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RNA form baker's yeast cultured with and without lipopolysaccharide (LPS) modulates gene transcription in an intestinal epithelial cell model, RTgutGC from rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The objective of this study was to evaluate if the intestinal RTgutGC cell line could be suitable for research on dietary ingredients and their function as modulators of inflammation during lipopolysaccharide (LPS) induced stress.

The RTgutGC cells cultured together with RNA from baker's yeast, reached confluency after 72 h. The cells were grown in either compete L-15 (CM) or nutrient deprived L-15 (DM). Then, the RTgutGC cells were exposed to LPS or RNA from baker's yeast, either alone, or in combination, in CM or DM. All cultures were harvested following LPS challenge for 48 h and 72 h.

LPS induced transcription of Interleukin 1β (*IL-1β)*, Interleukin −8 (*IL-8)*, Toll like receptor 3 (*TLR3)*, interferon regulating factor 3 (*irf3)*, Nuclear factor κβ *(NFκβ)*, one of the multidrug transporters, *ABCC2,* and glutamine synthase 1 (*GLS01*) in RTgutGC cells at one or both sampling points (48 h and/or 72 h post LPS challenge). RNA from baker's yeast in culture alone, (cultured 120 h and 144 h with RTgutGC cells and harvested at the respective LPS sampling points) induced transcription of *INF1*, *TNFα* and *ticam/trif*, not induced by LPS. In addition, RNA from baker's yeast affected *IL-1β*, *TLR3*, *irf3* and *NFκβ,* comparable to the responses triggered by LPS. RNA from baker's yeast alone did not affect *ABCC2* or *GLS01* transcriptions in this set up. So, LPS and RNA from baker's yeast affects distinct but also common gene transcripts in this intestinal cell line.

Culturing RTgutGC cells in DM, adding a combination of LPS and RNA from baker's yeast, reduced *IL-1β* transcription compared to cells grown in CM, 48 h and 72 h post LPS challenge. Also, in RTgutGC cells, grown in DM, the LPS induced transcription of *ABCC2* declined, measured 48 h post LPS challenge. Possibly indicating that optimal transcription of *IL-1β* and *ABBC2* in RTgutGC cells, cultured over time, requires access of adequate nutrients under stressful condition.

RNA from baker's yeast induced *INF1* transcription in the RTgutGC cells, regardless if the medium was complete or deprived of nutrients. However, culturing RTgutGC cells in DM enriched with RNA from baker's yeast for a longer period of time (120 h, 144 h), seemed beneficial for *INF1* transcription.

1. Introduction

The increasing demand for high-quality feed ingredients for aquaculture and how these nutrients are affecting the fish intestine and the immune cells residing in this channel, can be studied in the recently developed RTgutGC cell line from rainbow trout (*Oncorhynchus mykiss)*. The RTgutGC cell line origin from the distal portion of the intestine isolated from a female rainbow trout and is the only fish derived intestinal epithelial cell line available for *in vitro* research purposes [1]. The RTgutGC cell line is considered as a tool for a broad variety of research purposes like ecotoxicology [2]; Minghetti et al. 201,7), inflammation [1] and nutrient deprivation [3].

Fish intestine interacts with the environment during nutrient intake and osmoregulation and possible contaminants in the feed and water. Teleostan gut is different from mammalian gut as they lack organized lymphoid tissues such as lymph nodes, Peyers's patches and isolated lymphoid follicles. Instead fish intestine has mucosa-associated lymphoid tissue called GALT (gastrointestinal lymphoid tissue) included in lamina propria and intraepithelial structures [4]. These structures produce leukocytes and are important for antigen recognition

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and local immune responses. In the intestine of rainbow trout the presence of two types of B cells, executing phagocytic activity [5], T lymphocytes [6–9], dendritic cells co-expressing CD8α and MHCII and also executing a high phagocytic activity [10,11] have been demonstrated. These structures are probably regulators of gut immune tolerance [12]. Microorganims are in constant contact with the intestinal mucosa, and when harming the mucosa, the underlaying glycocalyx and finally the integrity of the epithelium would represent a way for the entry of pathogens [13,14]. Research on fish intestines and host-microorganism interaction has increased over the years [15–18] and a method for GALT leukocyte isolation from salmonid intestine has recently been established [10] and refined [18]. It has been demonstrated that fish intestine can produce a diversity of cytokines upon challenge [10,15,18].

LPS is the main component of external membrane of gram-negative bacteria is recognized by a highly conserved pathogen coded molecular structures termed pathogen associated molecular patterns (PAMPs). The PAMPs are recognized by pattern recognizing receptors (PRRs) and are the first line of cellular defense against pathogens. PRRs triggers downstream signaling pathways like transcription factors adaptors and kinases. There are five major families of PRRs investigated in vertebrates: the Toll like receptors, (TLRs), retinoic acid inducible gene I (RIG I)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD) like receptors (NLRs), C-type lectin receptors (CLRs) and absent in melanoma 2 (AIM2)-like receptors (ALRs) [19–21]. There are functional similarities between PRRs in fish and mammals, but there are distinct features in some downstream signaling mechanisms [22–24]. The presence and type of specific immune cells and PRRs residing in RTgutGC intestinal cell line are not well characterized. However, the cell line clearly responded towards lipopolysaccharide (LPS) and polyinosinic: polycytidylic acid (PIC) stress producing pro and anti -inflammatory cytokines and interferons [19,25]. Stimuli exposure also affected regulation of intestinal barrier markers [26,27].

Polynucleotides found in baker's yeast RNA can be a dietary source of bases, nucleosides and nucleotides. Main dietary sources of nucleotides are nucleoproteins and nucleic acids which are converted to nucleosides and bases which are transported into enterocytes via facilitated diffusion and specific Na + dependent carrier mediated mechanisms [28]. Nucleotides and their metabolites have received attention in recent years as they are active in various physiological functions and may be of particular significance for growth and development of tissues with rapid turnover, such as cells in the immune system and intestinal cells. Poly-ribonucleotides encode genetic information, have a role in energy metabolism and signal transduction [29,30]. Requirements for nucleotides are by a large part met by endogenous metabolic pathways of synthesis and salvage [29,30]. However, in some conditions endogenous synthesis of nucleotides is not enough to fulfil physiological demands [31,32]. In rodent nucleotide free diets decrease cellular and humoral immune responses [33,34] and resistance to bacterial and fungal pathogens [35,36]). Dietary nucleotides ingested by humans and terrestrial animals has been shown to enhance immune responses towards bacterial and viral infections [37–41]. Oral administration of nucleotide supplementation for fish such as Atlantic salmon (*Salmo salar*) [42,43]) common carp (*Cyprinus carpio)* [44], Nile tilapia (*Oreochromis niloticus*) [38], turbot (*Scophtalmus maximus*) [45], and rainbow trout [43] has shown beneficial effects on immune functions and disease resistance. Also, proliferation of salmonid cell lines was stimulated by adding bases, nucleosides and nucleotides to culture media [46,47]. [48]; using germ free zebrafish, showed that dietary nucleotides directly can stimulate the immunity of zebrafish independent of the intestinal microbiota.

So, functioning as immunostimulants, nucleotides in the shape of oligonucleotides, ssRNA from baker's yeast, imitations of dsRNA virus (poly I:C) and synthetic structures from DNA based bacteria and virus (CpG-ODN) can be used as ligands for receptors in the immune system. In fish different receptors can recognized nucleotides besides the purine and pyrimidine receptors. Toll like receptors like TLR3, 7, 8, 9 and 22 can recognize different structures of nucleotides [49].

In the current trial, the effect of RNA from baker's yeast, with and without the presence of the inflammation inducer LPS, were examined on the rainbow trout, RTgutGC intestinal cell line. Depriving nutrients from the culture media were introduced as an additional stressor in some of the cultures.

2. Materials and methods

2.1. Fish cell line

The RTgutGC cell line was isolated from rainbow trout (*Oncorhynchus mykiss)*. This cell line origin from the distal portion of the intestine, isolated from a female rainbow trout, and is the only fish intestinal epithelial cell line available for research purposes [1]. The cell line was routinely cultivated as described by Ref. [1]. In short, cells were cultured in Leibovitz −15 medium (L-15) without phenol red (21083027, Gibco, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS, F7524, Merck, Darmstadt, Germany), 2% 2 mM glutamax™ 100X (Gibco cat # 35056) and 1% Antibiotic Antimycotic Solution (A5955, Merck, Darmstadt, Germany). The cells were grown in Nunc EasY 75 cm^2 flasks (156499, ThermoFisher Scientific) at 19 °C, and split 1:2 when confluent by 0.25% trypsin in PBS (P10-021100, Pan Biotech, Aidenbach, Germany).

600 000 cells/well were seeded into 6 well plates (CS3506, Corning Costar, Arizona, USA) for this experiment. After 24 h, media were changed to either complete medium (CM) L15 (L5520, Sigma-Aldrich, Buchs, Switzerland) containing 10% FBS (F7524, Merck, Darmstadt, Germany), 2% 2 mM glutamax and 1% Antibiotic Antimycotic Solution (A5955, Merck, Darmstadt, Germany), or a nutrient deprived (DM) version containing 5% FBS (F7524, Merck, Darmstadt, Germany), no added glutamax, and 1% Antibiotic Antimycotic Solution (A5955, Merck, Darmstadt, Germany). After medium change, some of the cell cultures were exposed to 1 mg/mL RNA from baker's yeast (R6750, Sigma-Aldrich, Buchs, Switzerland).The cells reached 90–95% confluency after 72 h, cultured with and without RNA from baker's yeast, and LPS 100 μg/mL (L7018, Merck, Darmstadt, Germany) was added to selected wells. Control cultures without addition of RNA from baker's yeast or LPS were included. The cells were harvested 48 h and 72 h following LPS challenge.

2.2. Harvesting for RNA isolation

RTgutGT cells were homogenized directly in 600 μl RTL-Plus buffer (RNeasy ®Plus kit Qiagen) using a syringe and frozen at −80 °C before RNA extraction.

2.3. RNA extraction and conversion into cDNA

Total RNA was extracted using RNeasy ®Plus kit (Qiagen) according to the manufacturer's instructions, and frozen at −80 °C. The quantity and quality of RNA was assessed using the NanoDrop ND-1000 UV Spectrophotometer (NanoDropTechnologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA integrity was assessed using the RNA 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto, CA, USA) following the instructions from the supplier. The samples used in this experiment had 260/280 nm absorbance ratios of 2.0 ± 0.1 and 260/230 nm ratios of 2.4 ± 0.1 (mean ± STDEV, n = 24) and RIN-values between 8 and 10 indicating RNA samples suitable for RT-qPCR. Reverse transcriptase was used to convert the RNA template into the more stable cDNA for use in quantitative PCR. A standard curve was made of pooled samples and serial dilutions from 500 to 10 ng were run in triplicates into 96 well PCR plates (VWR, AB06000). The remaining samples were individually diluted and added the RT reaction mix (TaqMan reverse transcription

reagents) prepared. A nonamplification control (nac) and a non-template control (ntc) were added to the PCR plates. The RT reaction was performed with a CFX96™ Thermal Cycler (Bio-RAD system) starting with an incubation step for 10 min at 25 °C, continuing with RT reaction at 48 °C for 60 min by using oligo dTprimers (2.5 μM) in 30 μL total volume, and finally with 5 min inactivation at 95 °C. The PCR plates were stored at −20 °C.

