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Comparative susceptibilities and immune-related gene expressions of brown trout strains and their hybrids infected with *Lactococcus garvieae* and *Yersinia ruckeri*

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ABSTRACT

Brown trout are polymorphic salmonid species, and it is of importance to investigate whether hybridization affects disease resistance. In this study, susceptibility of brown trout (*Salmo trutta* Abant, Anatolian, Black Sea, and Caspius) strains and their hybrids to *Lactococcus garvieae* and *Yersinia ruckeri* as well as their immune-related gene expression profiles were studied. Results indicated that reciprocal hybridization did not affect disease resistance in brown trout strains. Purebred Black Sea strain of brown trout was the most resistant group against *Y. ruckeri*, followed by other Black Sea strain hybrids. On the other hand, purebred Anatolian strain was the most resistant group to *L. garvieae*, followed by other Anatolian strain hybrids. Expression pattern of target genes differed in families, but the overall gene expression was comparatively high in *Y. ruckeri* infected families. Upregulations were mainly significant at 7 and 28 d post infection while marginal regulations were observed 8 h after infection. Disease resistance status of strains was supported by high expression of immune-related genes such as major histocompatibility complex class I (*MHC-I*), immunoglobulin light chain (*IgL*), and antioxidant- and hemoglobin-related gene expression. Therefore, our findings suggest that Black Sea and Anatolian strains could be used to develop fish stock that are resistant for yersiniosis and lactocaccosis, respectively.

1. Introduction

Changes in environmental conditions, high stocking rate, handling, and any other human intervention can participate the emergence of infectious diseases [1,2], which restrict aquaculture development and cause serious economic losses [3,4]. Yersiniosis caused by *Yersinia ruckeri* and lactococcosis caused by *Lactococcus garvieae*, are common fish diseases that affect a wide variety of fish species including salmonids [2,3,5,6] when fish get stressed due to environmental conditions and handlings. Control measures for yersiniosis and lactococcosis in fish culture include use of antibiotics, vaccines, probiotics, and immunostimulants [5,6]. Development of disease-resistant fish by breeding is considered the best fish health management practice. Pure breeding and cross-breeding have been practiced in aquaculture to improve desired traits including increased growth rate, disease resistance, and environmental tolerance [7]. While pure breeding relies on the additive component of genetic variance, cross-breeding between populations, families, strains, and lineages benefit from hybrid vigor due to additive and non-additive components. For example, coho salmon and rainbow trout hybrids showed an increased disease resistance against IHN virus [8]. Hybrid catfish (female channel catfish, *Ictalurus punctatus* × male blue catfish, *I. furcatus*) were more resistant to columnaris disease compared to parent strains (Arias et al., 2012). Therefore, it is possible to increase resistance of fish against disease by selective breeding or hybridization since different strains or ecotype of fish species can vary in disease resistance.

The host's immunological and physiological conditions play a vital role in disease susceptibility. It is well known that susceptibility to diseases varies between species and strains within the family of *Salmonidae* [9–11]. Heritable variation in resistance to infectious diseases has been identified in brown trout and Atlantic salmon populations [12–14] indicating the potential for selective breeding and hybridization towards increased disease resistance in farmed fish and thus reducing requirements for therapeutics. Antibiotics are commonly used to treat yersiniosis and lactococcosis diseases. However, microorganism can develop resistance to antimicrobials and pass antimicrobial resistance genes to other environmental or pathogenic bacteria [15,16]. Therefore, hybridization or selective breeding would not only improve

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disease resistance and reduce losses due to diseases but also reduce or eliminate antimicrobial usage in aquaculture.

Taxonomic status of *Salmo trutta* belonging to the Danubian lineage was evaluated recently. Based on biological and genetic findings, four former subspecies of brown trout, *Salmo trutta abanticus* (Abant strain), *S.t. caspius* (Caspian Strain), *S.t. fario* (Anatolian Strain) and *S.t. labrax* (Black Sea Strain), constitute a single biological entity and recognized as strains [17]. In the present study, we aimed to compare disease resistance profiles of brown trout strains by determining survival and immune-related gene expression after challenge with *Y. ruckeri* and *L. garvieae*. Immune-related gene expression were studies in the kidney and liver tissues as they are important lymphoid organs. The aims of this study were to determine resistant strains or hybrids, to develop quantitative Real-Time PCR assays for evaluating the changes in immune-related genes, and to compare expression profiles of strains and hybrids after experimental infection with *Y. ruckeri* and *L. garvieae.*

2. Materials and methods

2.1. Experimental fish

Abant (A), Anatolian (N), Black Sea (B), Caspian (C) strains of *Salmo trutta* belonging to Danubian lineage were crossed reciprocally and all possible conspecific and heterospecific families were produced as described in Ref. [17]. When fish reached to 5 g, they were tagged behind the eye with visible implant elastomer tags (Northwest Marine Technology Inc.) to identify individuals of each family and stocked in 10 m^3 flow-through circular fiberglass tanks with aeration. Fish were maintained at ambient temperature and a natural photoperiod until bacterial challenge and gene expression tests. While 16 families were used for the challenge test, purebreds and one of each reciprocal hybrid families were used in gene expression assay (Table 1) since no significant differences were found between reciprocals such as AB and BA etc.

2.2. Bacteria

Yersinia ruckeri RB0708 and *L. garvieae* LgPer strains were cultured in TSA agar for 24 h at 25 °C and 30 °C, respectively. To ensure the purity of culture, bacteria were characterized by biochemical and molecular tests [15,18]. A single colony of each bacteria was isolated from TSA agar and suspended in BHI broth. After overnight incubation, bacteria were harvested by centrifuging at 5000 × *g* for 10 min, and the pellet was resuspended in PBS (pH 7.4). The final concentration of bacteria was adjusted to 1×10^{10} CFU by plate counting technique.

2.3. Fish challenges

The experiments described in this study were approved by the Institutional Animal Care and Use Committee at Karadeniz Technical University (approval #14/2013). Fish were transferred from outdoor fiberglass tanks to 40-L capacity flow-through spring-water supplied aquaria with continuous aeration. Fish were acclimated to experimental conditions for 15 days prior to challenge. Due to the insufficient number of tanks and space, *Y. ruckeri* and *L. garvieae* pathogenicity tests were performed separately at two different times. The mean water temperature during the *Y. ruckeri* and *L. garvieae* challenge test was 11.2 ± 1.1 °C and 17.7 ± 0.3 °C respectively.

Fish were anesthetized with benzocaine solution (250 mg/L) before experiments. A total of 40 fish from each family (2 × 20 fish/aquarium/per dose) were infected by intraperitoneal injection of 0.1 ml of five different bacterial doses (concentrations), 10^{10}, 10^8, 10^6, 10^4, and 10^2 CFU/ml. Negative control groups (2 × 20 fish/aquarium) were injected with 0.1 ml of PBS. Fish were fed with pellet fed twice a day. Cumulative mortality was monitored daily for 28 days, and dead fish were removed immediately for routine bacteriological examination. LD_{50} values for each family were estimated according to the method described by Ref. [18]. During the experiment, discharge water sterilized by UV filters to prevent bacterial contamination of the environment.

2.4. Gene expression assay

Gene expression assay of immune-relevant genes (n = 13) in each family following injection with *Y. ruckeri* and *L. garvieae* were conducted separately. Bacterial concentrations of *Y. ruckeri* and *L. garvieae* were adjusted to doses below the LD_{50} values, which were calculated at the end of the challenge experiment (Table 2). A total of 30 fish (2 × 15 fish/aquarium) from each family was anesthetized by benzocaine (250 mg/L) and injected intraperitoneally with 0.1 ml of the bacterial suspension. Negative control groups were injected with 0.1 ml of PBS. Water temperature was varied between 15 and 18 °C throughout the experiment. Three fish from each family including control group (one fish from each aquarium) were sampled at 0.3 (8 h), 1, 3, 7, 14, and 28 days post injection (dpi). The time zero samples were collected before the challenge experiment as unchallenged controls. Fish were sacrificed on ice, and 100 mg of kidney and liver tissue were taken from each fish and stored at −80 °C immediately until RNA isolation. Liver samples were used for antioxidant enzymes related genes expression while kidney samples were used for the expression of rest of the genes.

