Anti-nodavirus potential activity of R.A73 Enterococcus faecium strain, isolated from Tunisian freshwater fish mucus

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Abstract

R.A73 *Enterococcus faecium* (HG937697) was tested for investigating anti-nodaviral activity and immunomodulatory properties on E-11 fish cells. Non R.A73-related toxicity was confirmed. Likewise, *in-vitro* inhibition of nodavirus replication and gene expression of nodavirus capsid protein gene in addition to CCR3, IL-10 and TNFα genes were evaluated using qPCR. A preventive treatment of E-11 cells by the bacterial suspension generated a remarkable reduction in virus loads (RQ values from 0.1 to 0.9) at day 7. Interestingly, R.A73 *E. faecium* showed a significant gene expression upregulation of proinflammatory IL-10 cytokine whereas it did not significantly enhance CCR3 nor TNF-α immune gene expression in E-11 cells. Therefore, add-on its probiotic potential, R.A73 *E. faecium* is able to stimulate the immune system in the preventive phase throughout the differential expression of main inflammatory genes. This study provides the first evidence that R.A73 *E. faecium* strain can prevents effectively nodavirus replication, *in vitro*.

Highlights

- *R.A73 E. faecium* displays good probiotic potential to be use in fish
- *R.A73 E. faecium* has a cell protective effect against nodavirus co-infection, *in vitro*
- R.A73 *E. faecium* induces significant differential expressions of IL10, TNF-a and CCR3 genes

1. Introduction

Nervous Necrosis Virus (NNV) is considered as the main disease for European sea bass *Dicentrarchus labrax* causing viral encephalopathy and retinopathy (VER) and leading to high economical losses within Mediterranean fish farms (Toffan et al., 2017). The viral agent belongs to the Nodaviridae family which affects the nervous system of vulnerable fish and frequently involves mortalities in early life-stages: larvae and juveniles (Munday et al., 2002). In recent decades, the prevention and control of animal diseases have focused on the use of chemical additives and veterinary drugs, especially antibiotics (Gunal et al., 2006, Saxena et al., 2018). The interest in substituting such compounds with alternative natural products that do not harm either the host or the environment becomes more and more important. Recently, the novel lactic bacteria R.A73 *Enterococcus faecium* isolated from freshwater fish mucus has proven to be a good food-derived beneficial microorganism for probiotic application in fish (El-Jeni et al., 2015). R.A73 exhibits high inhibitory activities against food-borne pathogens and spoilage microbial species displaying a proteomic spectral signature (El-Jeni et al., 2019). More recently, its whole genome contents and gene functions further supported the antibacterial properties (El Jeni et al., 2020). The current study was performed to further evaluate the antiviral activity and immunomodulatory properties of the pre-selected R.A73 *E. faecium* strain. The ability to inhibit Nodavirus replication as well as to simulate the immune system are followed by *in vitro* assays on E-11 Nodavirus-sensitive cells.

2. Material And Methods

Bacterial strain source and characterization: The pre-selected *E. faecium* (R.A73) is a novel strain isolated from freshwater fish's mucus (*Mugil cephalis*) collected from Tunisian dams (El-Jeni et al., 2015). It was cultured (10^7 CFU/ ml) and prepared as previously described (El-Jeni et al., 2015). Briefly, the strain was inoculated in Man Rogosa Sharpe (MRS) medium broth and on MRS agar plates. After 48 h at 20°C incubation, culture was centrifuged at 12 000g for 10 min. R.A73 strain exhibited moderate heat resistance, adhesion ability to steel surfaces, and sensitivity to clinically relevant antimicrobial agents with specific proteomic profile (El-Jeni et al., 2019). The complete genome annotation (28 contigs consisting of 2,935,283 bps) has been studied and deposited into EMBL GenBank with the accession number EMBL: HG937697. A total of 2884 genes including 2834 coding sequences and 50 RNAs containing 3 rRNAs (one rRNA 16 s, one rRNA 23 s and one rRNA 5 s) and 47 tRNAs were identified within the R.A73 novel strain (El Jeni et al., 2020).

