

Effect of vitamin C on innate immune responses of rainbow trout  
(*Oncorhynchus mykiss*) leukocytes

Esther Leal<sup>1</sup>, Carlos Zarza<sup>2</sup>, Carolina Tafalla<sup>1\*</sup>

<sup>1</sup> *Centro de Investigación en Sanidad Animal (CISA-INIA). Valdeolmos (Madrid), Spain*

<sup>2</sup> *Skretting Aquaculture Research Centre, PO Box 48, Stavanger 4001, Norway*

\* Corresponding author: Carolina Tafalla. Address: Centro de Investigación en Sanidad Animal (CISA-INIA). Carretera de Algete a El Casar km. 8.1. Valdeolmos 28130 (Madrid). Spain. Tel.: 34 91 6202300; Fax: 34 91 6202247; E mail: tafalla@inia.es

Submitted to: Fish & Shellfish Immunology

June 2017

Revised version

## Abstract

Vitamin C, also known as ascorbic acid, is an essential micronutrient that influences a wide variety of physiological processes, including immunological functions. Although the positive effects of vitamin C supplementation on the immunological status of fish has been established in different species, the bases for these positive effects are still unknown. Hence, the aim of our study was to evaluate the *in vitro* effect of vitamin C on several innate immune functions of rainbow trout (*Oncorhynchus mykiss*) leukocyte populations. For this, we assessed the effects exerted on the established rainbow trout monocyte-macrophage cell line RTS11, and compared them to those observed in trout head kidney leukocytes. Our results demonstrate that vitamin C increases the production of reactive oxygen species and the percentage of phagocytic cells in both cell populations. On the other hand, vitamin C had no effect on the surface MHC II levels and only in the case of RTS11 cells increased the capacity of these cells to migrate towards the CK9 chemokine. Finally, vitamin C also increased the transcription of several pro-inflammatory and antimicrobial genes elicited by *Escherichia coli*, with some differences depending on the cell population studied. Our results contribute to further understand how vitamin C supplementation regulates the fish immune system.

Keywords: vitamin C; rainbow trout; RTS11 cells; head kidney leukocytes; phagocytosis; respiratory burst; antibacterial activities.

## 1. Introduction

Vitamin C, also known as ascorbic acid, is an essential micronutrient required for the normal metabolism of many animal species. Humans and most fish species have acquired defects on the L-gulonolactone oxidase, the final enzyme in the biosynthetic pathway for the production of vitamin C and consequently are incapable of synthesizing vitamin C by themselves and need to acquire it through the diet [1, 2]. In mammals, vitamin C is known to regulate many different cellular functions, including immune functions of different leukocyte subtypes. For example, vitamin C supplementation has been shown to increase natural antibody titers in humans [3]; shift the immune response to Th1 through the activation of dendritic cells (DCs) in mice [4]; or have stimulatory effects on human neutrophils with effects on the production of reactive oxygen intermediates or phagocytic capacities [5, 6]. Despite this, the exact mechanisms of the immunological effects of vitamin C have not been elucidated in most cases.

In fish, diet supplementation with vitamin C has been shown to have positive effects on growth in species such as Indian major carp (*Labeo rohita*) [7] Caspian roach (*Rutilus rutilus caspicus*) [8], grouper (*Epinephelus malabaricus*) [9] or red sea bream (*Pagrus major*) [10], while similarly vitamin C deficiencies provoked negative effects on weight gain in species such as grass carp (*Ctenopharyngodon idella*) [11] or Jian carp (*Cyprinus carpio* var. Jian) [12]. Vitamin C supplementation has also been proved to increase several immunological functions such as production of reactive oxygen intermediates (respiratory burst) [7, 9, 13-15], phagocytosis [7], lysozyme activity [9, 10, 13, 16] or production of complement factors [11, 15] in a wide range of fish species. Positive effects on the antimicrobial properties of skin mucus have also been reported [8, 16], as well as effects on

the intestinal microbial population [12] or percentage of circulating lymphocytes [17]. In concordance with these studies, increased survival to pathogen exposure after vitamin C supplementation has been revealed in some species [7, 18-20]. These experiments have dealt mainly with bacterial infections [7, 19, 20], although in some cases increased survival after parasitic infections has also been reported [18]. As in the case of mammals, the mechanisms through which vitamin C exerts its immunomodulatory effects are still unknown.