Table 1

Average length (L), and weight (W) of purebred brown trout [*Salmo trutta* Black Sea (B), Abant (A), Caspian (C) and Anatolian (N) strains] and their hybrids.

Fish Family	Abbr.	*Y. ruckeri*		*L. garvieae*	
		L (cm)	W (g)	L (cm)	W (g)
Black Sea X Black Sea	BB[a]	12.57 ± 0.84	18.19 ± 3.83	13.55 ± 0.83	32.11 ± 5.62
Black Sea X Abant	BA[a]	11.58 ± 2.27	14.96 ± 1.15	12.0 ± 0.71	22.95 ± 2.94
Black Sea X Caspian	BC[a]	9.85 ± 1.30	18.33 ± 2.99	16.15 ± 2.35	59.31 ± 11.03
Black Sea X Anatolian	BN[a]	8.29 ± 0.73	6.10 ± 1.56	8.18 ± 0.73	6.38 ± 1.70
Abant X Abant	AA[a]	7.62 ± 0.16	16.10 ± 2.14	12.4 ± 1.40	16.81 ± 3.00
Abant X Caspian	AC[a]	7.19 ± 0.87	11.04 ± 3.34	11.8 ± 0.71	19.62 ± 3.37
Abant X Anatolian	AN[a]	8.46 ± 0.59	6.31 ± 1.26	8.29 ± 0.66	6.20 ± 1.30
Abant X Black Sea	AB	8.93 ± 0.80	12.70 ± 3.54	10.75 ± 0.25	18.93 ± 1.62
Anatolian X Anatolian	NN[a]	8.22 ± 0.80	6.09 ± 1.34	8.22 ± 0.80	6.09 ± 1.34
Anatolian X Caspian	NC[a]	6.99 ± 3.29	11.27 ± 2.19	9.5 ± 0.90	8.56 ± 0.70
Anatolian X Black Sea	NB	7.97 ± 0.96	5.82 ± 0.96	7.92 ± 0.99	5.80 ± 1.85
Anatolian X Abant	NA	8.11 ± 0.24	6.01 ± 1.01	8.00 ± 0.50	5.94 ± 1.12
Caspian X Caspian	CC[a]	13.50 ± 2.36	29.47 ± 11.36	12.85 ± 0.59	20.53 ± 3.01
Caspian X Black Sea	CB	7.99 ± 2.59	10.27 ± 2.99	9.0 ± 0.70	9.16 ± 0.50
Caspian X Abant	CA	11.38 ± 2.36	19.18 ± 5.61	13.92 ± 1.42	28.19 ± 8.67
Caspian X Anatolian	CN	10.48 ± 2.36	20.08 ± 4.66	14.82 ± 1.42	29.09 ± 7.82

[a] Families used in gene expression study.

Table 2
Primer sequences including their GenBank accession numbers (Acc. No.) for the genes analyzed by qPCR. Primers are read from the 5′ end to the 3’ end.

Genes	Abbr.	Acc. No.	Primer Set(s)
Superoxide dismutase	SOD	BT057901	Fwd: CCACGTCCATGCCTTTGG Rev: TCAGCTGCTGCAGTCACGTT
Catalase	CAT	BE669040	Fwd: GAGGGCAACTGGGACCTTACT Rev: GGACGAAGGACGGGAACAG
Glutathione peroxidase	GPx	BG934453	Fwd: GATTCGTTCCAAACTTCCTGCTA Rev: GCTCCCAGAACAGCCTGTTG
Glutathione reductase	GR	BG934480	Fwd: CCAGTGATGGCTTTTTTGAACTT Rev: CCGGCCCCCACTATGAC
Cold-inducible RNA-binding protein	CIRBP	JZ713052	Fwd: TTCTAGGCCGGGGATCCTAC Rev: ATACAGCCAGCCAAGGGATG
Cyclin-dependent kinase inhibitor 2A	CDKN2A	JZ713054	Fwd: CGAAATGGCCGAGGGTGATA Rev: CCTCCTGGCCTTCCAATGTT
Prothymosin alpha 1	PTMA	BT057594	Fwd: CGAGCGTTTTGGAGGGAAAC Rev: ACGGACATTGGGTGTGTGTA
Transforming protein RhoA	TPRA	JZ713063	Fwd: ACTACGACAGACTACGGCCT Rev: GCTGTCGGGGCTATCTATGG
Immunoglobulin light chain	IgL	AF273017	Fwd: CAGACTCCGGTCACCAAGAC Rev: CTCAATCCAAGCCCCCTGTT
Major histocompatibility complex class I	MHC-I	JZ713068	Fwd: TGAAGAGAGAGGGAGCCGAT Rev: TGAAGCGATCCTTTGCCACT
Hemoglobin subunit beta	HBB	JZ713069	Fwd: ATCACGGTCTTTCCGTGCTT Rev: AGACACTTCAGCACCTTCGG
Stanniocalcin precursor	STC	JZ713071	Fwd: GCCATGACATCCCCGTTTTG Rev: GATGTCAAACCCCACCCACT
Beta-actin (internal control)	β-actin	BG933897	Fwd: GACAGCTACGTTGGAGACGA Rev: GTTGGCTTTGGGGTTGAGTG

2.5. RNA isolation

Total RNA was isolated from liver and kidney. 50 mg frozen tissue was homogenized in 1 ml Triazole with a homogenizer (Bioprep-24) at a setting of 700 m/s for a total of 3 × 30 s. Homogenate was transferred into a 1.5 ml Eppendorf tube, and 250 μl of chloroform was added to the sample. The mixture was shaken vigorously for about 15 s, aqueous phase removed and centrifuged at 14.000 × *g* for 5 min. The RNA was precipitated from the aqueous phase by mixing with 550 μl of isopropanol. Samples were incubated at room temperature for 5 min and centrifuged at 14.000 × *g* for 20 min. The supernatant was removed, and the RNA pellet was washed with 80% ethanol. The pellet was air-dried at room temperature, diluted with 40 μl DEPC treated water. The quality and quantity of the total RNA was assessed using a Nanodrop spectrophotometer (Nano-200, Hangzhou AllSheng) and stored at −80 °C.

2.6. cDNA synthesis

cDNA was synthesized by reverse transcription of RNA using the ABI cDNA Reverse Transcriptase Kit (Applied Biosystems, USA). PCR reaction was performed in a total volume of 10 μl with 5 μl of diluted template RNA (850 ng/μl), 1 μl 10X RT Buffer, 0.4 μl 10 nM dNTP, 0.5 μl RNase inhibitor, 0.5 μl reverse transcriptase, 1 μl 50 μM random primer and DEPC-treated water. Reverse transcription was performed in T100 Thermal Cycler (Bio-Rad Laboratories, California, USA) with incubation at 25 °C for 10 min, incubation at 37 °C for 2 h and inactivation of the enzyme at 85 °C for 15 min. The synthesized cDNA samples were diluted 1:10 with ultrapure water and stored at −20 °C until use.