2.4. RT-QPCR

Gene expression was quantified with qPCR on the Light Cycler 480 (Roche Applied Sciences, Basel Switzerland). Real Time plates were run on qPCR with a CF384™ Real-Time system (Bio-RAD system, C1000 Touch Thermal Cycler) and the following program: 5 min activation and denaturizing step at 95 °C followed by 45 cycles of 10s denaturizing step at 95 °C, 20s annealing step at 60 °C and a 30s synthesis step at 72 °C, followed by a melt curve analysis and cooling to 4 °C. The Bio-RAD CFX MAESTRO system was used to determine a normalization factor from two reference genes and used to calculate mean normalized expression for the target genes. The stability of the reference genes was calculated by the Bio-RAD system. *Elf1* and *mucin* were the most stable genes in this experiment and used as reference genes (Table 1).

2.5. Statistical analyses

Gene transcription differences between RTgutGC cells cultured in complete L15 medium (cL15), in L-15 medium deprived of nutrients and treatment differences under these conditions, were analyzed by Two Ways ANOVA, with Tukey's multiple comparison test ($\alpha = 0.05$) as indicated. GraphPad Prisms version 8.0 software was used. Significant differences between CM and DM and between treatments are indicated by letters: a≠b≠ *c*. Treatment differences compared to respective controls not treated with LPS or RNA from baker's yeast, are indicated by *.

3. Results

At the cell harvesting points, 48 h and 72 h post LPS challenge, RNA from baker's yeast (with or without LPS) had been cultured with RTgutGC for 120 h (5 days) and 144 h (6 days) respectively.

3.1. Transcription of immune related genes (IL-1β, IL-8, INF1, TNFα, TLR3) in RTgutGC cells

Challenging RTgutGC cells with LPS for 72 h induced significantly ($p = 0.0062$) *IL-1β* transcription above the respective control cultures. RNA from baker's yeast induced *IL-1β* transcription when measured 48 h ($p = 0.0098$) and 72 h ($p = 0.0467$) post LPS challenge. Combining RNA from baker's yeast and LPS, further increased *IL-1β* transcription 48 h and 72 h post LPS challenge, but only in RTgutGC cells grown in CM ($p < 0.0001$). Interaction $p = 0.0002$. (Fig. 1A).

LPS was the only treatment that induced *IL-8* transcription in these cells, significantly only 72 h ($p < 0.0001$) post LPS challenge. Combining LPS and RNA from baker's yeast did not increase the LPS induced transcription but the responses were significantly different from respective controls both at 48 h ($p = 0.0001$) and 72 h ($p = 0.0005$) post LPS challenge (Fig. 1B).

RNA from baker's yeast, with or without the presence of LPS, induced *INF1* transcription ($p < 0.0004$) in RTgutGC cells measured 48 h and 72 h following LPS treatment. RTgutGC cells grown in DM and added RNA from baker's yeast increased *INF1* expression 72 h post LPS challenge when compared to cells grown in CM (a≠ *b*, p = 0.0159, b ≠ c, p < 0.0001). Interaction p = 0.0020. (Fig. 1C).

TNFα was transcribed in RTgutGC cells when exposed to RNA from baker's yeast and in the combination RNA from baker's yeast + LPS following 48 h and 72 h post LPS challenge ($p < 0.0001$) (Fig. 1D).

TLR3 transcription was induced by LPS or RNA from baker's yeast, and in combination, in RTgutGC cells when measured 48 and 72 h following LPS challenge ($p < 0.0054$) (Fig. 1E).

3.2. Gene transcription of immune related signaling molecules (irf3, Myd88, Nfκβ, ticam/trif) in RTgutGC cells

In RTgutGC cells, the interferon regulatory factor, *irf3,* was equally induced by LPS or RNA from baker's yeast, or in combination, compared to the respective controls ($p < 0.0004$), measured 48 h and 72 h following LPS treatment (Fig. 2A).

RTgutGC cells transcribed *Myd88* constitutively, but the transcription was not affected by any of the treatments measured at 48 h and 72 h post LPS treatments (Fig. 2B).

RNA from baker's yeast cultured with or without LPS induced significant *Nfκβ* transcription in RTgutGC cells when measured 48 h and 72 h post LPS challenge ($p < 0.0155$) compared to respective controls.

Table 1
Primer sequences.

Primers	Forward	Reverse	Accesion number
INF1[a]	CTTGAGCGCAGAATACCTT	TCCTCAAACTCAGCATCATC	FJ184371.1 AY788890.1
GST-π	TATTGTGGGCTAATGTGTAAGAT	CCCYGAAGAGCTTTGTCG	AB026119.1
ABCC2	CGCTTCCTCAAACACAACGAG	GAACTCTAGACGGATGGCCAG	NM_001124655
IL-1β	TCCCCATTGAGACTGAAGCC	TTGAGACGGAAAGCAGACGA	AJ223954.1
FAS	ATGCGTATCCAAGCCCAAA	CCCACCAATCCTGGTCATCC	BT073300
GDH	TGTCGGTCGATGAGGTGAAA	TGGCTCCTCCAAATGGAACA	AJ556997
GLS01	TGGGCCATGTGAAGGTATA	ACGCCAAAGTCTTCACACAC	AF390021
IL-8	ATTGAGACGGAAAGCAGAC	CTCAGAGTGGCAATGATCTC	NM_001140710.2 NM_001124362.1
tnfα	GTGATGCTGAGTCCGAAAT	GTCTCAGTCCACAGTTTHTC	AJ277604.2 AJ401377.1
TLR3	CTCATCCTCAGCCCTATGT	GCTTGAACGGAGAGGTATTC	DQ459470.1
irf3	AAGCTCACTTCAGGGTTTC	CAGAAGCGGTTGTGTAAGT	NM_001257262.1 HF565492.1
Myd88	GATGCCTTCATCTGCTACTG	CAAACACACACAGCTTCAAC	AJ878918.1 NM_001124421.1
nfκβ	CACAGCCAGTTCAGTAACC	TTGCCTCCTCTTCTCATCTC	[27]
trif	GCTAACCATCTGGCTGAAA	CACGGTACACTCTTGGAAAG	[27]
Elf1	TGCCCCTGGACACAGAGATT	CCCACACCACCAGCAACAA	NM_ 001124339
mucin	TCAACACATTCTCTGACACC	GGCAGTTACTGTACCAAGTC	[27]

[a] Primer pair detects long and short forms of interferons [27].

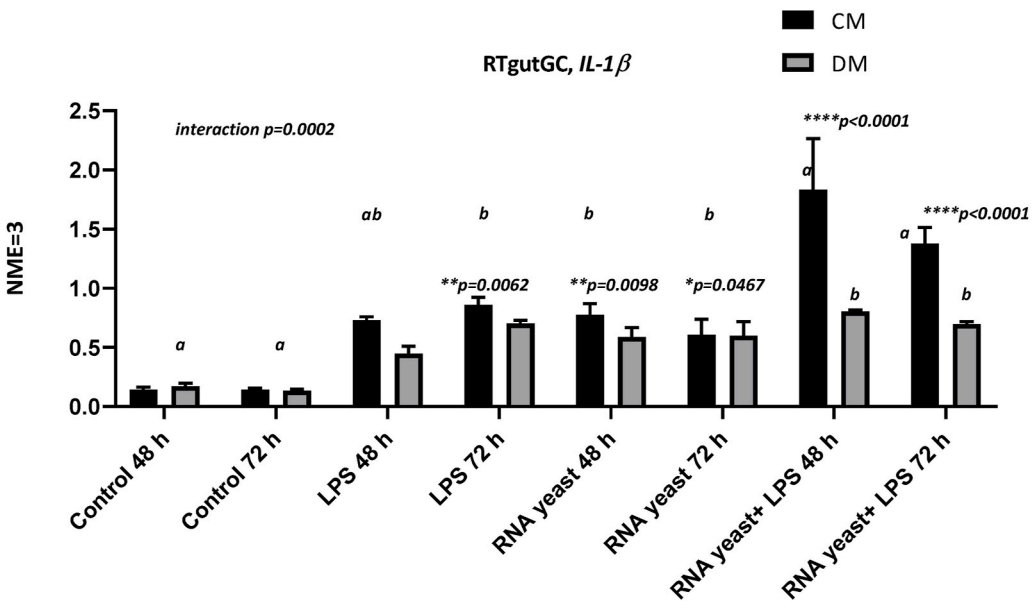

Fig. 1a. Transcription of immune related genes in RTgutGC cells. **(A)** Challenging RTgutGC cells with LPS for 72 h induced significantly ($p = 0.0062$) *IL-1β* transcription above respective control cultures. RNA from baker's yeast induced *IL-1β* transcription when measured 48 h ($p = 0.0098$) and 72 h ($p = 0.0467$) post LPS challenge. Combining RNA from baker's yeast and LPS further increased *IL-1β* transcription 48 h and 72 h post LPS challenge, but only in RTgutGC cells grown in CM ($p < 0.0001$). Interaction, $p = 0.0002$.

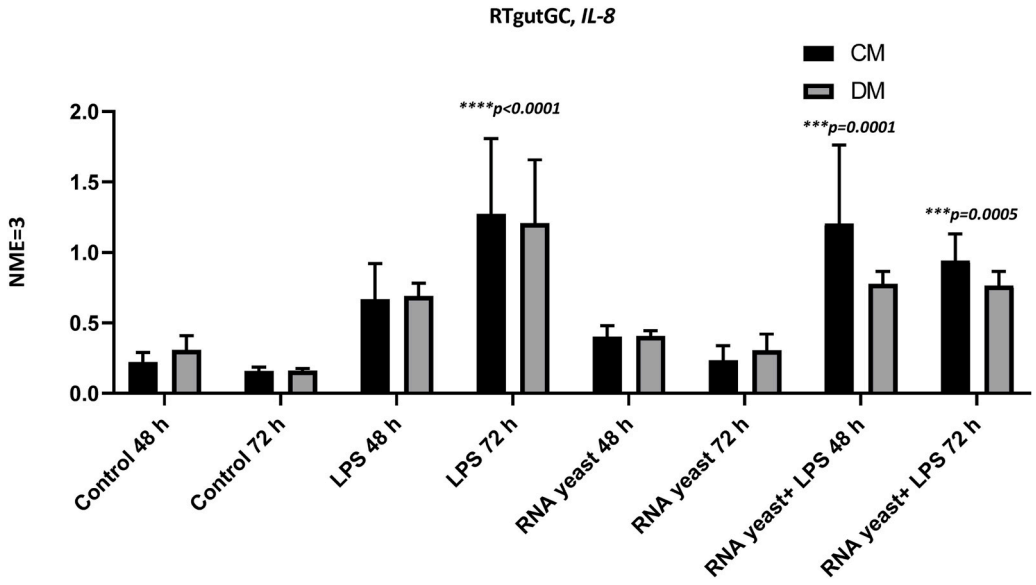

Fig. 1b. LPS was the only treatment that induced *IL-8* transcription in these cells, significantly only 72 h ($p < 0.0001$) post LPS challenge. Combining LPS and RNA from baker's yeast did not increase the LPS induced transcription but the responses were significantly different from respective controls both at 48 h ($p = 0.0001$) and 72 h ($p = 0.0005$) post LPS challenge.