Cell culture, in-vitro infection and cytotoxicity test by MTT assay

The fibroblastic clone E-11, derived from the SSN-1 cell line of snakehead fish, (Iwamoto et al., 2000) was used to study the cytotoxic and antiviral effects of R.A73 strain. Cells were cultured in $25cm^2$ flasks at 25°C in Leibovitz's L-15 medium (Eurobio), with 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin solution (100 mg/ml streptomycin and 100 IU/ml penicillin). The Tunisian nodavirus strain, RGNNV-131/Aq/10 isolated from sea bass (Chérif et al., 2010), was used for inoculation. Cell culture supernatants were clarified by low-speed centrifugation (3000 x g for 15 min) and stored in aliquots at -80°C. To titrate the isolate, viral supernatant diluted 10^{-1} to 10^{-8} was used to infect E-11 cells and the $TCID_{50}$/ml determined by the Spearman-Karber method (Kärber, 1931). Cytopathic effect (CPE) was observed each day and the titer determined on day 7. Freshly growing 96 well plates of E-11 cells were treated with 50µl of R.A73*E. faecium* suspension at 10^7 CFU/ ml. After 1h, cells were then inoculated with 50 µl of the nodavirus preparation of 1.0×10^7 TCID50/ml^{-1}, which resulted in a MOI of 0.1. Control cells were mock inoculated with L-15 medium. Additionally, R.A73 *E. faecium* was inoculated on E-11 cells solely. The appearance of specific NNV cytopathic effect within E-11 cells was examined and photographed using an inverted microscope (Motic AE2). The supernatants from virus inoculated wells, R.A73 *E. faecium* treated wells and negative control wells were collected and stored at -80 ºC prior to RNA extraction. The cells were harvested after 6, 24, 48 and 72h post infection for the assessment of viral and cellular gene expression.

Additionally, the R.A73 *E. faecium* effect on E-11 cells growth was tested for 7 days under *in vitro* conditions using the MTT assay as described by Mosmann (1983). The test is based on the reduction of soluble yellow MTT tetrazolium salt to a blue insoluble MTT formazan product by mitochondrial succinic dehydrogenase. After 24h of exposure, the test medium was replaced by 10 µl of 5 mg/ml MTT (Sigma) in PBS. After incubation for 4 h at 25°C, the staining solution was carefully removed by aspiration and the cells were rinsed twice with PBS, then 200 µl/well of DMSO was added to solubilize the blue formazan crystals produced. The absorbance of each well was measured at 570 nm (test wavelength) in the microplate reader (Fluostar Optima, BMG Labtech).

RNA extraction and Relative quantification of target genes by ΔΔCT: RNeasy Mini Kit (Qiagen) was used to purify total RNA from cell supernatants according to the manufacturer's instructions. Nucleic acid concentration was obtained directly using Nanodrop 2000 (Thermo scientific, USA) in terms of ng/µL and the 260: 280 ratios used to estimate the purity of the isolated RNA. Random hexamers were used to synthesize the first strand cDNA using the SuperScript first strand synthesis system for RT-PCR as recommended (Invitrogen). The 7500 Real Time PCR System (Applied Biosystems) and the sets of primers used for the viral and immune gene expression assays are published by Dalla Valle et al. (2005) and Azeredo et al. (2015), respectively. For the PCR reaction, cDNA was amplified using the SYBR Green Mastermix (Applied Biosystems). The specificity was determined by a first-derivative melt curve. Two negative controls using diethylpyrocarbonate (DEPC)-treated water as template (no RNA template) were included within each experiment. In addition, nodavirus free samples were first tested with the present protocol and found to confirm the specificity of the primers (data not shown). Each sample was analysed in duplicate and C_T values were plotted against the logarithm of the amount of template and a linear regression was performed. Normalization of the results was applied using the ratio between the mean C_T values obtained with the target gene primers and those obtained with the β-actin primers and a calibrator gene (sample with no LAB treatment). RNA quantities were estimated by the application of the formula of Livak and Schmittgen (2001). Differences in the response between infected and mock groups were represented as means ± SD. Data were analyzed by one-way analysis of variance (ANOVA) using Statistical Package for Social Sciences Software (SPSS) version 20.0 (SPSS Inc., Chicago, Illinois, US) and when statistically significant differences were observed ($P < 0.05$) a comparison of mean test was applied.