2.7. Real-time PCR assay

The Real-time PCR (RT-qPCR) analysis of potential immune-relevant genes expression was performed with Exicycler 96 (Bioneer). CAT, GPx, GR and Beta-actin genes were amplified by using primers developed by Hansen et al. [19]. While the rest of the primers were designed using Primer Express 2.0 software to amplify target genes (Applied Biosystems, Foster City, CA, USA) (Table 2). RT-qPCR was carried out in a total volume of 25 μl containing 1 μl of diluted cDNA, 1 μl of EvaGreen (Biotium, Inc. Parkway Fremont, CA, USA), 12.5 μl of hot start Taq 2X Master Mix (NEB). The cycling conditions were 95 °C for 60 s followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 58 °C for 20 s, extension at 68 °C for 45 s and melting curve program (59–94 °C with a gradual heating rate of 0.5 °C/10 s and continuous fluorescence reading). Expression of genes encoding catalase (*CAT*), superoxide dismutase (*SOD*), glutathione peroxidase (*GPx*), glutathione reductase (*GR)*, cyclin-dependent kinase inhibitor 2A (*CDKN2A*), cold-inducible RNA-binding protein (*CIRBP*), prothymosin alpha 1 (*PTMA*), major histocompatibility complex class I (*MHC*-I), immunoglobulin light chain (*IgL*), transforming protein RhoA (*TPRA*), hemoglobin subunit beta (*HBB*) and stanniocalcin precursor (*STC*) were determined. β-actin was used as a reference gene. qPCR products were visualized on 2% agarose gel to verify amplification.

qPCR efficiency was calculated using 10-fold cDNA dilution series. qPCR was performed by using the target gene primers or beta-actin primer as described above. C_T (y-axis) and log cDNA dilution (x-axis) values were plotted, and the slope of the line was determined.

The gene expression results were analyzed using the $2^{-\Delta\Delta CT}$ method [20]. Beta-actin gene was used as an internal control (reference gene) for normalization.

$$2^{-\Delta\Delta CT} = [(C_T \text{ target gene - } C_T \text{ internal control})_{\text{sample}} \text{ - } (C_T \text{ target gene - } C_T \text{ internal control})_{\text{control}}]$$

$$E = 10^{-1/slope}$$

Amplification efficiencies (E) of each PCR product were determined according to the formula:

$$\text{\% Amplification efficiency} = (E-1) \text{ x } 100 \text{ [20]}$$

A value more than one indicates upregulation while a value lower than one indicates downregulation of relative gene expression.

2.8. Statistical analyses

LD_{50} values of *Yersinia ruckeri* and *L. garvieae* for each family were calculated by probit analysis (IBM SPSS 23, SPSS Chicago, IL, USA). Survival curves were plotted by the Kaplan Meier method and verified by the Cox-Mantel (log-rank) test with Statistica (StatSoft, Tulsa, OK, USA). Changes in gene activity ($2^{-\Delta\Delta CT}$), slope, and amplification efficiency (E) were calculated using Microsoft Excel. Differences between each family were determined by One-way ANOVA and Cox-Mantel test (IBM SPSS 23). Partial Least Squares (PLS) regression method was performed to model the gene expression results of fish families by using XLSTAT Version 2018 [21].

3. Results

3.1. Challenge experiment

Initial mortality was started at 3 dpi when fish were exposed *L. garvieae* and *Y. ruckeri* and continued until the 11 and 15 dpi, respectively. Pure culture of bacteria was isolated from liver, kidney, and spleen of dead fish. While highest mortality was observed at a dose of 1×10^9 CFU/fish in both challenges, no fish died at a dose lower than 1×10^4 CFU/fish and 1×10^2 CFU/fish in any family when fish exposed to *L. garvieae* and *Y. ruckeri*, respectively. No mortality was observed during the 28-d experiment in control group. While BB family was the most resistant group against *Y. ruckeri* infection ($10^{7.571}$ CFU/fish), the most susceptible family against *Y. ruckeri* infection was the NA family ($10^{2.124}$ CFU/fish) (Table 3). CA family ($10^{6.54}$ CFU/ml) found as the least resistant while NN family ($10^{9.66}$ CFU/fish) was the most resistant to *L. garvieae* infection (Table 3). Statistically significant differences were found between some of the families according to the survival analysis (Table 4).

3.2. Gene expression

The expression of 12 genes and internal control (beta-actin) was comparatively profiled in each fish families (n = 10) infected with *Y. ruckeri* and *L. garvieae*. Expression analysis indicated that 10 out of 12 genes were upregulated in *Y. ruckeri* infected families compared to *L. garvieae* infected families, whereas *PTMA* and *HBB* genes were expressed at a similar level. Generally, significant regulations were observed at 7 dpi and 28 dpi, whereas marginal regulations were observed at 0.3 dpi (Figs. 1–8). Amplification efficiencies of PCR were ranged between 96% and 101% depending on the genes.

3.2.1. Antioxidant enzymes

Significant variations among families were observed with regard to the expression profiles of *CAT, SOD, GPx* and *GR* genes following the *Y. ruckeri* infection. Significant upregulation of *CAT* was observed at 7 dpi in all families except for CC. The highest level of expression was appeared at 14 dpi in BC (55.39 fold), and BB (30.52 fold) family whereas it decreased to low levels at 28 dpi (Fig. 1). *SOD* was upregulated in all families at 7 dpi. The highest changes in gene expression level were observed between 3 and 28 dpi. The highest level of gene expression appeared at 28 dpi in BB (9.79 fold) and NN (6.76 fold) families (Fig. 1). *GPx* was significantly upregulated at 7 dpi in most of the families except for NB and CC, which were downregulated at that time point. Expression level reached to the highest level (15.41 fold) in NN family at 3 dpi, and the expression returned to the control group level at 14 dpi (Fig. 1). Overall regulation of *GR* was marginal. Significant upregulation was observed in families except for BB, AN, and NN at 1 dpi. Down-regulation of *GR* expression was observed in families except for CC, BN, and NC. Expression level reached the highest level at 3 dpi in the BN family, which was downregulated over time until reaching the control group level at 28 dpi (Fig. 1).

Table 3

Susceptibility of brown trout strains and their hybrids to *Y. ruckeri* and *L. garvieae*.

Family	*Y. ruckeri*	*L. garvieae*
	LD_{50} (CFU/fish)	LD_{50} (CFU/fish)
BB	$10^{7.57(7.37-8.34)*}$	$10^{6.94(6.07-8.59)*}$
BA	$10^{7.20(6.82–7.59)}$	$10^{8.56(7.41–8.92)}$
BC	$10^{6.74(6.67–7.21)}$	$10^{7.45(6.89–9.13)}$
BN	$10^{3.11\ (1.41–4.13)}$	$10^{7.78(7.06–10.69)}$
AA	$10^{5.05(2.59–7.09)}$	$10^{7.47(7.01–9.88)}$
AC	$10^{4.67(4.27–5.07)}$	$10^{7.77(7.14–10.01)}$
AN	$10^{2.85\ (1.21–3.79)}$	$10^{6.80(4.86–8.37)}$
AB	$10^{6.68(6.68–8.10)}$	$10^{6.89(6.42–7.67)}$
NN	$10^{4.61(4.09–5.12)}$	$10^{9.66(8.93–10.50)}$
NC	$10^{3.01\ (2.52–3.80)}$	$10^{7.67(7.13–8.49)}$
NB	$10^{4.27(3.62–4.83)}$	$10^{7.41(6.70–8.11)}$
NA	$10^{2.12\ (1.01–3.42)}$	$10^{8.12(7.32–9.24)}$
CC	$10^{4.23(2.19–6.00)}$	$10^{8.44(7.48–9.58)}$
CB	$10^{6.69(6.60–9.49)}$	$10^{6.84(6.29–7.45)}$
CA	$10^{5.38(4.34–6.48)}$	$10^{6.54(5.82–7.32)}$
CN	$10^{3.57\ (2.94–4.18)}$	$10^{7.47(7.01–8.19)}$

Table 4

Statistical differences between families with regard to the survival rate of the fish infected with *Y. ruckeri* and *L. garvieae* based on Cox-Mantel test. When the P value is greater than 0.05, the (−) sign is used in groups.