LPS alone induced significant *Nfκβ* expression when measured 72 h post LPS challenge ($p = 0.0038$) (Fig. 2C).

RNA from baker's yeast added to RTgutGC cells significantly induced *ticam/trif* transcription when harvested 48 h post LPS challenge. In these cells, the general *ticam/trif* transcription seemed to decline when measured 72 h post LPS challenge, but only significantly in control cultures ($p = 0.0027$) (Fig. 2D).

3.3. Gene transcription of a multidrug transporter (ABCC2), detoxification enzyme glutathione transferase (GST-π), glutamate dehydrogenase (GDH), fatty acyl synthase (fas) and glutamine synthase 1 (GLS01) in RTgutGC cells

Gene transcription of the transporter protein *ABCC2* was induced at 48 h ($p < 0.0001$) and 72 h ($p < 0.0001$) after LPS treatments, compared to controls at 48 h and 72 h. When the RTgutGC cells were cultured in CM, LPS induced transcription of *ABCC2* was significantly higher than gut cells cultured in DM ($a \neq b, p = 0.0038$) 48 h post LPS treatment. Interaction p = 0.0472. (Fig. 3A).

GST-π transcription was down-regulated ($p = 0.0004$) when RTgutGC cells were treated with LPS for 48 h (p = 0.0004) compared to the respective controls. Reducing L-15 medium of glutamine and less FBS seemed to give a general reduction in the transcription of *GST*, but significantly only in control cultures measured at 48 h ($a \neq b, p < 0.0001$) and 72 h ($a \neq b$, $p = 0.0098$) post LPS challenge. Interaction $p = 0.031$ **(Fig. 3B).**

Glutamate dehydrogenase (*GDH*) and fatty acyl synthase (*fas*) was transcribed constitutively and equally in all cultures tested (Fig. 3C and Fig. 3D, respectively).

GLS01 was induced at 48 h ($p = 0.0121$) and 72 h ($p < 0.0001$) following LPS challenge as compared to control RTgutGC cultures (Fig. 3E).

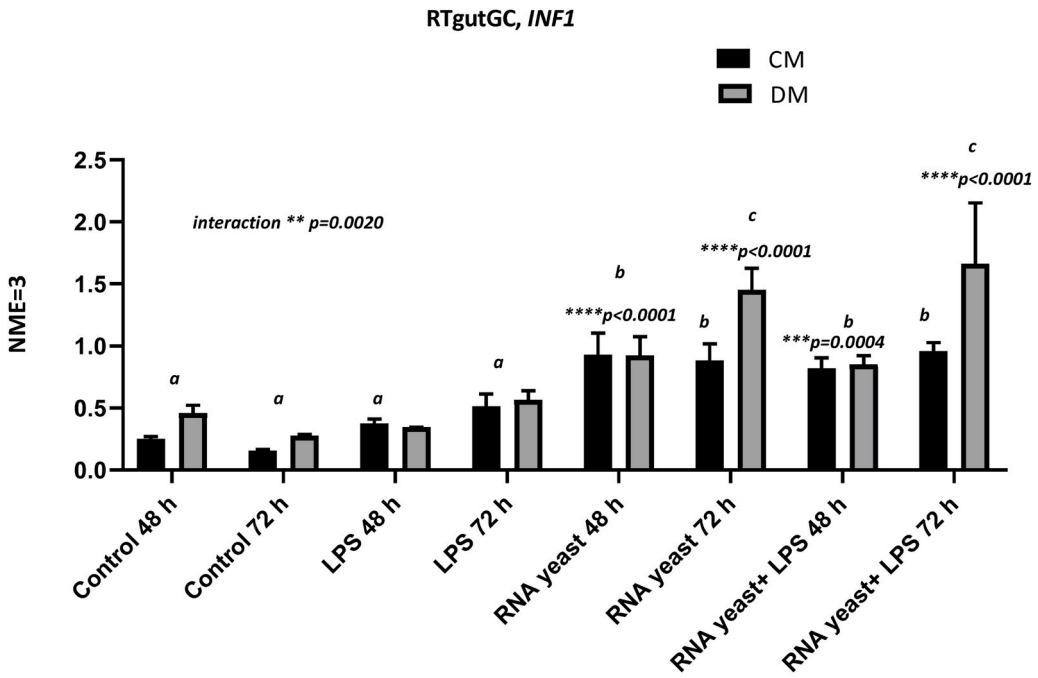

Fig. 1c. RNA from baker's yeast, with or without the presence of LPS, induced *INF1* transcription ($p < 0.0004$) in RTgutGC cells measured 48 h and 72 h following LPS treatment. RTgutGC cells grown in DM and supplemented with RNA from baker's yeast, with or without LPS, increased *INF1* expression 72 h post LPS challenge when compared to cells grown in CM ($a \neq b.p = 0.0159$) (b≠c, $p < 0.0001$). Interaction p = 0.0020.

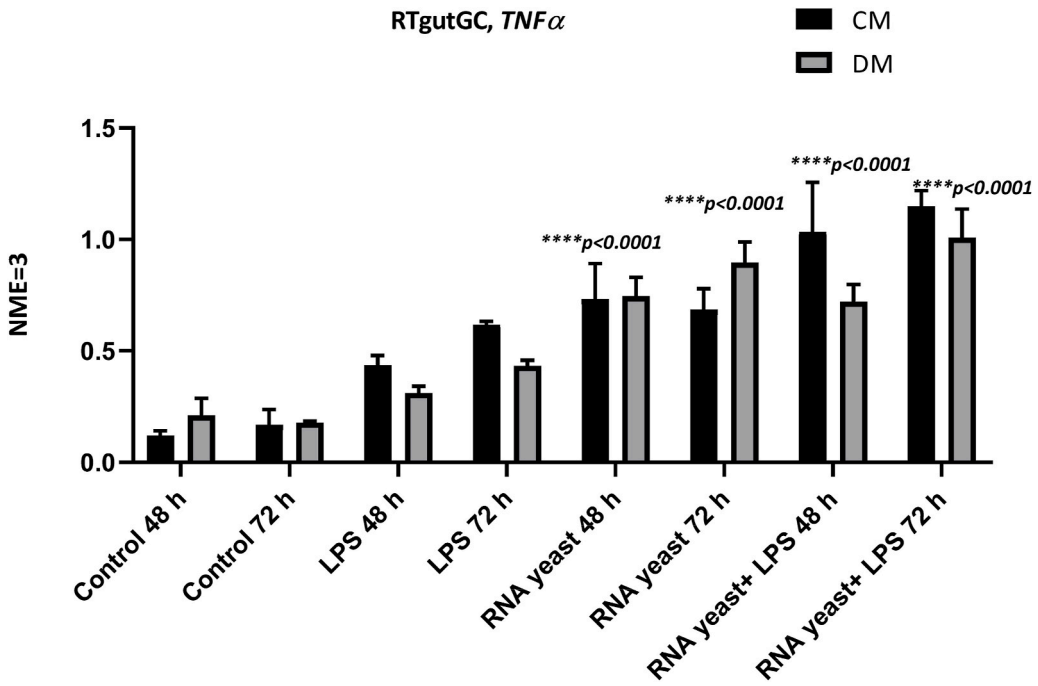

Fig. 1d. *TNFα* was transcribed in RTgutGC cells when exposed to RNA from baker's yeast and in the combination RNA from baker's yeast + LPS following 48 h and 72 h post LPS challenge ($p < 0.0001$).

4. Discussion

The lack of cell lines for studying signaling pathways in fish make the intestinal cell line a valuable tool. Especially since experiments in whole fish are to be avoided due to fish welfare. Signaling pathways cannot be evaluated *in vivo* in a controllable way, which means that too many other biological cues influence the results, making it difficult to understand the pathways.

Antigen uptake in the gastrointestinal tract may induce tolerance, lead to an immune response or to infections [37,49–52].

In this experiment, both RNA from baker's yeast and LPS induced expression of several mRNA transcripts in the RTgutGC cell line. LPS stimulated transcription of *IL-1β*, *IL-8*, *TLR3*, *NFκβ*, *irf3*, and the efflux transporter *ABCC2* and glutamine synthase 1, *GLS*01. LPS signaling in teleost seems to differ from mammals as mammalian TLR4 binding to microbial ligand requires MD-2 and CD14 costimulatory molecules [53].

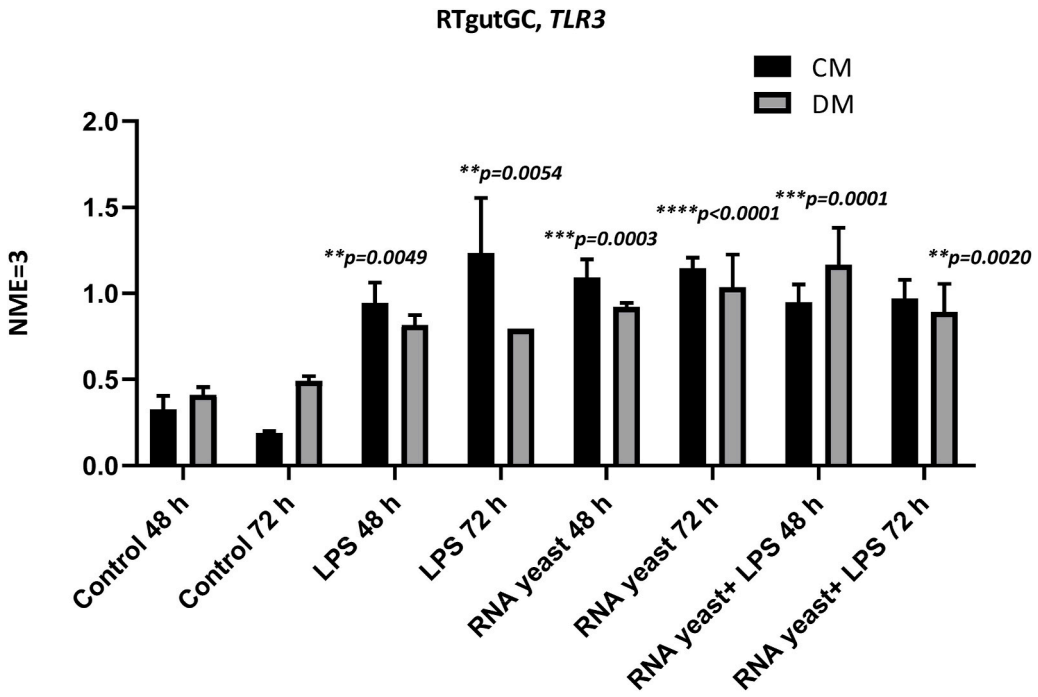

Fig. 1e. *TLR3* transcription was induced by LPS or RNA from baker's yeast, and in combination, in RTgutGC cells when measured 48 and 72 h following LPS challenge ($p < 0.0054$). (LPS 72 h had only one suitable value therefore no SD in this particular measurement).