3. Results

Effect of *E. faecium* suspension on Cell Viability

The potent cytotoxic property of R.A73 *E. faecium* suspension on the E-11 cell line was checked *via* the MTT test showing that the inoculum at 10^7 CFU/ ml did not alter the morphological appearance of the monolayer during seven monitoring days and optical densisity (OD) values indicated that cell viability was approximately 300% compared to the negative control (un-treated E-11 cells) after 24 hours of incubation (Fig. 1D).

Effect of R.A73 E. faecium **suspension on cytopathic effect (CPE) after nodavirus inoculation**: In nodavirus infected wells, CPE with typical multiple vacuolation was observed in cells after 3 to 4 dpi (Fig. 1A). Initially, the specific CPE developed as localized areas of rounded and refractile cells that later spread over the monolayer to form a network of degenerating cells. The monolayer was completely disintegrated after 7 days. No CPE was observed in mock cells (Fig. 1B). R.A73 *E. faecium* suspension treated wells showed a clear inhibition of nodavirus proliferation which was supported by the complete absence of the development of CPE during the observation period (Fig. 1C).

Effect of R.A73 *E.faecium* treatment on viral capsid gene expression

The expression of the viral capsid protein gene in infected cells with the nodavirus isolate increased steadily over the 72 hours post inoculation, demonstrating that viral replication took place. Interestingly, the pre-treatment of E-11 cells with R.A73 *E. faecium* during 1h right before virus inoculation, induced a reduction in virus loads which was measured by a subexpression of the viral capsid protein (CP) gene and the increase in RQ values from 0.1 to 0.9 at 7 days post inoculation (Fig. 2). Statistical data analysis showed that the expression of the viral capsid gene was significantly lower in well with treated cells compared to control wells ($p \leq 0.05$).

Expression Level Changes in Cell Mediator Genes in Response to Nodavirus Infection

The gene expression patterns of CCR3, IL-10 and TNFα genes were evaluated *in vitro* using qPCR after inoculation of E-11 cells with RGNNV isolate. Data were presented as the average of the RQ of the target genes after normalization by the reference gene β-actin ± SD in Figs. 3D, 3E, 3F. The expression of the three genes in infected cells increased steadily over the 72 hours post inoculation compared to the day 1 post inoculation where RQ values increased from 0.18 to 0.78, from 0.24 to 0.47 and from 0.2 to 3.6 in the case of IL-10, CCR3 and TNFα genes, respectively. The difference between treated and untreated assays were significant ($P < 0.05$).

Expression Level Changes in Cell Mediator Genes in Response to *E. faecium* Treatment

Similarly, the gene expression patterns of CCR3, IL-10 and TNFα genes were evaluated *in vitro* using qPCR after treatment with R.A73 *E. faecium* cell supernatant. Data were presented as the average of the RQ of the target genes after normalization by the reference gene β-actin ± SD. As depicted in Fig. 3, changes with respect to control groups were observed in all three target gene transcripts. IL-10 as an important anti-inflammatory cytokine, it may counteract the production of proinflammatory cytokines, such as TNF-α. In this study, R.A73 *E. faecium* enhanced the production of IL-10, *in vitro*. Indeed, Fig. 3A shows a significant increase in the expression of the IL-10 gene assessed three days post E-11 treated cells, compared to un-treated control cells in which RQ values increases from to 2 to 18. Furthermore, the R.A73 *E. faecium* enhanced the expression of the CCR3 gene (Fig. 3B) as well (RQ values increases from to 1 to 3.75), but significantly decreased the *in vitro* expression of TNFα gene (RQ values increases from to 1 to 0.2) suggesting an anti-inflammatory potential (Fig. 3C).