Family	*Y. ruckeri*	*L. garvieae*	Family	*Y. ruckeri*	*L. garvieae*
AA-AC	0.02	0.03	NN-CB	–	0.00
AA-AB	–	0.02	NN-FB	0.01	–
AA-BB	0.02	–	NN-BA	0.01	–
AC-BB	0.03	–	NN-BC	0.00	0.00
AN-AA	–	0.00	NN-BB	0.00	0.00
AN-AC	–	0.00	BA-AA	–	0.00
AN-AB	0.00	–	BA-AC	–	0.00
AN-CC	0.04	–	BA-CB	–	0.02
AN-CB	–	0.00	BC-CB	–	0.02
AN-NN	0.00	–	BC-BB	0.01	0.04
AN-BA	0.03	–	BF-AA	–	0.03
AN-BC	0.02	–	BF-AC	–	0.00
AN-BB	0.00	0.00	BF-AB	0.02	–
AB-AA	–	0.02	BF-CB	–	0.00
AB-AC	0.01	–	BF-NA	–	0.02
AB-CB	0.04	–	BF-NN	0.00	–
AB-BB	0.01	–	BF-BA	0.02	–
CB-BB	0.00	–	BF-BC	0.01	–
NA-AA	0.01	–	BF-BB	0.00	0.00
NA-NN	0.00	–	BB-AA	–	0.00
NA-NB	0.00	–	BB-AC	–	0.02
NN-AA	0.00	–	BB-AN	0.00	–
NN-AC	–	0.00	BB-NA	0.00	–
NN-AB	0.00	–	BB-NB	0.00	–
NN-CC	0.02	0.03			

Antioxidant enzyme genes expression in families following the *L. garvieae* infection were similar. *CAT* was significantly upregulated in the BN and NN families whereat downregulated in the rest of the families at 0.3 dpi. The highest levels of expression were appeared in NN (12.25 fold) and NC (11.24 fold) families at 28 dpi (Fig. 2). *SOD* was significantly upregulated (almost 2-folds) in BC, BN, AN, NC and NN families at 0.3 dpi. *SOD* was significantly upregulated in CC and NC at 1dpi, CC at 3 dpi, CC, NC and NN families at 7 dpi. While expression was increased at 28 dpi in AN, NC and NN families, downregulation was observed in the rest of the families (Fig. 2). *GPx* was significantly upregulated only in BC and AN family at 0.3 dpi. *GPx* gene expression was significantly upregulated in CC and AN family at 1 dpi, CC and NC families at 3 dpi, CC, BN and NC families at 7 dpi whereas, downregulation appeared in rest of the families. Families of CC, AN, NC, and NN showed a trend for upregulation at 14 and 28 dpi whereas the rest of the families showed a trend for downregulation compared to control group (Fig. 2). The expression of *GR* was significantly upregulated in BC and NN families at 0.3 dpi, CC, AN and NN families at 1dpi, BC, CC, AN and NC families at 3 dpi, CC, NC, and NN families at 7–14 dpi. Gene expression was peaked in the BN family (4.7 fold) at 7 dpi and NC family (4.65 fold) at 14 dpi (Fig. 2).

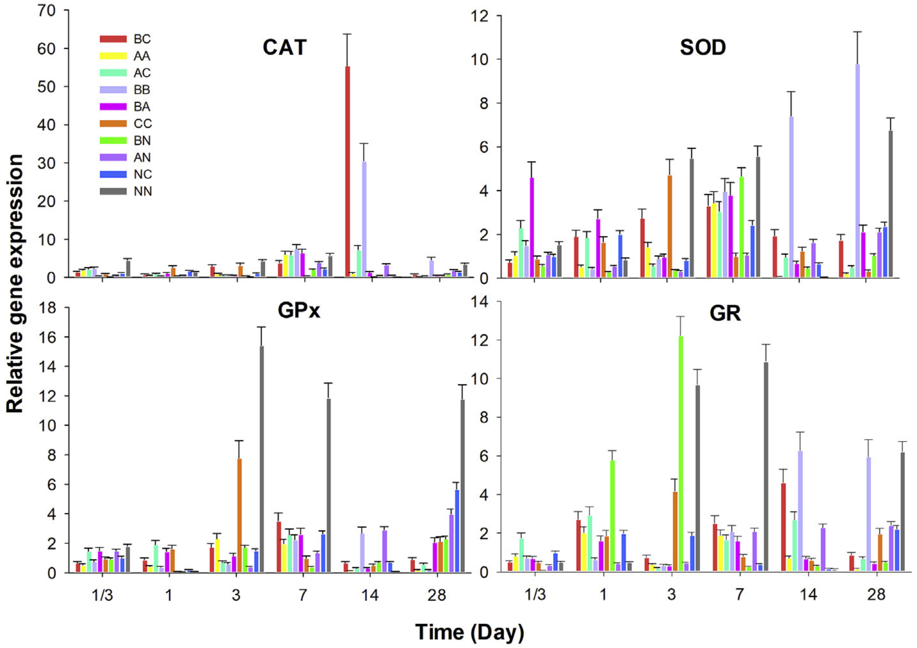

Fig. 1. Relative gene expression profiles of *CAT*, *SOD*, *GPX* and *GR* genes in the liver of brown trout (*Salmo trutta*) strains and their hybrids infected with *Yersinia ruckeri*. Samples were taken at 1/3, 1, 3, 7, 14 and 28 days post exposure. Data are expressed as the mean of three independent samples ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2.2. Cell regulation, cell survival, and cell proliferation

The levels of *CDKN2A* expression in the families were subjected to changes without a clear trend after *Y. ruckeri* infection. Significant upregulation was observed at 1 dpi in the families except for NN and downregulated at 3 dpi. Significant upregulations were continued in BN, NC and NN families. Expression was peaked at 14 dpi in BC family (11.66 fold) which subsequently decreased just above the control group level by 28 dpi. While expression in AA and CC families downregulated at 14–28 dpi, expression in BB family was peaked at 28 dpi (17.2-fold) (Fig. 3). Expression of *CIRBP* was most significant in BC, AA, AC, BB, BA and CC families at all sampling time points throughout the experiment except for 28 dpi. The highest expression was observed in AA (16.72 fold) and BA (21.09 fold) at 7 dpi, AC (22.29 fold) and BB (34.74 fold) at 14 dpi. A sharp decrease in *CIRBP* gene expression was noted at 28 dpi in all families (Fig. 3). Upregulation was started in *PTMA* at 0.3 dpi in families except for BN, AN, and NN. The maximal fold-increase in *PTMA* expression occurred on day 14 in BB (15-fold) and 28 in BB (10.69) and NC (9.20-fold) (Fig. 3).

The highest level of *CDKN2A* regulations was observed in BC and NN families 0.3 days after *L. garvieae* infection. Families of CC, AN, NC, and NN showed a trend for upregulation at 1, 3 and 7 dpi. A clear upregulation trend was observed in the NC family between 7 and 28 dpi (Fig. 4). *CIRBP* gene regulation was marginal in BC, AC, BA, NC and NN families at 0.3 dpi after *L. garvieae* infection. The level of *CIRBP* in NC family increased with the time after challenge and peaked at 28 dpi (8 fold). Expression in families except for NC, NN and AN was decreased to the control group level by 28 dpi (Fig. 4). Significant expression of *PTMA* gene was started at 1 dpi in BB, CC, AN and NC families. The maximal fold increase occurred at 3 dpi in BC family and downregulated over time until reaching the control group level by 14 dpi.

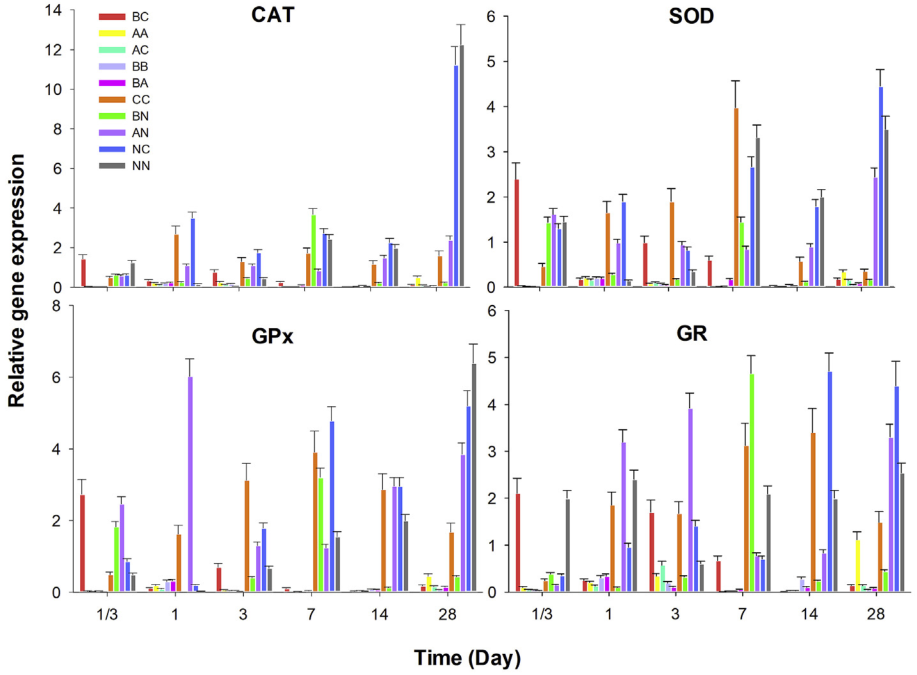

Fig. 2. Relative gene expression profiles of *CAT*, *SOD*, *GPX* and *GR* genes in the liver of brown trout (*Salmo trutta*) strains and their hybrids infected with *Lactococcus garvieae*. Samples were taken at 1/3, 1, 3, 7, 14 and 28 days post exposure. Data are expressed as the mean of three independent samples ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