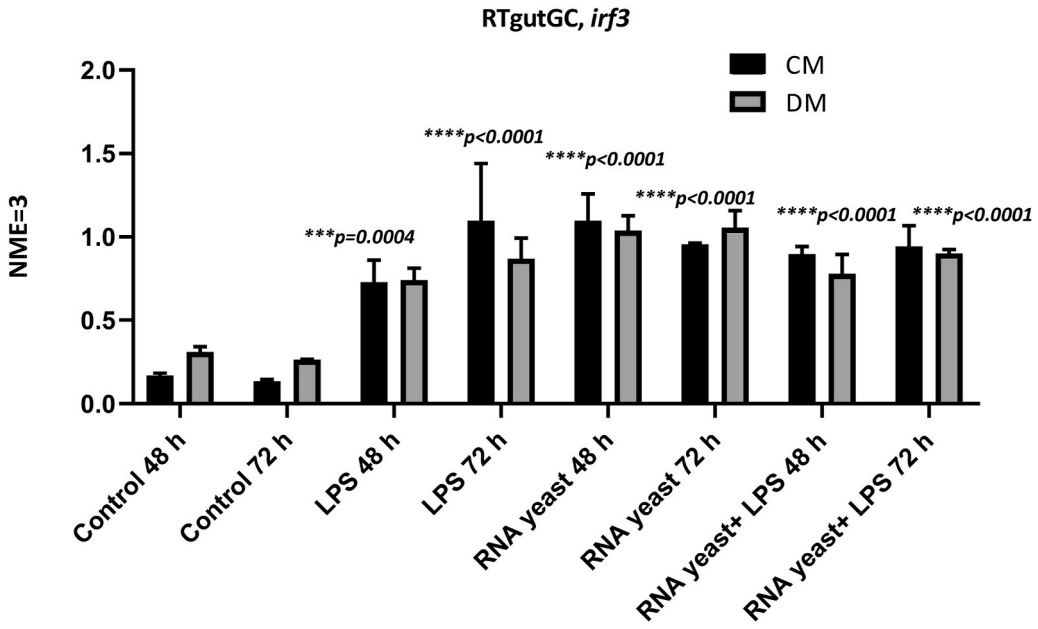

Fig. 2a. Transcription of immune related signaling molecules in RTgutGC cells. **(A)** In RTgutGC cells, *irf3* was equally induced by LPS or RNA from baker's yeast, or in combination, compared to respective controls ($p < 0.0004$), measured 48 h and 72 h following LPS treatment.

MD2 and CD14 are absent from all fish and amphibian genomes examined to date [54,55]. Interestingly, TLR4 has recently been cloned and characterized in several fish species [56–60]. Still, it seems that LPS is not recognized by fish TLR4 but may be recognized via other, presently unidentified receptors [61]. In mammals LPS activates TLR4 connected to MD2 and CD14 and activates myeloid differentiation adaptor molecule, Myd88, which activates MAPK and NFκβ downstream. Myd88 is a key adaptor protein required for signaling through all Toll like receptors except Toll like receptor 3 [62,63]. Also, the TIR domain containing adaptor inducing interferon β (TRIF) -dependent pathway is initiated from TLR4 containing endosomes and results in irf3 activation [64]. In fish the signaling pathway starting with LPS binding to its unknown ligand and possible adapters has not yet been fully understood. However, it is well known that LPS can activate Myd88 inducing downstream activation of MAPK and NFκβ. In the present experiment, *Myd88* transcription was not activated by LPS although *NFκβ* transcription was significantly upregulated when measured 48 h and 72 h post LPS challenge. These observations could be due to the prolonged RTgutGC cell culturing and harvesting as LPS challenge for 24 h was reported to trigger *Myd88* induction in RTgutGC cells [27]. Neither did LPS induce

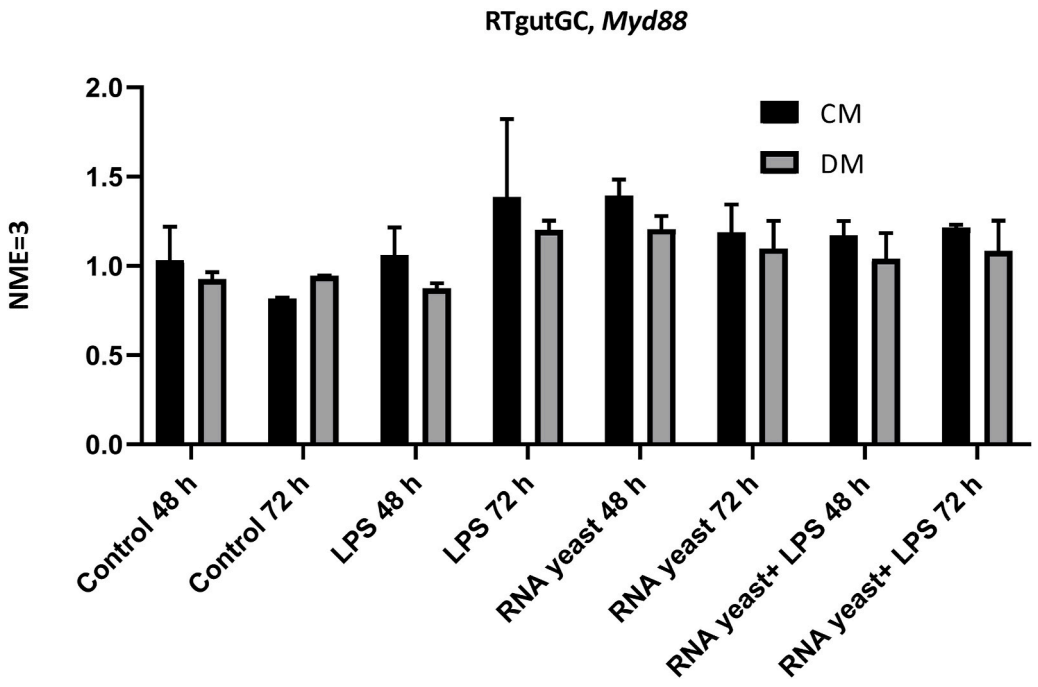

Fig. 2b. RTgutGC cells transcribed *Myd88* constitutively, and the transcription was not affected by any of the treatments measured at 48 h and 72 h post LPS treatments.

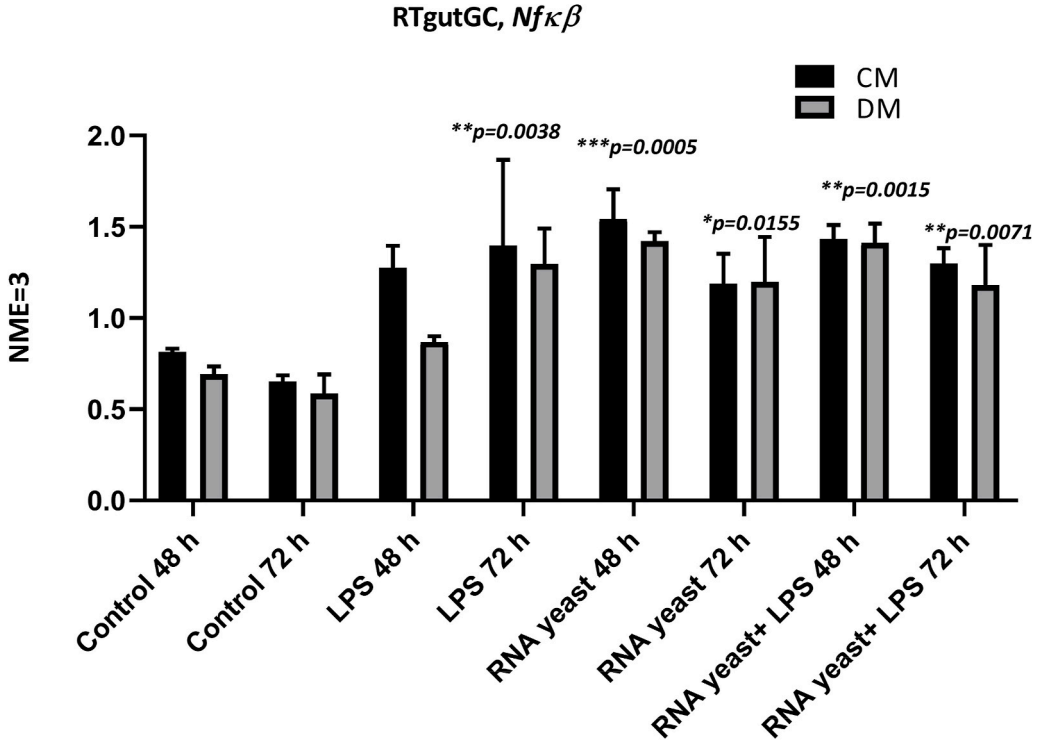

Fig. 2c. RNA from baker's yeast cultured with or without LPS induced significant *Nfκβ* transcription in RTgutGC cells when measured 48 h and 72 h post LPS challenge ($p < 0.0155$) compared to respective controls. LPS alone induced significant *Nfκβ* expression when measured 72 h post LPS challenge ($p = 0.0038$).

any *trif* transcription at 48 h and 72 h post LPS challenge, even though *irf3* transcription was detected at these points. Again, these observations may be due to prolonged culturing as control cultures and RNA from baker's yeast exposed cells harvested at 72 h (144 h for baker's yeast) downregulated *trif* expression compared to the expression at 48 h, respectively. Different immune genes have also different "peaks" depending on specie, tissue, stimulant and stimulation time. Other groups of genes may have different time frames for optimal expression.

