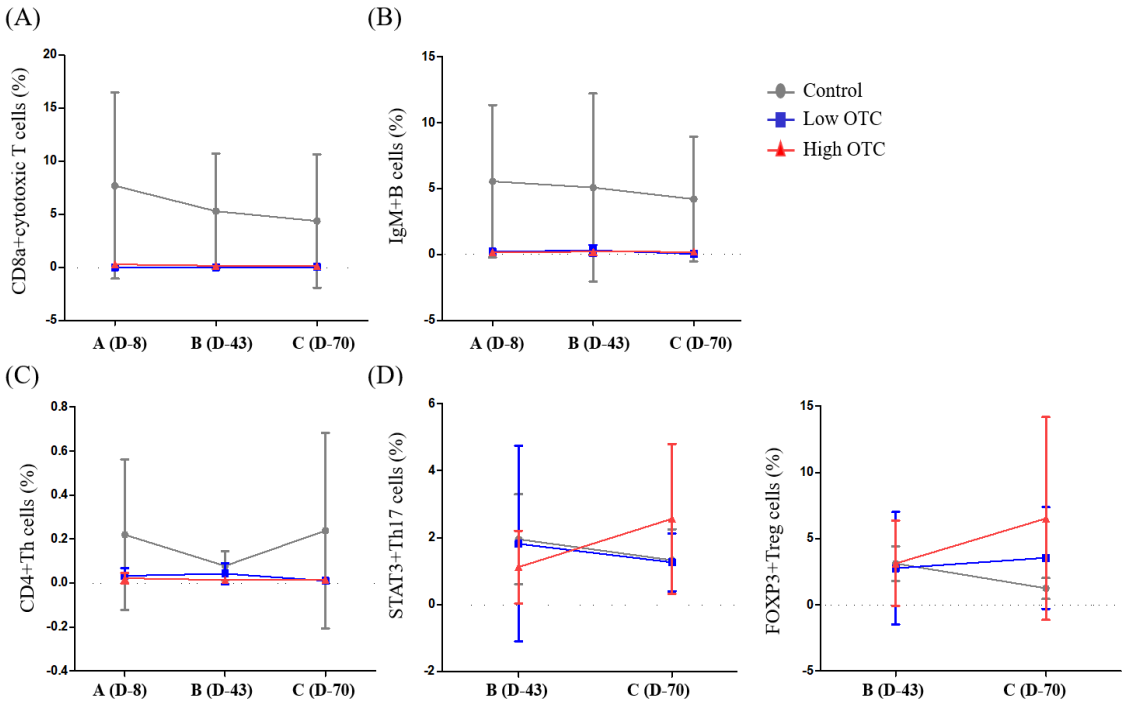

Figure 3. The immunophenotyping results in splenocytes. Summary graphs ($n = 6$ per group) of B and T cell frequencies (mean ± SD) are shown. The average percentages (%) of CD8α-positive T cells (A), IgM-positive B cells (B), CD4-positive T cells, STAT3-positive T cells and FOXP3-positive T cells were represented with SD.

In order to examine the effect of oxytetracycline on Immunoglobulin M genes, RT-qPCR was performed (Figure 4). The RT-qPCR results shown that there are no significant differences between OTC treated and control groups due to the high individual differences. Overall, OTC treatment might have no effects on the immune cell populations after first OTC administered red seabream juveniles.

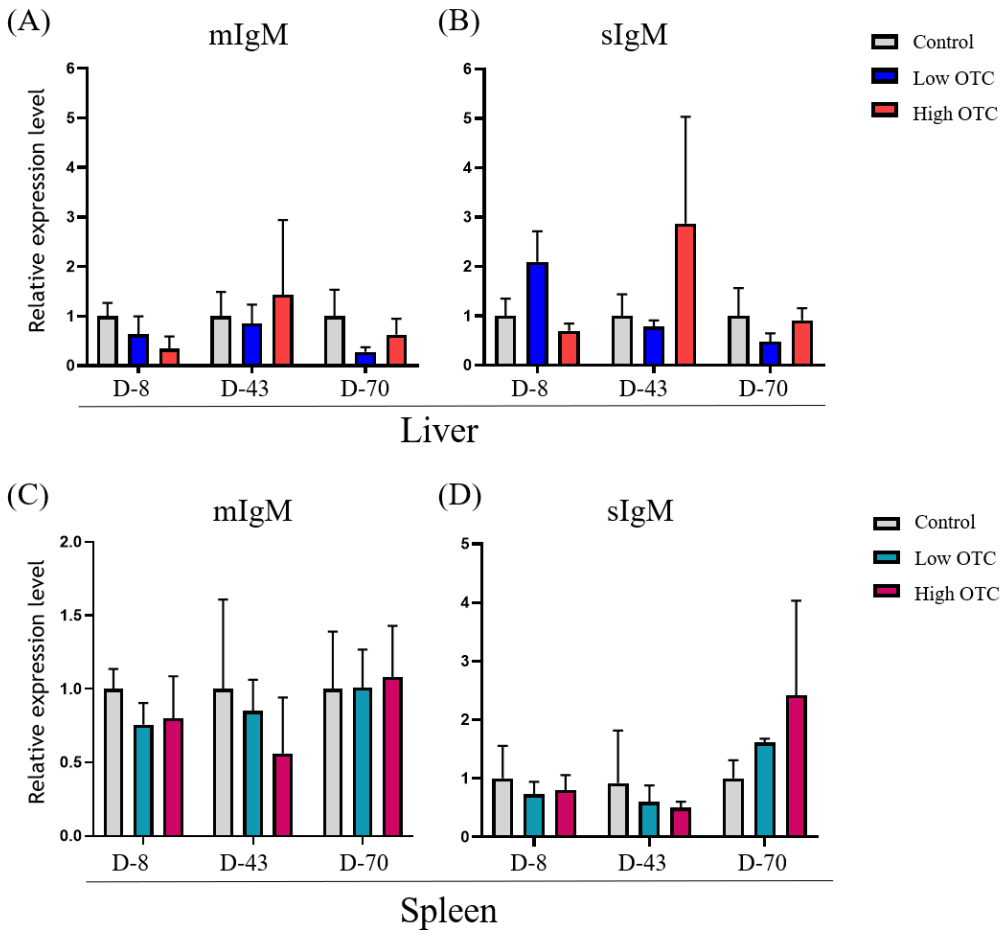

Figure 4. Relative expression levels of immunoglobulin M genes from OTC administrated red seabream. Relative expression levels of mIgM (A) and sIgM (B) from liver and mIgM (C) and sIgM (D) from spleen were represented with SD ($n = 3$ per group). Asterisk indicated significantly difference compare to the control group at same sampling point using one-way analysis of variance followed by Dunnett's multiple comparison test ($p < 0.05$). ND represented that are not detectable due to the under the detection limit.

Publication/conference presentation:

In progress:

- With improved data, this study would be presented with other researchers in the conference and published as a paper.

Perspectives in future:

This study could contribute to

- The understanding of the immunomodulatory effects in OTC administrated fish
- Provides basic immunological knowledge on fish disease prevention for the development of immunoactive agents
- The establishment of appropriate regulations and management of OTC usage in aquaculture.
- Solving the problem of drug-resistant bacteria occurrence caused by misuse and abuse of antibiotics

Also, by reducing the use of antibiotics, environmental and ecological side effects such as residual effects could be alleviated.