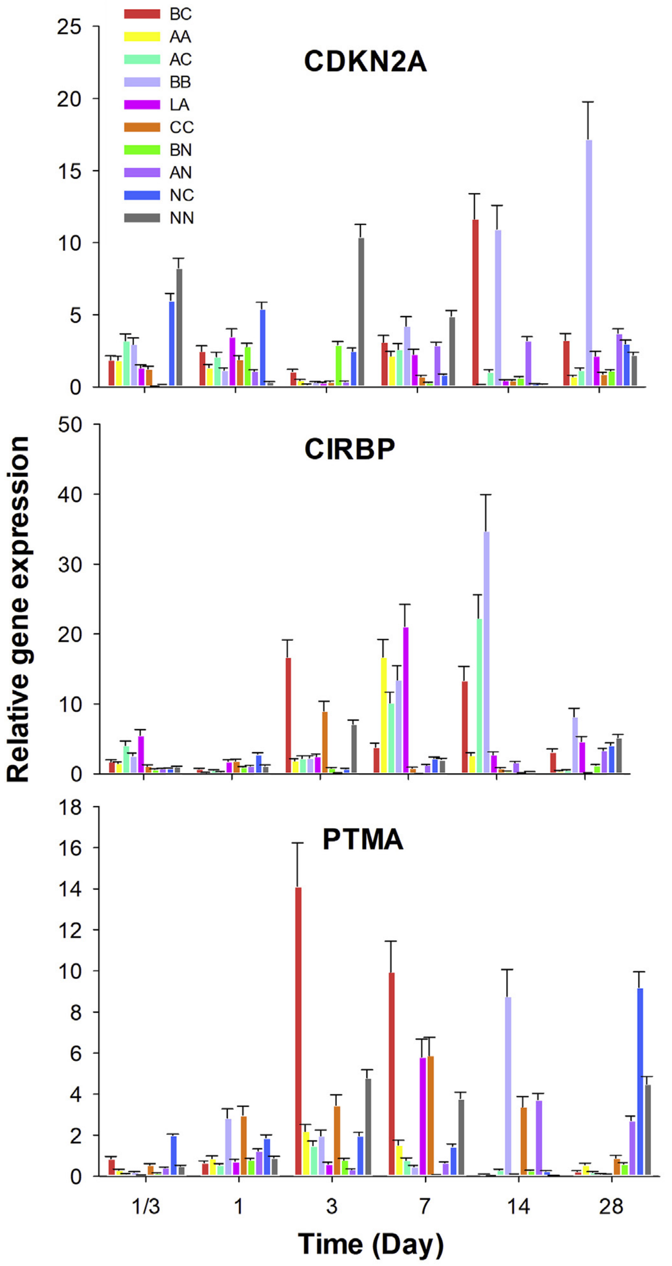

Fig. 3. Relative gene expression profiles of *CDKN2A, CIRBP* and *PTMA* genes in kidney of brown trout (*Salmo trutta*) strains and their hybrids infected with *Yersinia ruckeri*. Samples were taken at 1/3, 1, 3, 7, 14 and 28 days post exposure. Data are expressed as the mean of three independent samples ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

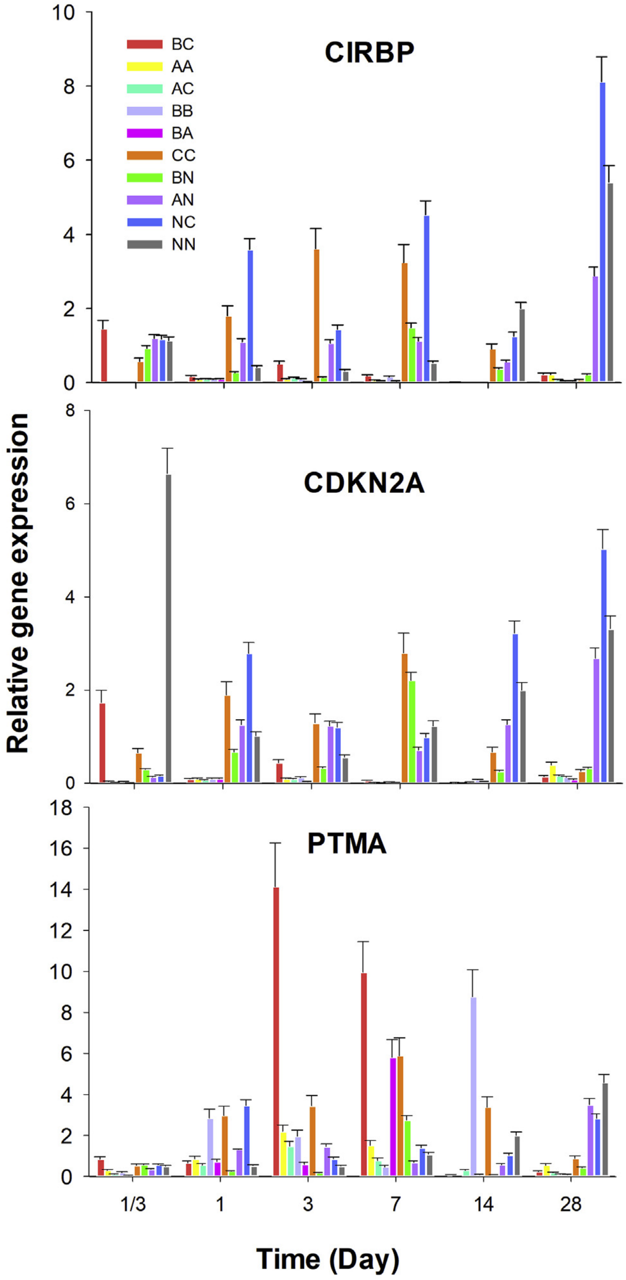

Fig. 4. Relative gene expression profiles of *CDKN2A, CIRBP* and *PTMA* genes in kidney of brown trout (*Salmo trutta*) strains and their hybrids infected with *Lactococcus garvieae*. Samples were taken at 1/3, 1, 3, 7, 14 and 28 days post exposure. Data are expressed as the mean of three independent samples ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Marginal increases in upregulation were observed in BA, CC and BN families at 7 dpi whereafter expression was descended to control levels at 28 dpi (Fig. 4).

3.2.3. *Humoral and cellular immune response*

A modest level of significant expression of *MHC*-I was observed at 1 dpi in families except for BN, NC, and NN after *Y. ruckeri* infection. Expression of *MHC*-I was significant in families except for BA and AA at 7 dpi. Expression was peaked at 14 dpi in BN (34.42 fold), BB (28.84 fold) and AC (13.36 fold) families whereafter expression was descended to marginal levels at 28 dpi (Fig. 5). Significant upregulation of *IgL* gene was observed at 1dpi in all of the families except for CC. The maximal *IgL* upregulation was observed in BB (36.34 fold), NN (34.23 fold) and BN (25.12 fold) families. No clear trend for upregulation observed in *IgL* gene among fish families (Fig. 5).