In rainbow trout (*Oncorhynchus mykiss*), while different studies have addressed the effect of vitamin C on diverse reproductive parameters [21, 22], its effects on the immune system have not been properly addressed in this species. Interestingly, vitamin C supplementation increased the survival of rainbow trout infected with the ciliate parasite *Ichthyophthirius multifiliis* [18], but the mechanisms responsible for this increased survival are still unknown. In this context, in the current work, we have studied the effect of vitamin C on several immune functions of rainbow trout using both the established monocyte-macrophage cell line RTS11 and head kidney primary leukocytes. We have conducted different *in vitro* tests to establish the effects of vitamin C on the production of reactive oxygen species (respiratory burst); phagocytosis; surface MHC II levels; migration to the CK9 chemokine; and gene expression in response to a bacterial stimulation. Our results provide additional experimental evidence to understand why vitamin C supplementation increases the survival of fish infected with pathogens.

## **2. Materials and Methods**

### *2.1. Experimental fish*

Rainbow trout (*Oncorhynchus mykiss*) adults of ~100 g were obtained from Piscifactoría Cienfuentes (Guadalajara, Spain) and maintained at the animal facilities of the Animal Health Research Center (CISA-INIA) in a recirculating water system at 16°C, with 12:12 h light/dark photoperiod. Fish were fed twice a day with a commercial diet (Skretting Spain, S.A., Burgos, Spain). Prior to any experimental procedure, fish were acclimatized to laboratory conditions for at least 2 weeks. All of the experiments described comply with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals and have been approved by the Instituto Nacional de Investigación Agraria y Alimentaria (INIA) Ethics Committee.

### *2.2. Leukocyte isolation and cell lines*

Rainbow trout were killed by an overdose of benzocaine (Sigma) and the head kidney was removed and placed in cold L-15 medium (Invitrogen) supplemented with 100 I.U./ml penicillin plus 100 µg/ml streptomycin (P/S, Life Technologies), 10 units/ml heparin (Sigma) and 5% FCS (Life Technologies). Single cell suspensions were then obtained using 100 µm nylon cell strainers (BD Biosciences) and placed onto 30/51% discontinuous Percoll (GE Healthcare) density gradients and centrifuged at 500 x g for 30 min at 4°C. The interface cells were collected and washed twice in L-15 containing 5% FCS. Experiments were always performed on cells from individual fish (4-9 fish depending on the immune assay).

RTS11, a continuous rainbow trout macrophage-like cell line, originally isolated from a long-term spleen hematopoietic culture [23] was maintained at 20°C in L-15 medium supplemented with P/S and 20% FCS. Cells were grown at a high cell density (approximately  $1 \times 10^6$  cells per ml) and passaged at a 1:2 ratio as described previously [23].

### *2.3. Cell viability assay*

Prior to determining the effect of vitamin C on RTS11 and head kidney leukocyte functions, we determined the effect of different vitamin C doses on the cell viability. To undertake this, RTS11 cells or head kidney leukocytes obtained from 3 different fish were seeded in 96-well plates in triplicate at a cell density of  $2 \times 10^5$  cells per well and incubated at 20°C with different vitamin C doses (0.1, 0.5, 1, 5 and 10  $\mu\text{M}$ ). After 3, 24, 48 or 72 h, cell viability was checked by Propidium Iodide (PI) staining on a FACSCalibur flow cytometer (BD Biosciences).

### *2.4. Respiratory burst activity*

To analyze the effect of vitamin C on the respiratory burst activity of RTS11 macrophages or head kidney leukocytes, we used the Nitro Blue Tetrazolium (NBT, Sigma) method. For this, cells were seeded in 96-well plates (Nunc) at a concentration of  $2 \times 10^5$  cells per well, washed through repeated centrifugation ( $500 \times g$  for 10 min at 4°C) and resuspended in 1X Phenol Red-Free Hank's Balanced Salt Solution (HBSS, Life Technologies). Then, NBT was added to the cells at a final concentration of 1  $\mu\text{g}/\mu\text{l}$  in the presence or absence of different vitamin C doses (0.1, 0.5 and 1  $\mu\text{M}$ ). To assess the specificity of the reaction, 300 units/ml of Superoxide Dismutase (SOD, Sigma) were

added to parallel wells in triplicate. Cells were incubated for 1 h at room temperature, in the dark, and then centrifuged. The supernatant was discarded and cells were incubated with absolute methanol for 5 min at room temperature. Methanol was then discarded and the cells were air-dried for 30 min. The reaction was developed by the addition of a developing buffer (120  $\mu$ l of 2 M KOH and 140  $\mu$ l of DMSO per well). Samples were incubated for 5 min and optical density at 620 nm measured, to determine reduction of the NBT substrate.