Expression Level Changes in Cell Mediator Genes in Response to a simultaneous inoculation of *E. faecium* and RGNNV nodavirus

Unexpectedly, the monitoring of a potential anti-nodavirus inhibitory activity of R.A73 *E. faecium* on pre-treated E-11 cell line expression during seven days showed that the simultaneous inoculation of E-11 cells by R.A73 *E. faecium* and nodaviral suspensions resulted in an under-expression of IL-10, CCR3 and TNFα genes (Fig. 3D, 3E, 3F) compared to their expression in response to nodavirus alone. RQ values decreased from 0.19 to 0.02, from 0.3 to 0.01 and from 0.19 to 0.02 in the case of IL-10, CCR3 and TNFα genes, respectively. The difference between treated and untreated assays were significant ($P < 0.05$).

4. Discussion

The *Nodaviridae* family has been causing major damage affecting species such as grouper, Asian seabass/barramundi, European sea bass, gilthead sea bream, Japanese and barfin flounder, Atlantic and Pacific cod and Atlantic halibut (Bandín and Souto, 2020). The knowledge on antiviral activity of probiotics has been raised in recent years (Lakshmi et al., 2013). For example, Pseudomonas, Vibrio, Aeromonas spp. and Coryneforms had antiviral activity against infectious hematopoietic necrosis virus (IHNV) (Kamei et al., 1988). Previously, Li et al. (2009) demonstrated that feeding with a Bacillus megaterium strain increased the resistance to white spot syndrome virus (WSSV) in the shrimp *Litopenaeus vannamei*. Application of *Latic Bacteria* probiotics (LAB) as a single strain or mixed with Sporlac improved disease resistance against lymphocystis viral disease in olive flounder (*Paralichthys olivaceus*) (Harikrishnan et al., 2010). Among invertebrates, probiotic bacteria have been found to induce both cellular and humoral immune responses in the shrimp (Castex et al., 2008, Farzanfar, 2006, Vargas-Albores et al., 2017, Knipe et al., 2021). This includes the stimulation of pro-inflammatory cytokines, increasing the phagocytic activity of leucocytes (Pirarat et al., 2006), increasing the levels of antibodies complements (Balcázar et al., 2007), cytokines (interleukin-1 (IL-1), IL-6, IL-12, tumor necrosis factor a (TNF-a), gamma interferon (IFN-g), IL-10 and antimicrobial peptides (Mukherjee et al., 2012). In the current study, assays were implemented in order to further refine work initiated to demonstrate the potential use of novel lactic acid bacteria isolated from mucus of freshwater fish (*Mugil cephalis*) and involved as probiotic in aquaculture (El-Jeni et al., 2019) seeking to address more gaps such as exploring the anti- nodavirus RGNNV activity. By primary analyses, the first experiment did not detect any toxicity following R.A73 *E. Faecium* application on E-11 fish cell line and no morphological differences were observed, giving evidence of cell biosafety. This has been seen in the case of Chai et al. (2013), at concentrations up to 1.00E + 07 CFU/ml of *E. faecium* NCIMB 10415 in animal nutrition. In addition, the release of TNF-α, cytokine IL-10 and CCR3 genes were monitored to determine the underlying cellular mechanism. The E-11 cells were treated with R.A73 *E. faecium* resulting an over-expression of the IL-10 gene. We can assume that the response of the innate immune system, characterized by the increase of the anti-inflammatory cytokine IL-10 caused the beneficial physiological effects of the probiotic *Enterococcus* strain. This result is in agreement with Tarasova et al. (2010) findings which showed the influence of probiotic *E. faecium* strain on cytokines expression in rats and as an optimal supplementation level of dietary for tilapia (Li et al., 2020). In contrast to earlier findings, however, no evidence of TNF-α secretion was detected in the present study, the ratios obtained from control cells compared to R.A73 *E. faecium*-inoculated wells revealed a decline in TNF-α expression levels. These results reflect those of Kern et al. (2017) who also found that upregulation of TNF-α at the mRNA level was prevented when cells were co-incubated with *E. faecium*. On the other hand, the assessment of gene expression coding for the chemokine receptor C-C chemokine receptor 3 (CCR3) from cells inoculated with R.A73 *E. faecium* showed increased expression of the CCR3. In previous study, the probiotic treatment altered markers such as CCR3, resulting in an overall increase in gene expression, which was particularly noticeable in fish fed diets (Moroni et al., 2021).