The expression of *IgL* following the *L. garvieae* infection after 8 h post infection was significantly down regulated in all of the families except for BC. BC, AA, AC, BB, and BA families *IgL* gene was down regulated up to 10 fold while rest of the families *IgL* gene were upregulated at 1 dpi. Similar gene expression continued to 3 dpi except AN which was upregulated at 1 dpi and down regulated at 3 dpi. Significant upregulation of *IgL* gene was observed in CC, BN, AN, NC and NN families at 7 dpi while *IgL* expression was downregulated in the rest of the families at all sampling points. Maximal gene expression occurred in NC (9.16 fold) and NN (12.26 fold) families at 28 dpi. The *IgL* gene in CC,

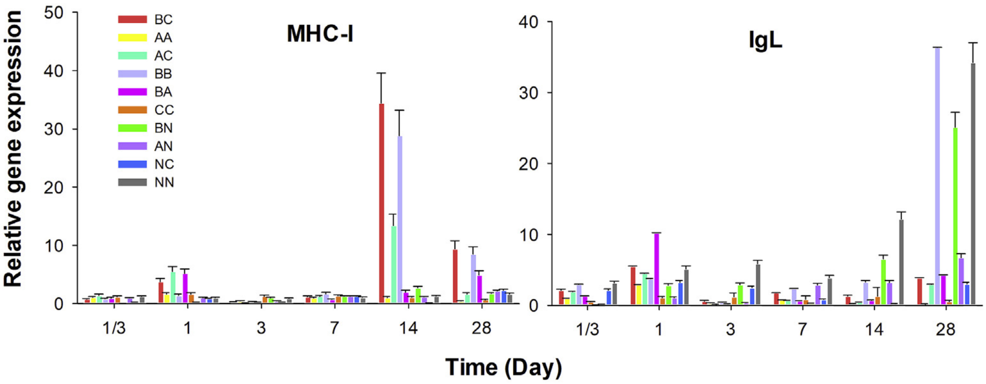

Fig. 5. Relative gene expression profiles of *MHC*-I and *IgL* genes in kidney of brown trout (*Salmo trutta*) strains and their hybrids infected with *Yersinia ruckeri*. Samples were taken at 1/3, 1, 3, 7, 14 and 28 days post exposure. Data are expressed as the mean of three independent samples ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

BN, NC and NN families showed a trend for upregulation starting from 1 dpi (Fig. 6). After 8 h post infection of fish with *L. garvieae, MHC*-I gene expression was significantly downregulated in all families except BN family upregulated and BC family was not affected. *MHC*-I gene expression was down regulated at 1 dpi except CC family and started to upregulate at 3 dpi in the BC, BN, AN, and NN families. The expression of *MHC*-I following the *L. garvieae* infection showed an upregulation trend starting from 7 dpi in the NN, NC and AN family, which were significant at 7, 14 and 28 dpi. The highest expression was obtained at 3 dpi in BC (5.92 fold) and at 7 dpi in CC (5.23 fold) (Fig. 6).

3.2.4. *Signal transduction, calcium homeostasis, hemoglobin*

The highest level of *TPRA* following the *Y. ruckeri* infection was observed in BC family (44.37 fold) at 14 dpi and BB family (24.34 fold) at 28 dpi after *Y. ruckeri* infection. *TPRA* was down-regulated in BB (0.24 fold) and BA (0.27-fold) families at 3 dpi. Expression was significant only in BC and BB families at 28 dpi. The time-dependent changes in expression were low in AA, CC and NC families (Fig. 7). The general trend of the gene expression pattern of *HBB* following *Y. ruckeri* infection differed among families. Expression of *HBB* was significantly up-regulated in BC family from 0.3 dpi to 14 dpi, and the expression was down-regulated at 28 dpi. Expression levels in AA, AC, CC, and BA families fluctuated throughout the experiment. The maximal up-regulations occurred in BC (35.51 fold) at 14 dpi, BB (16.86 fold) and AN (20.35 fold) families at 28 dpi (Fig. 7). Significant *STC* up-regulation was observed in all families at 7 dpi except for AN. The highest expression was seen in BC (35.51 fold) at 14 dpi and AN (20.35 fold) at 28 dpi (Fig. 7).

Expression of *TPRA* gene following the *L. garvieae* infection was up-regulated at 0.3 dpi only in BC family that was significantly different from the others. Expression in BN (10.06 fold) and NC families (15.67 fold) peaked at 7 dpi and 28 dpi respectively. Expression was only significant in NC and NN families at 28 dpi (Fig. 8). No clear *HBB* up-regulation trend was observed in families following the *L. garvieae* infection. Significant up-regulation was observed in CC, AN, and NC at 1 dpi, CC and AN at 3 dpi, CC at 7 dpi, NN at 14 dpi, BA, NC and NN at 28 dpi. The highest expression rates were observed in NC (7.52 fold) at 1 dpi. *HBB* gene expression was only modestly up-regulated in AN, NC and NN families at 28 dpi. Up-regulation of the *HBB* gene was observed only in AN, NC and NN genes at 28 dpi that were also significant (Fig. 8). Meanwhile, expression was down-regulated in the rest of the families. Down-regulation of *STC* was observed in families except for BC and NN at 0.3 dpi. Expression was only modestly up-regulated (1.89–5.99 fold) in the NC family between 1 and 28 dpi. *STC* up-regulation was only observed in AN (4.58 fold), NC (5.11 fold) and NN (2.78 fold) at 28 dpi, which were significant (Fig. 8).

4. Discussion

Interspecific hybridization (crossbreeding) have been practiced to improve desired traits such as increased growth rate, disease resistance, environmental tolerance, better food conversion in aquaculture [7]. The success of hybridization depends on the genetic structure of parental species, crossing patterns and directions. The expected output of hybridization is a hybrid with the desired trait or better characteristic than its parents. While pure breeding relies on the additive component of genetic variance, cross-breeding between populations, families, strains, and lineages benefit from heterotic effect, additive and non-additive components. Hybrid fish species with increased resistance against parasites [22,23], viral [24,25] and bacterial pathogens [26] were reported previously. Up to now, only a few studies investigated whether disease resistance is affected by inter and intraspecies hybridization in salmonids. In this study, we have evaluated disease resistance of brown trout strains and their hybrids against *Y. ruckeri* and *L. garvieae*.

Yersinia ruckeri is the causative agent of enteric redmouth disease or yersiniosis. Mortalities caused by *Y. ruckeri* is dependent on the type of bacterial strain, fish species, fish size, and water temperature. Austin

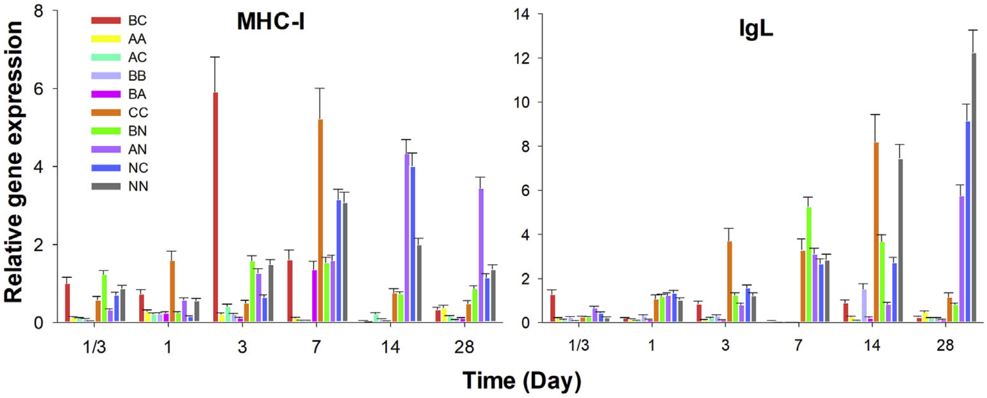

Fig. 6. Relative gene expression profiles of *MHC*-I and *IgL* genes in kidney of brown trout (*Salmo trutta*) strains and their hybrids infected with *Lactococcus garvieae*. Samples were taken at 1/3, 1, 3, 7, 14 and 28 days post exposure. Data are expressed as the mean of three independent samples ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