### *2.5. Phagocytic activity*

For the analysis of phagocytosis, RTS11 cells or head kidney leukocytes were seeded in 96-well plates at a cell density of  $2 \times 10^5$  cells per well and incubated for 3 h at 20°C with fluorescent beads (FluoSpheres® Microspheres, 1.0  $\mu$ m, Crimson Red Fluorescent 625/645, 2% solids; Life Technologies) at a cell:bead ratio of 1:10 in the presence or absence of different vitamin C doses (0.1, 0.5 and 1  $\mu$ M). Cells were harvested and non-ingested beads were removed by centrifugation (100 x g for 10 min at 4 °C) over a cushion of 3% (weight/volume) BSA (Fraction V; Fisher Scientific) in PBS supplemented with 4.5% (weight/volume) D-glucose (Sigma). Cells were resuspended from the pellet in staining buffer (PBS containing 1% FCS and 0.5% sodium azide), labelled with anti-IgM [1.14 mAb mouse IgG<sub>1</sub> coupled to fluorescein (FITC), 1  $\mu$ g/ml] as previously described [24] when needed, and analyzed on a FACSCalibur flow cytometer. In another set of experiments, the cells were previously incubated with the different vitamin C doses for 24 h at 20°C before the addition of the fluorescent beads. Afterwards, the experiments were conducted as described above.

## 2.6. MHC II expression

The levels of MHC-II expression on the surface of RTS11 cells or head kidney leukocytes exposed to different vitamin C doses were measured through flow cytometry using a mAb against trout MHC-II [25]. Cells seeded in 96-well plates at a cell density of  $2 \times 10^5$  cells per well were incubated with different vitamin C doses (0.1, 0.5 and 1  $\mu\text{M}$ ) or with media alone (control) for 24 h at 20°C. After that time, cells were washed in FACS staining buffer (PBS containing 1% FCS and 0.5% sodium azide) and stained with Alexa 647-conjugated anti-MHC-II antibody for 30 min at 4°C protected from light. Finally cells were washed twice with the same buffer and analysed by flow cytometry.

## 2.7. CK9 chemotaxis assay

CK9 is a rainbow trout chemokine with chemoattractant capacities towards RTS11 cells and head kidney leukocytes [24]. Thus, we also assessed whether vitamin C had an effect on the capacity of these cells to migrate to CK9. For this, cells seeded in 96-well plates at a cell density of  $2 \times 10^5$  cells per well were incubated with different vitamin C doses (0.1, 0.5 and 1  $\mu\text{M}$ ) or with media alone (control) for 24 h at 20°C. Thereafter, chemotaxis was assessed using 3  $\mu\text{m}$  (for primary cultures) or 5  $\mu\text{m}$  (for RTS11 cells) pore polycarbonate Transwell chambers (Sigma), following the manufacturer's instructions. Briefly, 600  $\mu\text{l}$  of control medium (L-15 medium supplemented with P/S and 5% FCS) or medium containing 10 ng/ml recombinant CK9 were added to the bottom chambers. In the upper chambers, 100  $\mu\text{l}$  of RTS11 cells or head kidney leukocytes ( $2 \times 10^6$  cells/ml) were dispensed. Cells in migration chambers were incubated for 2 h at 20°C, then the 600  $\mu\text{l}$  of the bottom chamber were harvested and the migrated cells were analyzed based on side and

forward light scatter (SSC/FSC) parameters on a FACSCalibur flow cytometer at a constant flow time (1 min).

## 2.8. *Transcriptional response to E. coli*

Finally, we have also studied the effect of vitamin C on the transcriptional response triggered in RTS11 or head kidney leukocytes in response to bacterial exposure. For this, cells seeded in 96-well plates at a cell density of  $2 \times 10^5$  cells per well were exposed to heat-inactivated *Escherichia coli* (cell:bacteria ratio 1:10) in the presence or absence of 0.5  $\mu\text{M}$  vitamin C. Controls with vitamin C alone or media alone were also included as controls. After 24 h of incubation at 20°C, DNase I-treated total RNA was isolated from cells using Tri-reagent (Invitrogen) following the manufacturer's instructions. Total RNA was dissolved in RNase-free water, quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific) and stored at -80°C. Potential contaminating genomic DNA was removed from RNA samples using the RapidOut DNA Removal kit (Thermo Scientific) according to the manufacturer's instructions. For each sample, 1  $\mu\text{g}$  of total RNA was reverse transcribed using the RevertAid reverse transcriptase (Thermo Scientific) and oligo (dT)<sub>12-18</sub> (0.5  $\mu\text{g}/\text{ml}$ ), as indicated by the manufacturer. cDNA was diluted in nuclease-free water and stored at -20°C.