When investigating the antiviral activity, the R.A73 *E. faecium* was added for 1h right before the infection period by the nodavirus. The findings revealed a substantial decrease in the expression of IL-10, TNF-α, and the CCR3 genes compared to E-11 cells infected with nodavirus alone. We hypothesis the protective effect of the probiotic R.A73 *E. Faecium* on cells. Indeed, IL-10, a prototypical anti-inflammatory cytokine, produced by activated monocytes, T cells and other cell types such as keratinocytes, is considered to be a crucial factor in a major immune response and inflammation inhibitor (Brockmann et al., 2018). We speculated that the probiotic R.A73 *E. faecium* has a protective effect on the cells and reduces the inflammatory cytokine expression induced by nodavirus infection. Hence, by co-incubating with this probiotic strain, the stimulus for the release of IL-10 is blocked and induced an under expression of TNF-α and CCR3 compared to the virus-infected cells during the seven days of post-inoculation assuming the probiotic to have protective effects on cells.

Altogether, further studies are needed to support data obtained. The first evidence-based capability of R.A73 *E. faecium* novel probiotic strain to induce INF-a, IL-10 and CCR3 immune genes in E-11 cells may subsequently contributed to improving the antiviral response mounted by the cells against RGNNV nodavirus.

Declarations

Compliance with ethical standards

Ethics Statement: No animal experiments were performed during this study. Viral strains were isolated from fish specimens derived from the diagnostic and monitoring activities carried out at the National Institute of Sea Sciences and Technologies (INSTM) through its research project CapTunHealth (PEER Cycle 5) which was approved by the bioethics committee under the reference 2017/24/E/INSTM.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions: N.C conceived the study and wrote the manuscript. F.A. and R.J performed bacteria and cell culture experiments and cytotoxicity assays. M.B. and R.J. performed LAB isolation characterization. B.B contributed to data analysis and interpretation. N.C. and F.A. performed virological analysis. N.C. and R.J. prepared the original draft, B.B. and M.B contributed to manuscript editing and reviewing. All authors reviewed the manuscript.

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Data Availability statement: The data that support the findings of this study are available on request from the corresponding author. Some of the data are not publicly available due to the privacy or ethical restrictions.