RNA from baker's yeast did not induce *Myd88* transcription but triggered transcription of *TLR3*, *NFκβ*, *trif, irf3*, *TNFα* and *INF1*. In mammals, viral TLRs (TLRs 3, 7, 8, 9) are located within endosomes and recognize viral RNA and DNA, TLR 7/8 sense ssRNA and TLR3 recognize dsRNA [65]. However, in zebrafish the modulation of TLR3 gene expression was reported following infection with a single stranded virus

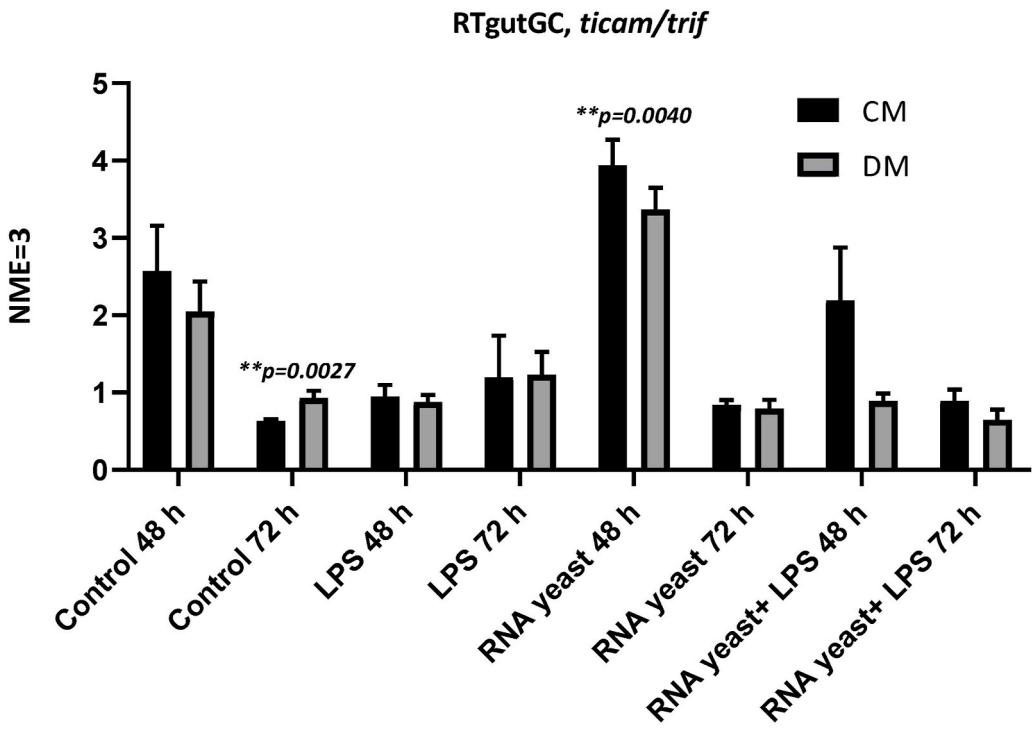

Fig. 2d. RNA from baker's yeast added to RTgutGC cells significantly induced *ticam/trif* transcription when harvested 48 h post LPS challenge. In these cells, the general *ticam/trif* transcription seemed to decline when measured 72 h post LPS challenge, but only significantly in control cultures ($p = 0.0027$).

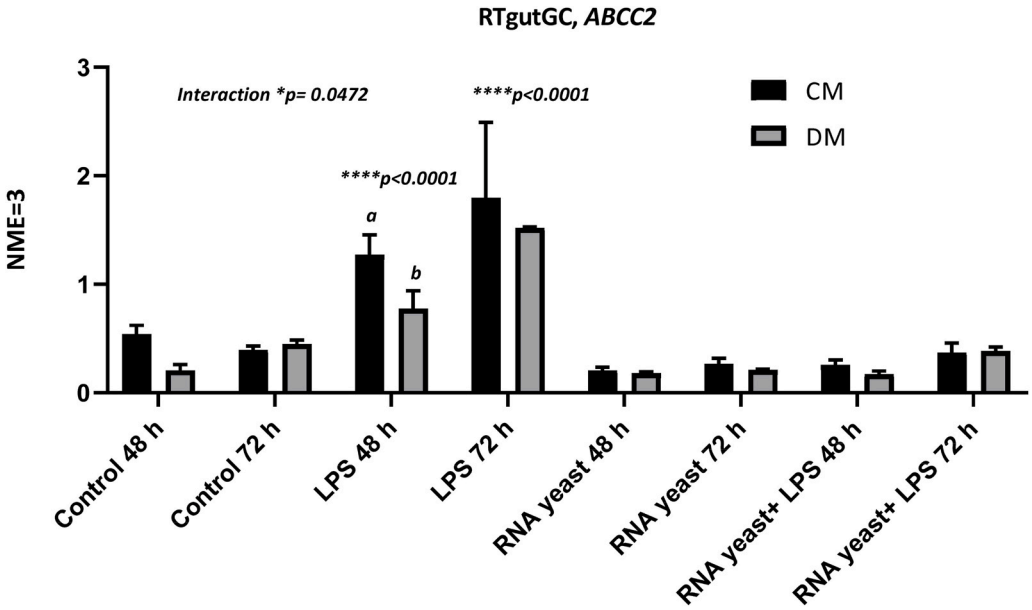

Fig. 3a. Transcription of transporter (*ABCC2*), detoxification enzyme (*GST*), glutamate dehydrogenase (*GDH*), Fatty acid metabolism enzyme (*Fas*) and glutamine synthase 1 (*GLS01*) in RTgutGC cells. (A) Gene transcription of the transporter protein *ABCC2* was induced 48 h ($p < 0.0001$) and 72 h ($p < 0.0001$) after LPS treatment, compared to controls at 48 h and 72 h. When the RTgutGC cells were cultured in CM, LPS induced transcription of *ABCC2* was significantly higher than gut cells cultured in DM ($a \neq b$, $p = 0.0038$). When harvested 48 h following LPS challenge. Interaction p = 0.0472.

(ssRNA hemorrhagic septicemia virus VHSV) [66] and the double stranded RNA mimic, Poly I:C, protected Japanese flounder (*P.olivaceous*) following VHSV infection as reported in Ref. [67]. Suggesting that RNA from baker's yeast may be recognized by TLR3 in this RTgutGC cell line and/or possibly to other TLRs [22]. Another option is that the TLR3 primer pairs used in this experiment could also be designed to recognize both single stranded and double stranded RNA. TRIF (TICAM-1) is the functional adapter for both TLR3 and TLR4 in mammals [67], and TLR3 in teleost [68] that may induce NFκβ, irf3, type1interferons and Myd88 independent dendritic cell maturation.

In RTgutGC cell line, only RNA from baker's yeast triggered transcription of *TNFα*, *trif* and *INF1* and maintained the transcription of *INF1* during prolonged culturing and when nutrients were depleted in the medium. For further studies, RNA from baker's yeast should be examined together with virus or virus mimic to elucidate the presence and role of different TLRs in the gut cell line and their role in the antiviral response.

Besides functioning in immune responses against pathogens, cytokines have been suspected to interact with the tight-junction complexes by rearrangements, down-regulation or internalization of tight junction related proteins. Changes in tight junction have been shown to affect the barrier permeability of the intestinal epithelium and often leads to

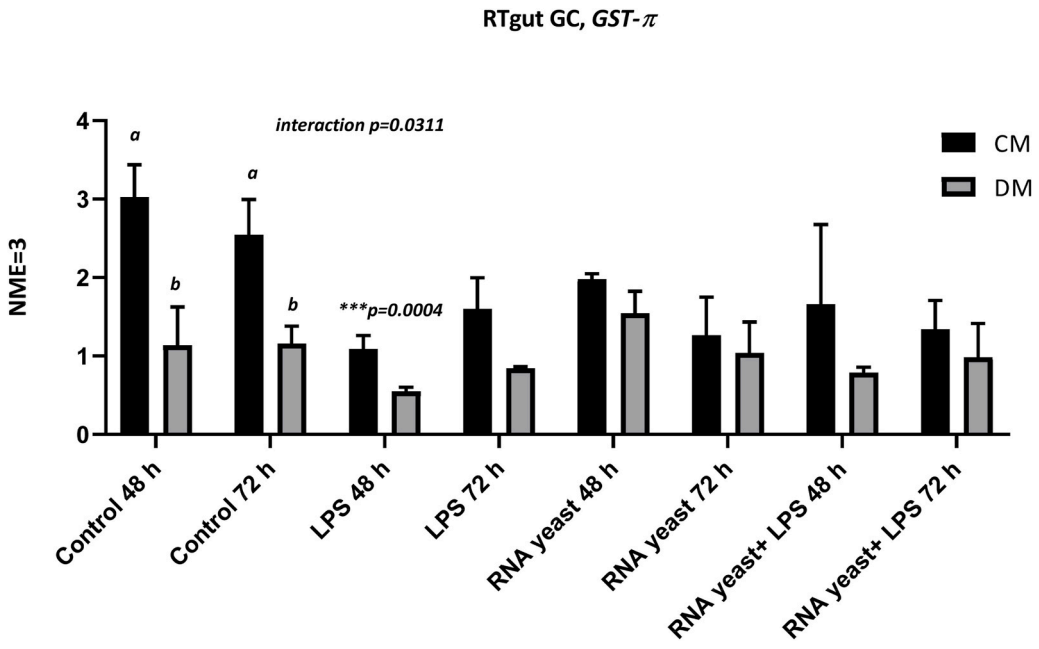

Fig. 3b. *GST-π* transcription was down-regulated ($p = 0.0004$) when RTgutGC cells were treated with LPS for 48 h (p = 0.0004) compared to respective controls. Depriving L-15 medium of glutamine and less FBS seemed to give a general reduction in the transcription of *GST π*, but significantly only in control cultures measured at 48 h ($a \neq b, p < 0.0001$) and 72 h ($a \neq b$, $p = 0.0098$) post LPS challenge. Interaction $p = 0.0311$.

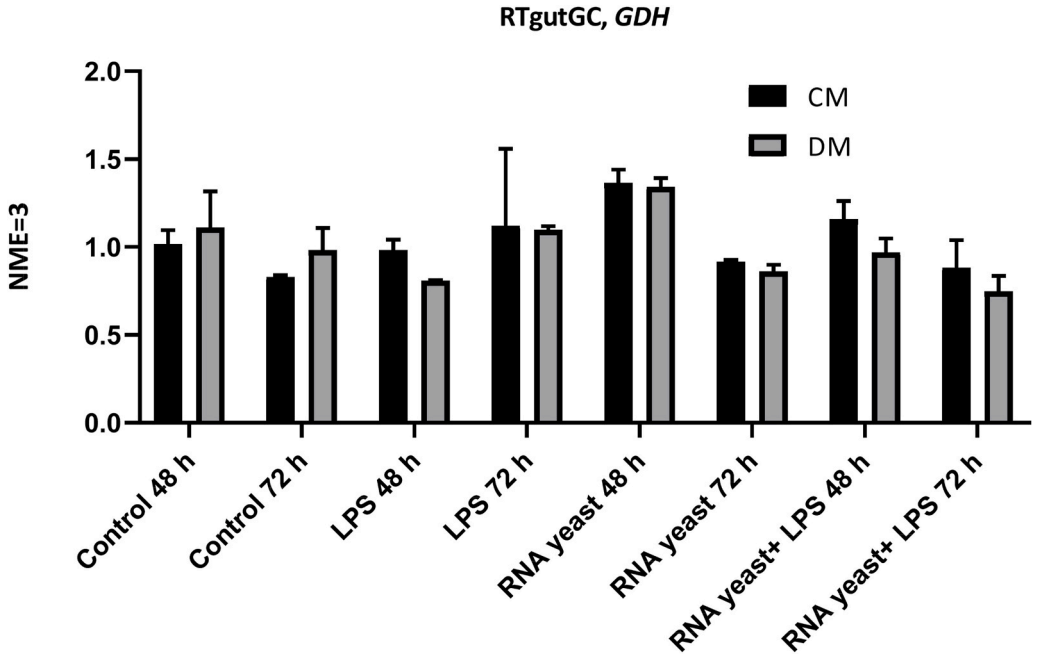

Fig. 3c. Glutamate dehydrogenase (*GDH*) was transcribed constitutively and equally in all cultures tested.

diseased intestinal states [69,70]. Information on the connection between immune parameters and intestinal barrier in the RTgutGC cell line as well in whole fish is scarce. However, the evidence of a functional barrier in the trout RTgutGC cell line [71] is emerging. In salmonids, whole fish, IL-1β and IL-6 decrease physical barrier tightness while interferons strengthen barrier functionality [72]. The effect of proinflammatory cytokines and how RNA from baker's yeast may modulate intestinal barriers functions should be investigated further in this cell line.