To evaluate the levels of transcription of the different genes, real-time PCR was performed in a LightCycler 96 System instrument (Roche) using FastStart Essential DNA Green Master reagents (Roche) and specific primers (shown in Table 1). The efficiency of the amplification was determined for each primer pair using serial 10 fold dilutions of pooled cDNA, and only primer pairs with efficiencies higher than 97.5% were used. Each

sample was measured in duplicate under the following conditions: 10 min at 95°C, followed by 40 amplification cycles (30 s at 95°C and 1 min at 60°C). The expression of individual genes was normalized to that of trout EF-1 $\alpha$  and expression levels calculated using the  $2^{-\Delta Ct}$  method, where  $\Delta Ct$  is determined by subtracting the EF-1 $\alpha$  value from the target Ct as described previously [26, 27]. EF-1 $\alpha$  was selected as reference gene according to the MIQE guidelines [28]. A statistical analysis determined there were no differences between the means of the expression of EF1 $\alpha$  among samples. Negative controls with no template were included in all experiments. A melting curve for each PCR was determined by reading fluorescence every degree between 60°C and 95°C to ensure only a single product had been amplified.

## 2.9. Statistics

Statistical analyses were performed using a two-tailed Student's *t* test with Welch's correction when the F test indicated that the variances of both groups differed significantly. The differences between the mean values were considered significant on different degrees, where \* means  $P < 0.05$ , \*\* means  $P < 0.01$  and \*\*\* means  $P < 0.005$ .

## 3. Results

### 3.1. Cell viability in the presence of vitamin C

Before determining the effect of vitamin C on immune functions of RTS11 and head kidney leukocytes, we studied the viability of these cell cultures when incubated with

a wide range of vitamin C doses for 24 h, establishing that doses above 5  $\mu\text{M}$  were highly toxic for both cell populations (Fig. S1, data not shown). Although the cell viability remained higher than 95% when cells were incubated with 5  $\mu\text{M}$  vitamin C; based on these results, we decided to use doses from 0.1 to 1  $\mu\text{M}$  to determine the effects of vitamin C on the different immune assays.

### *3.2. Effect of vitamin C on the respiratory burst activity of RTS11 and head kidney leukocytes*

We first determined the effect of vitamin C on the production of reactive oxygen species using an NBT assay. Vitamin C concentrations of 0.5 and 1  $\mu\text{M}$  were capable of inducing the production of reactive oxygen species in the absence of an additional stimulus in both RTS11 (Fig. 1A) and head kidney leukocytes (Fig. 1B). Vitamin C was not reacting directly with NBT as in the absence of cells no increased absorbance was observed (data not shown). Furthermore, the specificity of the reaction was confirmed by addition of SOD which significantly reduced the response to vitamin C in both cell populations (Fig. 1A, B).

### *3.3. Effect of vitamin C on the phagocytic capacity of RTS11 and head kidney leukocytes*

We then studied whether vitamin C could have an effect on the phagocytic capacities of RTS11 cells and head kidney leukocytes. For this, cells were incubated with fluorescent polystyrene beads for 3 h in the presence of different vitamin C doses (co-incubation) or were incubated with vitamin C for 24 h prior to the addition of beads (pre-incubation). When RTS11 cells were co-incubated with beads and vitamin C, only the 1  $\mu\text{M}$  dose was capable of significantly increasing the percentage of cells displaying

phagocytic activity (Fig. 2A). On the other hand, when cells were incubated for 24 h with vitamin C before exposing them to the beads, the percentage of cells showing phagocytic activity increased with all vitamin C doses used (Fig. 2A). In the case of head kidney leukocytes, vitamin C at doses of 0.5 and 1  $\mu$ M significantly increase the percentage of phagocytic cells either upon co-incubation or when cells were pre-incubated for 24 h with vitamin C (Fig. 2B). Despite these increases in the percentages of phagocytic cells in response to vitamin C, the mean fluorescent intensity (MFI) was not significantly affected (data not shown), revealing that vitamin C is able to increase the number of cells with phagocytic potential but not the number of beads that these cells can ingest.