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Figures

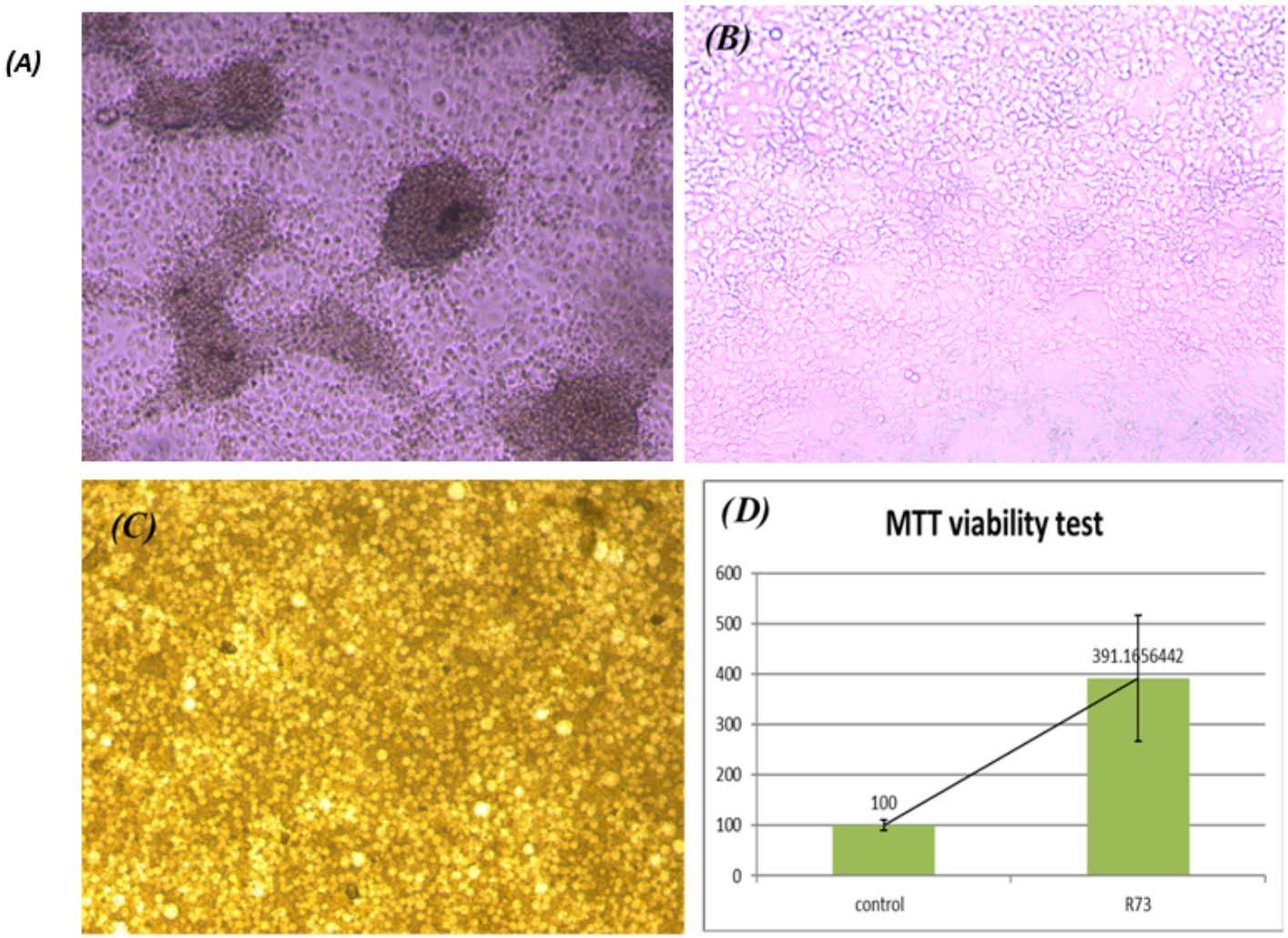

Figure 1

Susceptibility of E-11 cells to nodavirus and R.A73 *E. faecium* treatment (A) Extensive CPE with multiple vacuolation in E-11 cells infected with nodavirus. (B) Confluent uninfected E-11 cells. (C) Nodavirus infected E-11 cells treated with R.A73 *E. faecium*. (D) E-11 viability test using MTT measured at 590nm before and after treatment with R.A73 *E. faecium.*