Transcription of *ABBC2* transporter was induced 48 h and 72 h post LPS challenge in the RTgutGC cell line, indicating a function also in inflammatory responses. This was not observed challenging the cells with RNA form baker's yeast and LPS together. Probably RNA form baker's yeast inhibited the LPS induced response of this gene. This was also observed for the GLS01 transcript. Efflux activities are probably mediated by ABBC1-3 [73] and are catalogized as phase three cellular detoxification proteins. GST is also a detoxification protein present in rainbow trout intestines as GST activity was increased when arsenite III was included in the feed. The arsenite III was transported by ABCC and was expressed in rainbow trout middle intestine and liver [74]. However, cell lines, derived from different tissues have often a high constitutively expression of these transporters compared to respective tissues

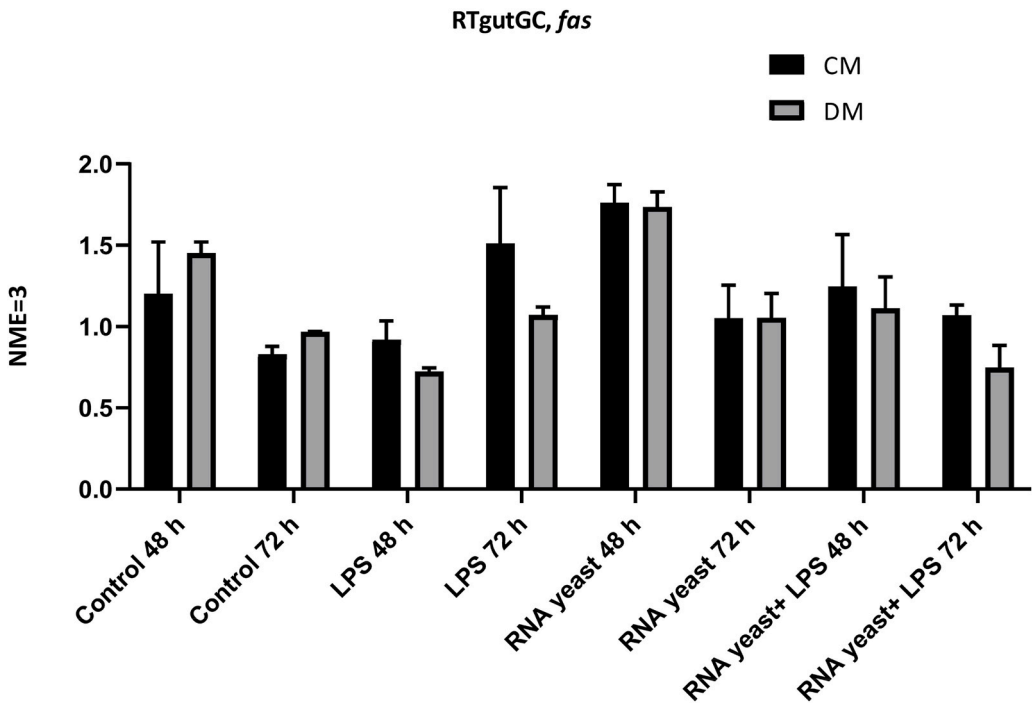

Fig. 3d. *fas* was transcribed constitutively and equally in all cultures tested.

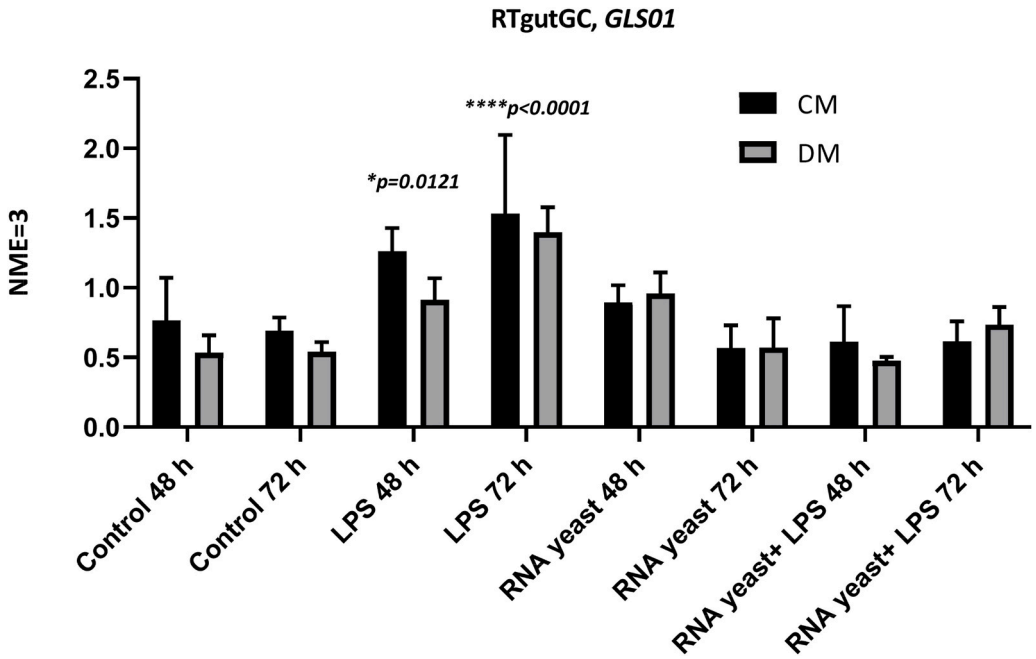

Fig. 3e. *GLS01* was induced 48 h ($p = 0.0121$) and 72 h ($p < 0.0001$), following LPS challenge as compared to control RTgutGC cultures.

[75]. The functional significance of these ABC efflux transporters must be further addressed particularly before using these cells for toxicological studies.

Previously it was noticed that after seven days of severe depletion of nutrient, only L-15 with salts, the RTgutGC cell line still adhered to the plastic surface where the cells were grown, indicating that the cell line not are particularly sensitive to nutrient depletion [76]. Although the cells got wounded, adding complete L-15 to the cells resulted in rapid repair. In the present experiment, culturing cells in DM reduced, and prolonged culturing with RNA from baker's yeast did not increase the *IL-1β* transcription. In contrast, culturing RTgutGC cells in DM but in the presence of RNA from baker's yeast for a prolonged period of time (120 h and 144 h) seemed beneficial for *INF1* transcription. Again, suggesting that RNA from baker's yeast may be protective during viral infections. The detoxification enzyme, *GST*, transcription was affected by DM but only significantly in control cultures compared to RTgutGC cells cultured in CM. This is interesting since the expression of this detoxification enzyme seemed to be dependent on optimal nutrition during LPS challenge, (which is known to interfere with detoxification signals [77, 78].

5. Summary

The results indicate that RNA from baker's yeast alone affects immune gene expression and can modulate bacterial and viral induced inflammatory responses.

The RTgutGC cell line provides a convenient, control-able and cost-effective way to study fish intestinal signaling pathways following intestinal cell challenging with dietary poly-ribonucleotides, the inflammatory inducer LPS and their interactions. In the RTgutGC cell line, LPS is recognized by its yet unknown ligand(s) and RNA from baker's yeast seem to be recognized by TLRs initiating downward signaling through MAPK and NFκβ, irf3 and ticam/trif, regulating the transcription of proinflammatory cytokines and markers.

Author contributions

Marit Espe and Elisabeth Holen were responsible for the experimental design, Maren Austgulen Hoff performed the cell experiment. Elisabeth Holen wrote the manuscript and manuscript was reviewed and edited by Marit Espe. All authors approved the final version.

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Author statement

Elisabeth Holen planned, wrote and edited the manuscript.
Marit Espe planned and edited the manuscript.
Maren Austgulen Hoff did the practical cell culture work.

Declaration of competing interest

This work contain no financial, commercial or any other conflicts of interest.

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References

[1] A. Kawano, C. Haiduk, K. Schirmer, R. Hanner, L.E.J. Lee, B. Dixon, et al., Development of a rainbow trout intestinal epithelial cell line and its response to lipopolysaccharide, Aquacult. Nutr. 17 (2011), https://doi.org/10.1111/j.1365-2095.2010.00757.x e241–e52.

[2] L.M. Langan, G.M. Harper, S.F. Owen, W.M. Purcell, S.K. Jackson, A.N. Jha, Application of the rainbow trout derived intestinal cell line (RTgutGC) for ecotoxicological studies: molecular and cellular responses follow exposure to copper, Ecotoxicology 26 (2017) 1117–1133, https://doi.org/10.1007/s10646-017-1838-8.

[3] P.G. Pumputis, V.R. Dayeh, L.E.J. Lee, P.H. Pham, Z. Liu, S. Viththiyapaskaran, et al., Responses of rainbow trout intestinal epithelial cells to different kinds of nutritional deprivation, Fish Physiol. Biochem. 44 (2018) 1197–1214, https://doi.org/10.1007/s10695-018-0511-3.

[4] R.K. Buddington, A. Krogdahl, A.M. Bakke-Mckellep, The intestine of carnivorous fish: structure and functions and the relations with diet, Acta Physiol. Scand. Suppl. 638 (1997) 67–80. PMID:9421581.

[5] J. Li, D.R. Barreda, Y.A. Zhang, H. Boshra, A.E. Gelman, S. LaPatra, et al., B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities, Nat. Immunol. 7 (2006) 1116–1124, https://doi.org/10.1038/ni1389.

[6] E.O. Koppang, U. Fischer, L. Moore, M.A. Tranulis, J.M. Dijkstra, B. Køllner, et al., Salmonid T cells assemble in the thymus, spleen and in novel interbranchial lymphoid tissue, J. Anat. 217 (2010) 728–739, https://doi.org/10.1111/j.1469-7580.2010.01305.x.