Because, in fish, B cells have potent phagocytic capacities, we next studied whether vitamin C was increasing the number of phagocytic cells in head kidney leukocyte cultures through effects on B cells or other hand was only affecting the phagocytic capacity of myeloid cells such as neutrophils or macrophages. For this, we repeated the phagocytosis experiments, in this case staining the cells with anti-IgM before analyzing the phagocytic activity in the flow cytometer. When head kidney leukocytes cultures were treated with vitamin C during the 3 h in which the cells were in contact with the beads, vitamin C had no significant effect on the number of phagocytic B cells (Fig. 3A). Alternatively, when cells were incubated with 0.5 or 1  $\mu$ M vitamin C for 24 h, the percentage of B cells that were phagocytosing beads significantly increased in the head kidney leukocyte cultures (Fig. 3A). In parallel to these analyses, we also established the number of phagocytic cells present within what has been commonly designated as the “myeloid gate” (cells with large size and high complexity). It is commonly accepted that these cells should correspond mostly to myeloid cells such as macrophages or neutrophils [29]. In this case, the

percentage of phagocytic cells within the myeloid gate significantly increased when cells were incubated with 0.5 or 1  $\mu\text{M}$  vitamin C either adding the vitamin at the same time than the beads or when cells were pre-incubated for 24 h with vitamin C before the beads were added (Fig. 3B). These results demonstrate that, within the head kidney, vitamin C has positive effects on the phagocytic potential of both B cells and myeloid cells.

#### *3.4. Effect of vitamin C on the surface MHC II expression of RTS11 cells and head kidney leukocytes*

Upon pathogen phagocytosis, antigen presenting cells will express the foreign antigens in an MHC II context. Thus, having observed a positive effect of vitamin C on the phagocytic capacity of RTS11 cells and head kidney leukocytes, we next examined whether vitamin C exposure affected the expression of surface MHC II. In this case, vitamin C had no effect on the levels of surface MHC II expression of RTS11 cells (Fig. 4A) or head kidney leukocytes (Fig. 4B).

#### *3.5. Effect of vitamin C on the migration of RTS11 and head kidney leukocytes towards CK9*

CK9 is a rainbow trout CC chemokine that has chemoattractant capacities over RTS11 cells and head kidney leukocytes [24], thus, we also studied the effect of vitamin C on the migratory capacity of these cells towards CK9. In the case of RTS11 cells, their pre-incubation for 24 h with doses of vitamin C ranging from 0.1 to 1  $\mu\text{M}$  significantly increased the capacity of these cells to migrate towards CK9 (Fig. 5A). On the contrary, vitamin C had no effect on the migratory capacities of head kidney leukocytes (Fig. 5B).

### 3.6. Effect of vitamin C on the transcriptomic response of RTS11 and head kidney leukocytes to bacterial exposure

Finally, we also established how vitamin C supplementation affected the transcriptomic response that is elicited in response to a bacterial exposure. For this, the levels of expression of different immune genes were studied in cells exposed to *E. coli* in the presence or absence of 0.5  $\mu$ M vitamin C. *E. coli* up-regulated the transcription of IL-1 $\beta$ , IL-8, COX-2B, TNF- $\alpha$ , cathelicidin 2 and hepcidin in RTS11 cells (Fig. 6). When the cells were exposed to *E. coli* in the presence of vitamin C, the transcription levels of IL-1 $\beta$ , COX-2B, TNF- $\alpha$ , cathelicidin 2 and hepcidin were further increased to significantly higher levels (Fig. 6). Vitamin C on its own had no effect on the transcription levels of these molecules (Fig. 6). In the case of head kidney leukocytes, *E. coli* up-regulated the mRNA levels of IL-1 $\beta$ , IL-8, TNF- $\alpha$ , COX-2B and hepcidin but had no significant effect on the levels of transcription of cathelicidin 2 (Fig. 7). When cells were exposed to *E. coli* in the presence of vitamin C, IL-1 $\beta$ , IL-8 and COX-2B transcription levels were significantly higher than those observed in cells exposed to *E. coli* alone (Fig. 7). As in the case of RTS11 cells, vitamin C alone had no effect on any of the genes studied (Fig. 7).