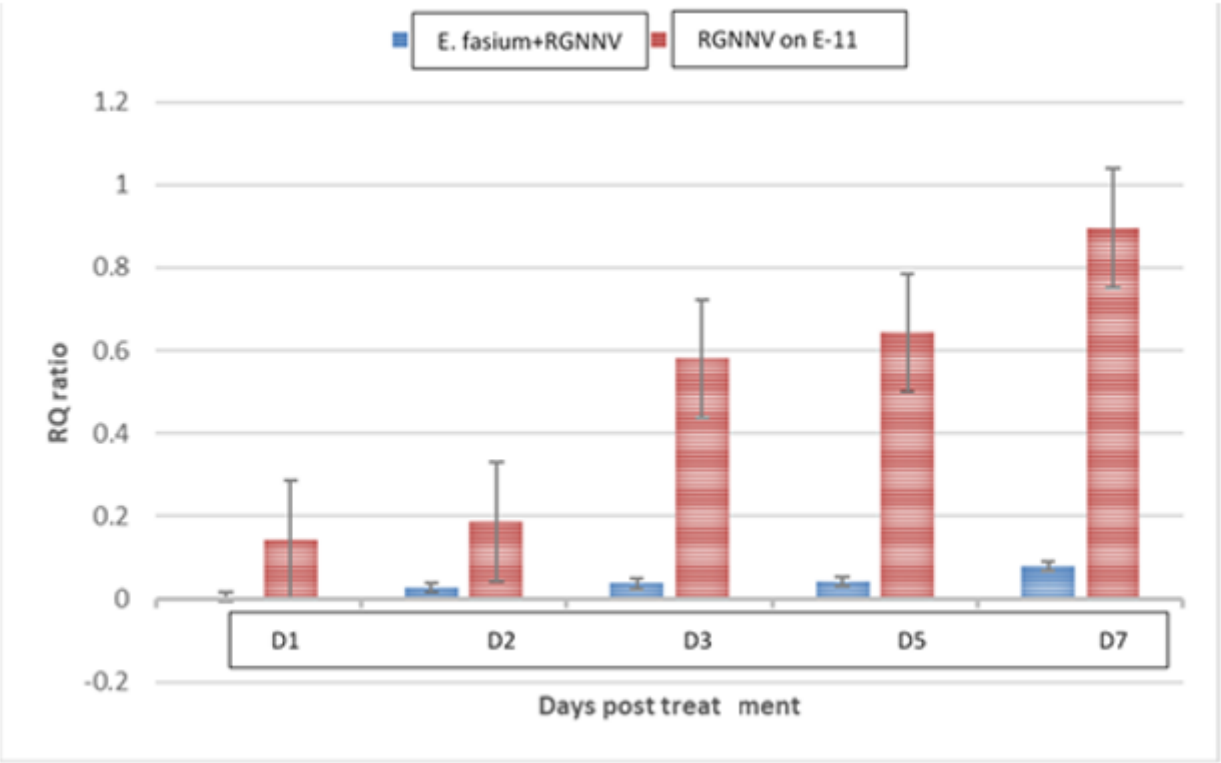

Figure 2

Expression level of nodavirus capsid protein (CP) gene in the E-11 cell line at various days post challenge with RGNNV nodavirus. Cells treated with LAB R.A73 *E. feacium* and nodavirus are shown in blue while control cells which are inoculated with nodavirus only are in red. Fold expression was calculated as $2^{-\Delta\Delta Ct}$. Control group (Day 0 post challenge) was used as the calibrator.