[7] H. Toda, Y. Saito, T. Koike, F. Takizawa, K. Araki, Y.T. Yabu, et al., Conservation of characteristics and functions of CD4 positive lymphocytes in teleost fish, Dev. Comp. Immunol. 35 (2011) 650–660, https://doi.org/10.1016/j.dci.2011.01.013.

[8] F. Takizawa, J.M. Dijkstra, P. Kotterba, T. Korytar, H. Kock, J. Køllner, et al., The expression of CD8α discriminates distinct T cell subsets in teleost fish, Dev. Comp. Immunol. 35 (2011) 752–763, https://doi.org/10.1016/j.dci.2011.02.008.

[9] T. Boardman, C. Warner, F. Ramirez-Gomez, J. Matrisciano, E. Bromage, Characterization of an anti-rainbow trout (Oncorhynchus mykiss) CD3ε monoclonal antibody, Vet. Immunol. Immunopathol. 145 (2012) 511–515, https://doi.org/10.1016/j.vetimm.2011.11.017.

[10] A. Attaya, T. Wang, J. Zou, T. Herath, A. Adams, C.J. Secombes, et al., Gene expression analysis of isolated salmonid GALT leukocytes in response to PAMPs and recombinant cytokines, Fish Shellfish Immunol. 80 (2018) 426–436, https://doi.org/10.1016/j.fsi.2018.06.022.

[11] E. Bassity, T.G. Clark, Functional identification of dendritic cells in the teleost model, rainbow trout (Oncorhynchus mykiss), PLoS One 7 (2012), e33196, https://doi.org/10.1371/journal.pone.0033196.

[12] I. Soleto, A.G. Granja, R. Simon, E. Morel, P. Diaz-Rosales, C. Tafalla, Identification of CD8α+ dendritic cells in rainbow trout (Oncorhynchus mykiss) intestine, Fish Shellfish Immunol. 89 (2019) 309–318, https://doi.org/10.1016/j.fsi.2019.04.001.

[13] D. Parra, T. Korytar, F. Takizawa, J.O. Suyer, B cells and their role in the teleost gut, Dev. Comp. Immunol. 64 (2016) 150–166, https://doi.org/10.1016/j.dci.2016.03.013.

[14] J.H.W.M. Rombout, L. Abelli, S. Pichette, G. Scapigliati, V. Kiron, Teleost intestinal immunology, Fish Shellfish Immunol. 31 (2011) 616–626, https://doi.org/10.1016/j.fsi.2010.09.001.

[15] I.E. Mulder, S. Wadsworth, J.C. Secombes, Cytokine expression in the intestine of rainbow trout (Oncorhynchus mykiss) during infection with Aeromonas salmonicida, Fish Shellfish Immunol. 23 (2007) 747–759, https://doi.org/10.1016/j.fsi.2007.02.002.

[16] J. Lokesh, J.M.O. Fernandes, K. Korsnes, Ø. Bergh, M.F. Brinchmann, V. Kiron, Transcriptional regulation of cytokines in the intestine of Atlantic cod fed yeast derived mannan oligosaccharide or β-Glucan and challenged with Vibrio anguillarum, Fish Shellfish Immunol. 33 (2012) 626–631, https://doi.org/10.1016/j.fsi.2012.06.017.

[17] Z. Han, J. Sun, A. Wang, A. Lv, X. Hu, L. Chen, Y. Guo, Differentially expressed proteins in the intestine of Cynoglossus semilaevis Günther following Shewanella algae challenge, Fish Shellfish Immunol. 104 (2020) 111–122, https://doi.org/10.1016/j.fsi.2020.06.013.

[18] A. Attaya, C.J. Secombes, T. Wang, Effective isolation of GALT cells: insight into the intestine immune response of rainbow trout (Oncorhynchus mykiss) to different bacterin preparations, Fish Shellfish Immunol. 105 (2020) 378–392, https://doi.org/10.1016/j.fsi.vaccine2020.06.051.

[19] T. Kawai, S. Akira, The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors, Nat. Immunol. 11 (2010) 373–384, https://doi.org/10.1038/ni.1863.

[20] H. Kumar, T. Kawai, S. Akira, Pathogen recognition by the innate immune system, Int. Rev. Immunol. 30 (2011) 16–34, https://doi.org/10.3109/08830185.2010.529976.

[21] S.W. Brubaker, K.S. Bonham, I. Zanoni, J.C. Kagan, Innate immune pattern recognition: a cell biological perspective, Annu. Rev. Immunol. 33 (2015) 257–290, https://doi.org/10.1146/annurev-immunol-032414-112240.

[22] Y. Palti, Toll like receptors in bony fish: from genomics to function, Dev. Comp. Immunol. 35 (2011) 1263–1272, https://doi.org/10.1016/j.dci.2011.03.006.

[23] E. Holen, S. Winterthun, Z.-Y. Du, A.V. Krøvel, Inhibition of p38MAPK during cellular activation modulate gene expression of head kidney leukocytes isolated from Atlantic salmon (Salmo salar) fed soybean oil or fish oil-based diets, Fish Shellfish Immunol. 30 (2011) 397–405, https://doi.org/10.1016/j.fsi.2010.11.017.

[24] D.B. Iliev, T. Hansen, S.M. Jørgensen, A. Krasnov, J.B. Jørgensen, CpG and LPS activated MAPK signaling in vitro cultured salmon (Salmo salar) mononuclear phagocytes, Fish Shellfish Immunol. 35 (2013) 1079–1085, https://doi.org/10.1016/j.fsi.2013.07.014.

[25] S.J. Pointer, J. Weleff, A.B. Soares, S.J. DeWitte-Orr, Class-A scavenger receptor function and expression in rainbow trout (Oncorhynchus mykiss) epithelial cell lines RTgutGC and RTgill-W1, Fish Shellfish Immunol. 44 (2015) 138–146, https://doi.org/10.1016/j.fsi.2015.01.028.

[26] M. Minghetti, C. Drieschner, N. Bramaz, H. Schug, K. Schirmer, A fish intestinal epithelial barrier model established from the rainbow trout (Oncorhynchus mykiss) cell line, RTgutGC. Cell Biol Toxicol. 33 (2017) 539–555, https://doi.org/10.1007/s10565-017-9385-x.

[27] H. Schug, Y. Yue, R. Krese, S. Fischer, T.M. Kortner, K. Schirmer, Time- and concentration-dependent expression of immune and barrier genes in the RTgutGC fish intestinal model following immune stimulation, Fish Shellfish Immunol. 88 (2019) 308–317, https://doi.org/10.1016/j.fsi.2019.02.036.

[28] J.D. Carver, W.A. Walker, The role of nucleotides in human nutrition, Nutr Biocehm 6 (1995) 58–72, https://doi.org/10.1016/0955-2863(94)00019-I.

[29] J.R. Bronk, J.G. Hatewell, The transport of pyrimidines into tissue rings cut from rat small intestine, J. Physiol. 382 (1998) 475–478, https://doi.org/10.1113/jphysiol.1987.sp016379.

[30] R. Aggett, J.L. Leach, R. Rueda, W.C. MacLean, Innovation in infant formula development: a reassessment of ribonucleotides in 2002, Nutrition 19 (2003) 35–384, https://doi.org/10.1016/s0899-9007(02)00999-1.

[31] J. Maldonado, J. Navarro, E. Narbona, A. Gil, The influence of dietary nucleotides on humoral and cell immunity in the neonate and lactating infant, Early Hum. Dev. 65 (2001) S69–S74, https://doi.org/10.1016/s0378-3782(01)00208-0.

[32] V.V. Trichet, Nutrition and immunity: an update, Aquacult. Res. 41 (2010) 356–372, https://doi.org/10.1111/j.1365-2109.2009.02374.x.

[33] H. Jyonouchi, Nucleotide actions on human immune response, Nutrition 124 (1994) 138S–143S, https://doi.org/10.1093/jn/124.suppl_1.138S.

[34] C.T. Van Buren, A.D. Kulkarni, F.B. Rudolph, The role of nucleotides in adult nutrition, J. Nutr. 124 (1994) 160S–164S, https://doi.org/10.1093/jn/124.suppl_1.160S.

[35] A.D. Kulkarni, W.C. Fanslow, F.B. Rudolph, C.T. Van Buren, Effect of dietary nucleotides on response to bacterial infections, J. Parenter. Enteral Nutr. 10 (1986) 169–171, https://doi.org/10.1177/0148607186010002169.

[36] A.A. Adjei, J.T. Jones, F.J. Enriques, S. Yamamoto, Dietary nucleotides and nucleosides reduce Cryptosporidium parvum infections in dexamethasone immunosuppressed adult mice, Exp. Parasitol. 92 (1999) 199–208, https://doi.org/10.1006/expr.1999.4415.

[37] J.D. Carver, Dietary nucleotides. Effects in the immune and gastrointestinal system, Acta Pædiatr Suppl 430 (1999) 83–88, https://doi.org/10.1111/j.1651-2227.1999.tb01306.x.

[38] R.M. Reda, K.M. Selim, R. Mahmoud, I.E. El-Araby, Effect of dietary yeast nucleotide on antioxidant activity, non-specific immunity, intestinal cytokines, and disease resistance in Nile Tilapia, Fish Shellfish Immunol. 80 (2018) 281–290, https://doi.org/10.1016/j.fsi.2018.06.016.

[39] C. Wu, Z. Yang, C. Song, C. Liang, H. Li, W. Chen, et al., Effects of dietary yeast nucleotides supplementation on intestinal barrier function, intestinal microbiota, and humoral immunity in specific pathogen-free chickens, Poultry Sci. 97 (2018) 3837–3846, https://doi.org/10.3382/ps/pey268.

[40] E.F. Mohamed, M.M. Hadny, N.F. Kamel, N.M. Ragaa, The impact of exogenous dietary nucleotides in ameliorating Clostridium prefingens infections and improving intestinal barrier gene expression in broiler chicken, Vet Animal Sci 10 (2020) 100130, https://doi.org/10.1016/j.vas.2020.100130.

[41] E. Holen, O. Bjørge, R. Jonsson, Dietary nucleotides and human immune cells. II. Modulation of cell growth and cytokine secretion, Nutrition 21 (2005) 1003–1009, https://doi.org/10.1016/j.nut.2005.03.003.

[42] G. Strandskog, S. Villoing, D.B. Iliev, H.L. Thim, K.E. Christie, J.B. Jørgensen, Formulation combining CpG containing oligonucleotides and poly I:C enhance magnitude of immune responses and protection against pancreas disease in Atlantic salmon, Dev. Comp. Immunol. 35 (2011) 1116–1127, https://doi.org/10.1016/j.dci.2011.03.016.