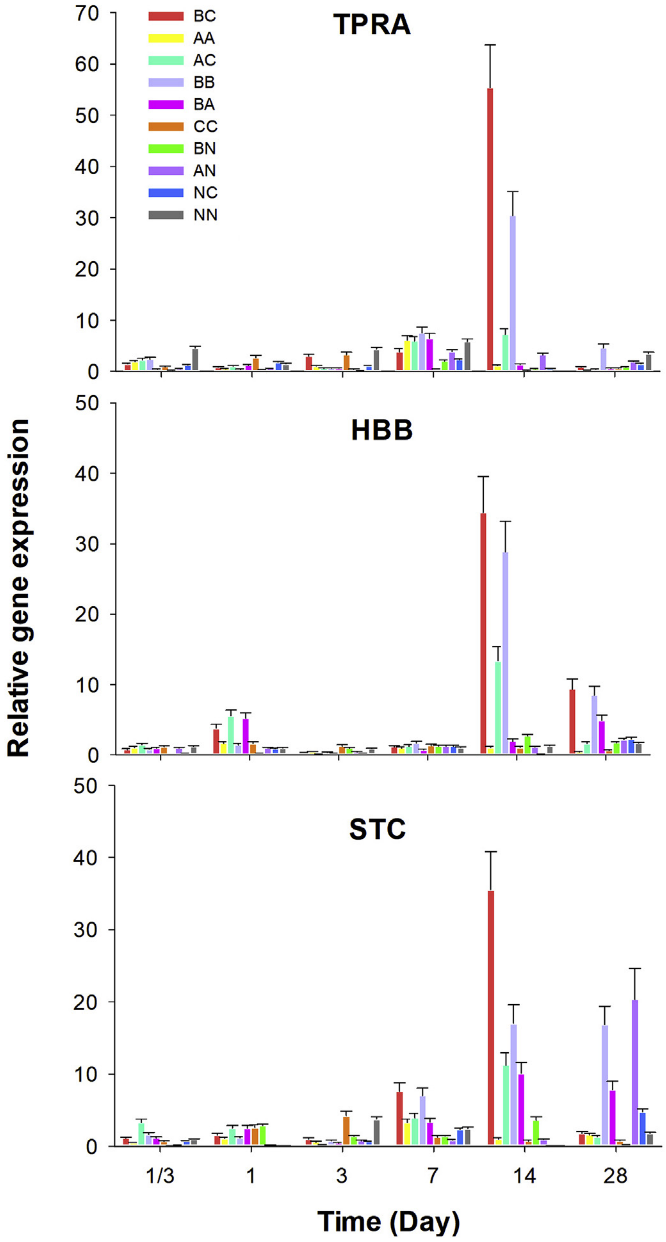

Fig. 7. Relative gene expression profiles of *TPRA, HBB* and *STC* genes in kidney of brown trout (*Salmo trutta*) strains and their hybrids infected with *Yersinia ruckeri*. Samples were taken at 1/3, 1, 3, 7, 14 and 28 days post exposure. Data are expressed as the mean of three independent samples ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and Austin [27] pointed out that *Y. ruckeri* infections in rainbow trout commonly affects fish with approximately 7.5 cm in length, and the disease gets less severe in larger fish. In addition, mortalities related with *Y. ruckeri* infection peaks at water temperatures between 15 and 18 °C. In rainbow trout, the LD_{50} dose was calculated as 3×10^5 CFU/ml (Type I) and 10^7 CFU/ml (Type II) [28]. In this study, the lethal concentration (LD_{50}) of *Y. ruckeri* for brown trout strains and their hybrids with similar size and length were between $10^{7.57}$ CFU/ml in BB and $10^{2.12}$ CFU/ml in NA. Anatolian strain and their hybrids exhibit remarkably lower tolerance, while Black Sea strain and their hybrids exhibit the highest tolerance to *Y. ruckeri* infection.

Lactococcus garvieae is the causative agent of lactococcosis. Water temperature is the leading environmental factor affecting the severity of *L. garvieae* infections. High mortalities in farmed trout generally occur when the water temperature reaches above 18 °C, which indicates an association of outbreaks with high water temperature [29]. In rainbow trout, LD_{50} dose of *L. garvieae* (Lgper strain) was calculated as 2×10^5 CFU/ml at 16 °C water temperature [30]. In this study, the lethal concentration (LD_{50}) of *L. garvieae* for brown trout strains and their hybrids with similar size and length were ranged between $10^{9.66}$ CFU/ml in NN and $10^{6.54}$ CFU/ml in CA at 17.7 ± 0.3 °C water temperature. While hybrids of Caspius, Abant and Anatolian strains were more susceptible to *L. garvieae* compared to their purebreds, hybrids of Black Sea strain were more resistant to *L. garvieae* compared to their pure breed strains. Overall levels of mortality among families

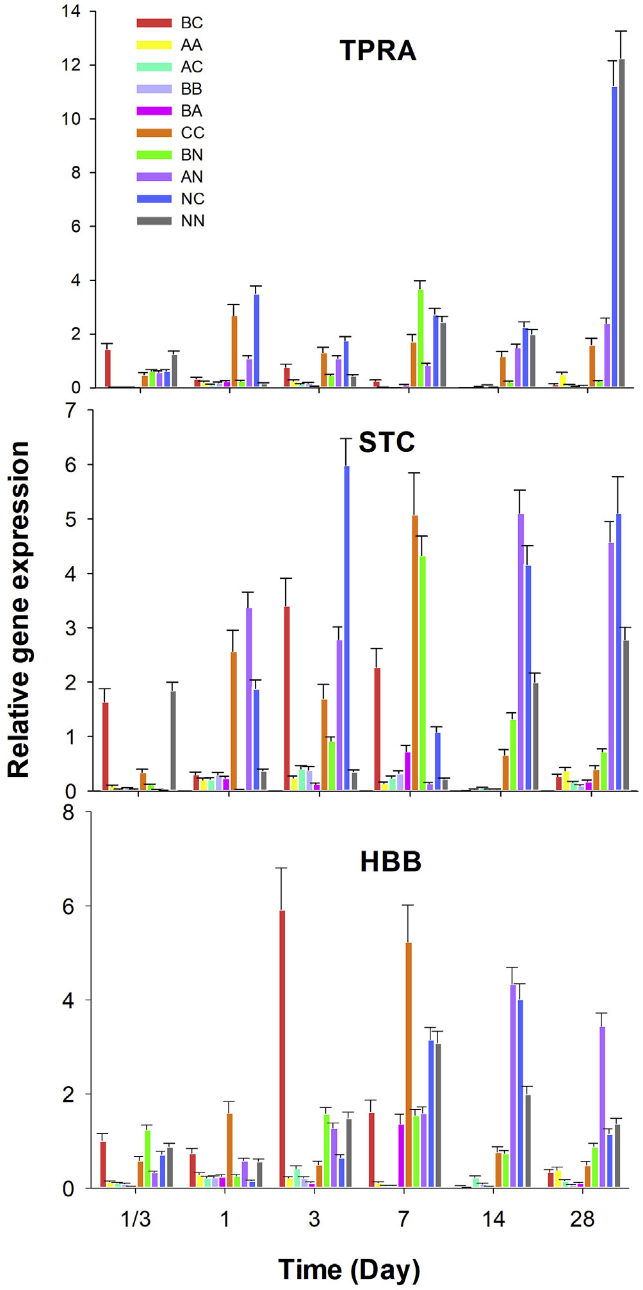

Fig. 8. Relative gene expression profiles of *TPRA, HBB* and *STC* genes in kidney of brown trout (*Salmo trutta*) strains and their hybrids infected with *Lactococcus garvieae*. Samples were taken at 1/3, 1, 3, 7, 14 and 28 days post exposure. Data are expressed as the mean of three independent samples ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

infected with *Y. ruckeri* and *L. garvieae* were different. The difference in mortality between the families indicates that hybridization and genetic background is involved in disease resistance against bacterial pathogens.