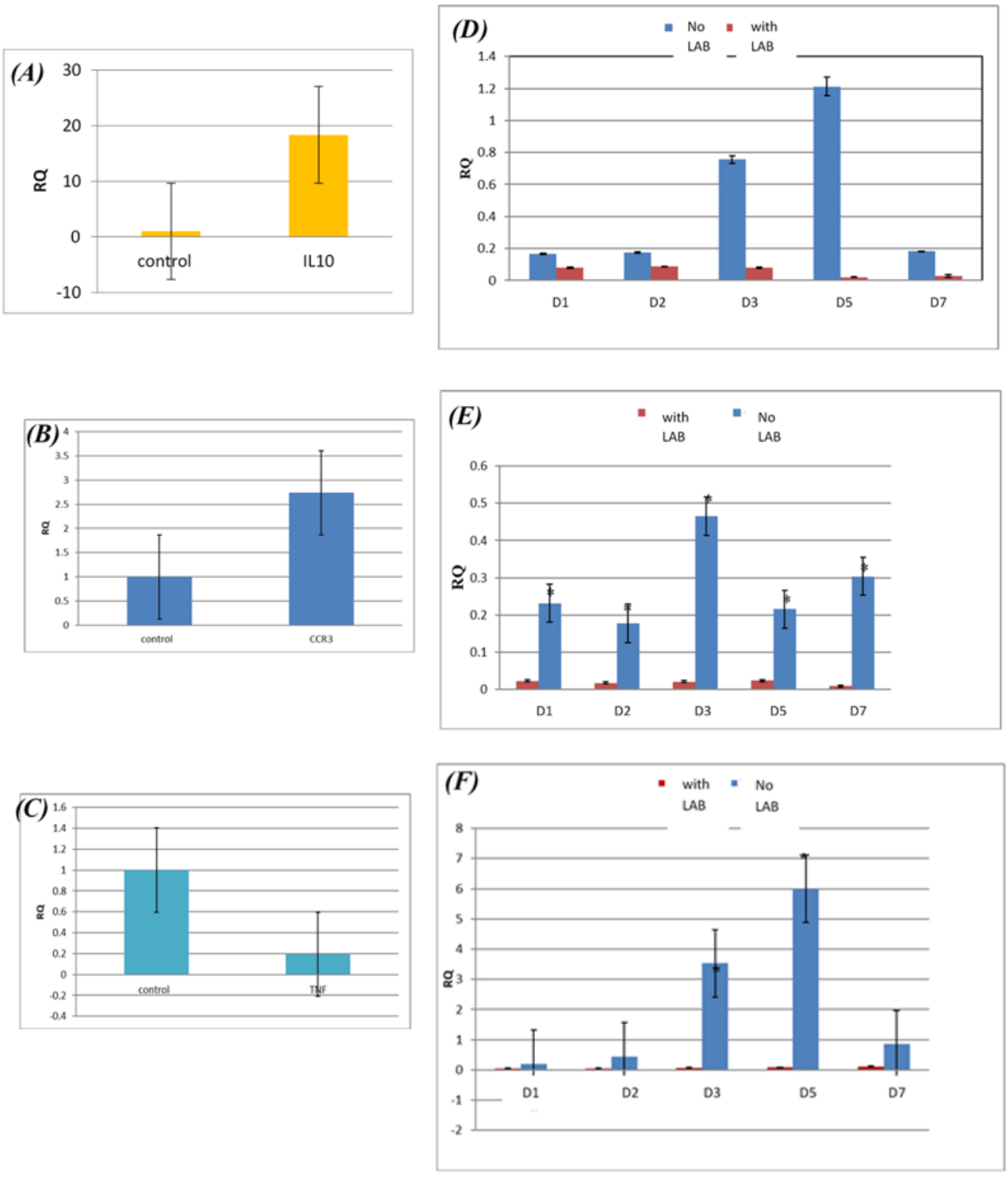

Figure 3

Expression levels of three immune genes in E-11 cells treated with R.A73 *E. faecium* and inoculated by RGNNV nodavirus for different time periods (D, day post challenge). A: IL-10 gene transcripts RQ values, B: CCR3 gene transcripts RQ values and C: TNF-α gene transcripts RQ values. Results D, E and F correspond to differential expression levels of IL-10, CCR3 and TNF-α , respectively. RQ results are shown in presence (w) of R.A73 *E. faecium* treatment (red) and in absence (w/o) of R.A73 *E. faecium* treatment

(blue). Fold expression was calculated as $2^{-\Delta\Delta Ct}$. Control group (Day0 post challenge) was taken as the calibrator.