[43] C. Burrels, P.D. William, P.F. Forno, Dietary nucleotides: a novel supplement in fish feed. Effects of resistance to diseases in salmonids, Aqacult 199 (2001) 159–169, https://doi.org/10.1016/S0044-8486(01)00577-4.

[44] M. Sakai, K. Taniguchi, K. Mamoto, H. Ogawa, M. Tabata, Immunostimulant effects of nucleotide isolated from yeast RNA on carp, Cyprinus carpio, J. Fish. Dis. 24 (2001) 433–438, https://doi.org/10.1046/j.1365-2761.2001.00314.x.

[45] C.-S. Liu, Y. Sun, Y-h. Hu, L. Sun, Identification and analysis of a CpG motif that protects turbot (Scopthalmus maximus) against bacterial challenge and enhances vaccine induced specific immunity, Vaccine 28 (2010) 4153–4161, https://doi.org/10.1016/j.vaccine.2010.04.016.

[46] R.C. Ganassin, N.C. Bols, Effect of purine supplementation on the growth of salmonid cell lines in different mammalian sera, Cytotechnology 8 (1992) 21–29.

[47] R.C. Ganassin, Q.H. Tran, T.F. Rabgey, N.C. Bols, Enhancement of proliferation in cultures of Chinook salmon embryo cells by interactions between inosine and bovine sera, J. Cell. Physiol. 160 (1994) 409–416, https://doi.org/10.1002/jcp.1041600303.

[48] X. Guo, J.L. Li, C. Ran, A. Wang, M. Xie, Y. Xie, et al., Dietary nucleotides can directly stimulate the immunity of zebrafish independent of intestinal microbiota, Fish Shellfish Immunol. 86 (2019) 1064–1071, https://doi.org/10.1016/j.fsi.2018.12.058.

[49] A. Rebl, T. Goldhammer, H.M. Seyfert, Toll-like receptor signaling in bony fish, Vet. Immunol. Immunopathol. 134 (2010) 139–150, https://doi.org/10.1016/j.vetimm.2009.09.021.

[50] G. Løkka, E.O. Koppang, Antigen sampling in the fish intestine, Dev. Comp. Immunol. 64 (2016) 138–149, https://doi.org/10.1016/j.dci.2016.02.014.

[51] A. Gil, C. Gomez-Leon, R. Rueda, Exogenous nucleic acids and nucleotides are efficiently hydrolyzed and taken up as nucleosides by intestinal explants from suckling piglets, Br. J. Nutr. 98 (2007) 285–291, https://doi.org/10.1017/S000711450770908X.

[52] J.R. Hess, N.A. Greenberg, The role of nucleotides in the immune and gastrointestinal system: potential clinical applications, Nutr. Clin. Pract. 27 (2012) 281–294, https://doi.org/10.1177/0884533611434933.

[53] D.R.E. Ranoa, S.L. Kelley, R.I. Tapping, Human lipopolysaccharide-binding protein (LBP) and CD14 independently deliver triacylated lipoproteins to Toll like receptor1 (TLR1) and TLR2 and enhance formation of the ternary signaling complex, J. Biol. Chem. 288 (2013) 9729–9741, https://doi.org/10.1074/jbc.M113.453266.

[54] D.B. Iliev, J.C. Roach, S. Mackensie, J.V. Planas, F.W. Goetz, Endotoxin recognition. In fish or not in fish, FEBS Lett. 579 (2005) 6519–6528, https://doi.org/10.1016/j.febslet.2005.10.061.

[55] J.D. Pietretti, H.P. Spaink, A. Falco, M. Forlenza, G.M. Wiegertjes, Accessory molecules for Toll-like receptors in teleost fish. Identification of TLR4 interactor with leucine-rich repeats (TRIL), Mol. Immunol. 56 (2013) 745–756, https://doi.org/10.1016/j.molimm.2013.07.012.

[56] C. Jault, I. Pichon, J. Chluba, Toll like receptor gene family and TIR-domain adapters in Danio rerio, Mol. Immunol. 40 (2004) 759–771, https://doi.org/10.1016/j.molimm.2003.10.001.

[57] J. Su, C. Yang, F. Xiong, Y. Wang, Z. Zhu, Toll like receptor 4 signaling pathway can be triggered by grass carp reovirus and Aeromonas hydrophilia infection in rare minnow Gobiocypris rarus, Fish Shellfish Immunol. 27 (2009) 33–39, https://doi.org/10.1016/j.fsi.2009.02.016.

[58] P. Kongchum, Y. Palti, E.M. Hallermann, G. Hulata, L. David, SNP discovery and development of genetic markers for mapping innate immune response genes in common carp (Cyprinus carpio), Fish Shellfish Immunol. 29 (2010) 356–361, https://doi.org/10.1016/j.fsi.2010.04.013.

[59] R. Huang, F. Dong, S. Jang, L. Liao, Z. Zhu, Y. Wang, Isolation and analysis of a novel grass carp Toll like receptor 4 (tlr4) gene cluster involved in response to grass carp reovirus, Dev. Comp. Immunol. 38 (2012) 383–388, https://doi.org/10.1016/j.dci.2012.06.002.

[60] S.M. Quiniou, P. Boudinot, E. Bentgen, Comprehensive survey and genomic characterization of Toll like Receptors (TLRs) in channel catfish, Ictalurus punctatus: identification of novel fish TLRs, Immunogenetics 42 (2013) 567–568, https://doi.org/10.1007/s00251-013-0694-9.

[61] J.D. Pietretti, G.F. Wiegertjes, Ligand specificities of Toll-like receptors in fish: indications from infections studies, Dev. Comp. Immunol. 43 (2014) 205–222, https://doi.org/10.1016/j.dci.2013.08.010.

[62] K.A. Lord, B. Hoffman-Liebermann, D.A. Liebermann, Nucleotide sequence and expression of cDNA encoding MyD88, a novel myeloid differentiation primary response gene induced by IL-6, Oncogene 5 (1990) 1095–1097. PMID:2374694.

[63] S.M. Miggin, L.A. O'Neill, New insights into the regulation of TLR signaling, J. Leukoc. Biol. 80 (2006) 220–226, https://doi.org/10.1189/jlb.1105672.

[64] K. Richard, D.J. Perkins, E.M. Harbert, Y. Song, A. Gopalakrishnan, K.A. Shirey, et al., Dissociation of TRIF bias and adjuvanticity, Vaccine 38 (2020) 4298–4308, https://doi.org/10.1016/j.vaccine.2020.04.042.

[65] S. Smith, C. Jefferies, Role of DNA/RNA Sensors and Contribution to Autoimmunity, 25pp, Cytokine Growth Factor Rev., 2014, pp. 745–757, https://doi.org/10.1016/j.cytogfr.2014.07.019.

[66] B. Novoa, A. Romero, V. Mulero, I. Rodrigues, I. Fernandez, A. Figueras, Zebrafish (Danio rerio) as a model for the study of vaccination against viral haemorrhagic septicemia virus (VHSV), Vaccine 24 (2006) 5806–5816, https://doi.org/10.1016/j.vaccine.2006.05.015.

[67] B.R. Sahoo, M. Basu, B. Swain, J. Maharana, M.R. Dikhit, P. Jayasankar, et al., Structural insights of rohu TLR3, its binding site analysis with fish reovirus dsRNA, Poly I:C and zebrafish TRIF, Int Biolog Macromol 51 (2012) 531–543, https://doi.org/10.1016/j.ijbiomac.2012.06.005.

[68] P.F. Zou, J.J. Shen, Y. Li, Z.P. Zhang, Y.L. Wang, TRAF3 enhances TRIF-mediated signaling via NFκβ and IRF3 activation in large yellow croaker, Larimichthys crocea, Fish Shellfish Immunol. 97 (2020) 114–124, https://doi.org/10.1016/j.fsi.2019.12.024.

[69] A. Fasano, J.P. Nataro, Intestinal epithelial tight junctions as targets for enteric bacteria-derived toxins, Adv. Drug Deliv. Rev. 56 (2004) 795–807, https://doi.org/10.1016/j.addr.2003.

[70] C.T. Capaldo, A. Nusrat, Cytokine regulation of tight junction, Biochim. Biophys. Acta Biomembr. 1788 (2009) 864–871, https://doi.org/10.1016/j.bbamem.2008.08.027.

[71] J. Wang, P. Lei, A.A.A. Gamil, L. Lagos, V. Yue, K. Schirmer, et al. Rainbow trout (Onchorhyncus mykiss) intestinal epithelia cells as a model for studying gut immune function and effects of functional feed ingredients. doi:10.3389/fimmu.2019.00152.

[72] L. Niklasson, Intestinal Mucosal Immunology of Salmonids-Response to Stress and Infection and Crosstalk with the Physical Barrier, University of Gotenburg, 2013. http://hdl.handle.net/2077/32780.

[73] S. Fischer, J. Loncar, R. Zaja, S. Schnell, K. Schirmer, T. Smital, T. Luckenbach, Constitutive mRNA expression and protein activity levels of nine ABC efflux transporters in seven permanent cell lines derived from different tissues of rainbow trout (Oncorhynchus mykiss), Aquat. Toxicol. 101 (2011) 438–446, https://doi.org/10.1016/j.aquatox.2010.11.010.

[74] J.C. Painefilu, M.M. Pascal, F. Bieczynshi, C. Laspoumaderes, C. Gonzales, S.M. M. Villaneueva, et al., Ex vivo and in vivo effects of arsenite on GST and ABCC2 activity in the middle intestine of the rainbow trout Onchorhynchus mykiss, Comp Biochem Phys C Toxicol Pharamcol 255 (2019) 108566, https://doi.org/10.1016/j.cbpc.2019.108566.

[75] K.S. Vellonen, E. Mannermaa, H. Turner, M. Hakli, J.M. Wolosin, T. Tervo, P. Honkakoski, A. Urtti, Effluxing ABC transporters in human corneal epithelium, J. Pharmacol. Sci. 99 (2010) 1087–1098, https://doi.org/10.1002/jps.21878.

[76] P.G. Pumputis, Development of RTgutGC as a tool for fish feed development. Master of science degree, biology, waterloo, ontario Canada, Available at: https://uwspace.uwaterloo.ca/bitstream/handle/10012/12150/Pumputis_PatrickGilles.pdf?sequence=1&isAllowed=y, 2017.

[77] E. Holen, P.A. Olsvik, Aryl hydrogen receptor protein and CYP1A1 gene induction by LPS and phenanthrene in Atlantic cod (Gadus morhua) head kidney cells, Fish Shellfish Immunol. 40 (2014) 384–391, https://doi.org/10.1016/j.fsi.2014.07.022.

[78] E. Holen, P.A. Olsvik, Naphthoflavone interferes with cyp1c1, cox2 and IL-8 gene transcription and leukotriene B4 secretion in Atlantic cod (Gadus morhua) head kidney cells during inflammation, Fish Shellfish Immunol. 54 (2016) 128–134, https://doi.org/10.1016/j.fsi.2016.03.043.