Pathological conditions often change immune-related gene expression profile. As a result, cells are obligated to coordinate adjustments in gene expression to protect cellular structures and repair damages in order to survive [31]. Cellular responses and immune genes have been examined previously for rainbow trout infected with bacterial and viral pathogens [32–36], but only a few studies performed gene expression in brown trout [37–39]. Herein we report the first comparative gene expression study in brown trout strains and hybrids that exposed to *Y. ruckeri* and *L. garvieae*. In order to investigate the mechanisms behind the possible differential susceptibilities of brown trout strains and their hybrids to common bacterial pathogens, we compared expression of four antioxidant-related genes in liver and eight genes responsible for oxidative stress, cell regulation, cell survival, cell proliferation, immune response, signal transduction, hemoglobin and calcium homeostasis in kidney.

Catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase are antioxidant enzymes which play a key role in redox balance, and control of reactive oxygen species (Alharby et al., 2016; Couto, Wood, and Barber 2016; Peterman et al., 2015). Reactive oxygen species (ROS) are generated to destruct pathogens by phagocytosis [45]. In other words, the pathogen infection can lead to overexpression of H_2O_2. However, excess amounts of ROS are harmful and may cause cellular damage and immune dysfunction [46]. Antioxidant enzymes have evolved to suppress or prevent the formation of excessive ROS [47]. Expereesion of these antioxidant enzymes indicate oxidative stress status of fish. Antioxidant enzyme expression in families after *L. garvieae* infection were similar. Among all families, AN, NC and NN exhibited the most significant modulation in oxidation resistance that indicates increased disease resistance to the pathogen. The level of antioxidant enzyme expression in families infected with *Y. ruckeri* appeared to be remarkably higher compared to *L. garvieae* infected families. Upregulation was observed in most of the families at different time points. Similarly, following the *Aeromonas salmonicida* infection, the expression levels of *CAT*, *SOD*, *GP*x were elevated in *Salmo salar* fed with probiotic containing diets [44]. A potential explanation of low expression of any of the antioxidant enzyme is that *CAT, SOD, GPx,* and *GR* are all effective at H_2O_2 elimination and any of them could be expressed to exert the role of H_2O_2 scavenger.

Cold-inducible RNA-binding protein, specific to cell types, stabilizes transcripts of genes involved in cell survival. *CIRBP* protein expression is mainly induced by temperature and UV [48]. However, recent studies indicate that *CIRBP* also has a function in cell protection under environmental stress at normal temperatures [49,50]. In the present study, higher *CIRBP* expression was detected in *Y. ruckeri* infected families compared to *L. garvieae* infected families. *Lactococcus garvieae* infection induced marginal increases in *CIRBP* expression in AN, NC, and NN families throughout the experiment whereat *Y. ruckeri* infection induced comparatively higher expression in all families except for CC and BN starting from 3 dpi. Upregulation of *CIRBP* was observed in the skin mucus of Atlantic cod infected with *Vibrio anguillarum* whereas downregulation was observed in skin tissue [51]. Furthermore, *CIRBP* upregulation was also noted in brown trout infected with a parasite, *Tetracapsuloides bryosalmonae* [39].

Cyclin-dependent kinase inhibitor 2A involves in cell cycle regulation and cellular senescence [52]. In the present study, *CDKN2A* is significantly upregulated only in hybrids of Anatolian strain and purebred Caspian strain, which were the most susceptible brown trout families against *L. garvieae* infection, meanwhile significant upregulation was observed in all of the families following the *Y. ruckeri* infection at different time points.Similarly, *CDKN2A* upregulation was reported following the exposures to DNA damaging agents [53] and parasite infection [54,55] in fish. Upregulation of *CDKN2A* gene after bacterial infections indicates that pathogens may activate genes that play a role in cell survival.

Prothymosin alpha 1 plays a role in numerous biological processes such as cell proliferation [39], cancer development [56], chromatin remodeling [57] and immune responses [58]. Differential upregulation of *PTMA* was observed in all families at a different level and dpi following both *Y. ruckeri* and *L. garvieae* infection. Upregulation of *PTMA* was also determined in brown trout following the *T. bryosalmonae* infection whereas downregulation was observed in rainbow trout [59]. Upregulation of *PTMA* suggests that bacterial infections do not constrain cell growth and proliferation processes.

Major histocompatibility complex class I is found on the cell surface and plays an important role in adaptive immune response [60]. Intracellular antigens are presented by MHC I. $CD8^+$ T cells are activated and they induce the lysis of infected host cells. As part of a cell-mediated immune response, a trend for upregulation was observed in families infected with *Y. ruckeri* especially between 7 and 28 dpi. Similarly, Peatman et al. [61] reported an upregulation of *MHC*-I in the liver of blue catfish at 3 days after *Edwardsiella ictaluri* infection [61]. However, *Lactococcus garvieae* infection did not induce *MHC*-I expression in families except for BC, NC, NN and BC families in which expression levels were marginal. Similarly, Tafalla et al. [62] did not record any *MHC*-I expression in kidney, spleen, and liver of rainbow trout following the VHSV infected fish. However, *MHC*-I expression was observed in gills of the rainbow trout following VHSV infection [63] which indicates the role of organs may change depending on the pathogen. Downregulation of *MHC*-I genes was also observed in zebrafish following the *Mycobacterium marinum* infection [64] and in Caspian trout following the VHSV infection [38].

Immunoglobulin (Ig) genes encode defense proteins which are known as antibodies [65]. Immunoglobulin light chain precursor participates in humoral immune response [39]. *IgL* regulation levels show that families infected with *Y. ruckeri* and *L. garvieae* have a different pattern of expression. While modest regulations were observed in AN, NC and NN families infected with *L. garvieae*, the higher trend for upregulation was observed in all families except for AA, CC following the *Y. ruckeri* infection.

Transforming protein RhoA is involved in GTPase mediated signal transduction (Kumar et al., 2014). *TPRA* gene expression was not responsive to *L. garvieae* infection in families except for NC and NN whereas *Y. ruckeri* infection leads high rates of expression in BC and BB families. Upregulation of *TPRA* was previously reported in zebrafish during *Mycobacterium marinum* infection and brown trout during *T. bryosalmonane* infection. Downregulation of GTPase enzymes paves a way to infectious diseases in humans (Salas-Vidal et al., 2005). However, the function of GTPase enzymes during bacterial infection in fish is not clear.

Hemoglobin subunit beta is involved in oxygen transport from gills to tissues. Oxidative stress upregulates *HBB* expression (Liu et al., 2011). *HBB* was upregulated at different time-points upon challenge with *Y. ruckeri* confirming its involvement in the host response to bacterial infection. Meanwhile, moderate upregulation was observed in CC, BN, AN, NC and NN families infected with *L. garvieae*. Stanniocalcin precursor (*STC*) regulates phosphate and calcium homeostasis [39]. Upregulation regime of *STC* following the *L. garvieae* and *Y. ruckeri* infections were similar to the regime of *HBB* regulation.

5. Conclusion

In the present study, we tested the hypothesis of whether selective breeding or hybridization affects disease resistance in brown trout strains and to find resistant strains or hybrids that could be used in aquaculture. Reciprocal hybridization did not affect disease resistance that is directly related to brown trout strains and diseases agents. While purebred Black Sea strain of brown trout is the most resistant group against yersiniosis followed by other Black Sea strain contained hybrids. On the other hand, the purebred Anatolian strain is the most resistant group against lactococcosis followed by other Anatolian strain contained hybrids. In general, disease resistance is supported by gene expression. Therefore, it can be suggested that brown trout strain can be selected according to the presence of disease where brown trout cultured. For example, if yersiniosis is most common where trout cultured, Black Sea strain can be used. Thus, fish culturist may not spend money on antibiotics, spend time to treat disease and lost money due to fish mortality.

Conflicts of interest

The authors declare that they have no competing interests.

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