## 4. Discussion

To kill pathogens, in association with their phagocytic activity, phagocytes activate a respiratory burst response that generates highly reactive oxidants such as superoxide or

hydrogen peroxide which are largely responsible for the microbicidal activity elicited [30]. Because vitamin C is a potent antioxidant [30], it was generally believed that its presence would protect cells from the products of the oxidative burst [31]. Conversely, studies performed on mammalian neutrophils have revealed that vitamin C directly elicits the production of reactive oxygen species in neutrophils [5, 6]. The mechanisms by which vitamin C enhances superoxide production are still uncertain in mammals and have been suggested to include effects on expression of the different oxidase subunits, or post-translational modifications of these subunits [6]. In our study, vitamin C significantly increased the production of reactive oxygen species in both RTS11 cells and head kidney leukocytes, revealing that this effect of vitamin C is conserved in rainbow trout. Our results correlate with many different studies that have reported an increased respiratory burst activity in response to vitamin C supplementation in the diet in different fish species [7, 9, 14, 15]. However, contradictory results have also been reported. In the case of groper (*Polyprion oxygeneios*), vitamin C decreased the production of superoxide [32]. In gilthead seabream (*Sparus aurata*) vitamin C failed to elicit a respiratory burst activity on its own and only increased it when combined with vitamin E when assayed *in vitro* [33]. Surprisingly, in this same species, an increased respiratory burst activity was detected in fish fed with a diet supplemented with vitamin C [14]. These results seem to indicate that there might be some differences between the effects that vitamin C exerts on different species, whereas some differences may also be a consequence of different models or experimental approaches.

In correlation with the increased respiratory burst activity, we also found a positive effect of vitamin C on the phagocytic capacity of RTS11 cells and head kidney leukocytes.

The effects were experienced on the percentage of phagocytic cells but not on the number of beads that these cells can ingest, suggesting that once these cells become phagocytic the number of beads they can ingest is somehow limited. These results also correlate with a previous report in which vitamin C supplementation was found to increase the phagocytic capacity of leukocytes [7]. In the case of head kidney leukocyte cultures, we further investigated which cells were increasing their phagocytic activity in response to vitamin C given that in fish B cells are known to have potent phagocytic capacities [34]. Interestingly, both B cells and myeloid cells responded to vitamin C, demonstrating that vitamin C regulates this function in a range of cell types. In mammals, incubation of peripheral blood leukocytes with vitamin C caused a significant increase in the number of latex particles internalized by each individual polymorphonuclear cell, but not by monocytes [35]. In this case, the number of cells with phagocytic capacities was not altered [35].

Since vitamin C has also been shown to increase the chemotactic capacity of human neutrophils [6], we also studied its effect on the capacity of rainbow trout cells to migrate towards CK9, a rainbow trout CC chemokine that specifically attracts monocytes/macrophages and B cells from spleen, kidney or blood [24]. In this case, different responses were observed in RTS11 cells and head kidney leukocytes, as vitamin C significantly up-regulated the migratory capacities of RTS11 cells towards CK9 but not that of head kidney leukocytes. These results suggest that, on the contrary to what occurred in phagocytosis, vitamin C regulates some immune functions in a cell-specific fashion. In mammals, it has been postulated that the regulation of neutrophil movement by vitamin C is due to rearrangements of the cytoskeleton, as no effect on specific chemokine receptors were observed [36]. In rainbow trout, because the receptor for CK9 still remains unidentified the mechanisms through which vitamin C regulates the migration of

monocytes/macrophages to CK9 remain unknown, and should be further investigated in the future.

Some differences were also found between the effects that vitamin C had on the transcriptomic responses to *E. coli* in RTS11 cells and head kidney leukocytes, again pointing to cell-specific effects. In RTS11 cells, when vitamin C was added, the transcription levels of IL-1 $\beta$ , COX-2B, TNF- $\alpha$ , cathelicidin 2 and hepcidin in response to *E. coli* were further up-regulated, but vitamin C had no effect on IL-8 transcription. On the other hand, when this assay was conducted in head kidney leukocytes, we found that vitamin C only up-regulated the levels of transcription of IL-1 $\beta$ , IL-8 and COX-2B but had no effect on the other genes studied. These differences could be due to the fact that in head kidney leukocyte cultures diverse leukocyte subtypes co-exist in which vitamin C might be having differential effects, while the positive effects are easier to be unveiled using a relatively homogenous cell line. Despite these differences, our results in RTS11 cells demonstrate a capacity of vitamin C to induce the transcription of antimicrobial peptides such as cathelicidin 2 and hepcidin. The effect of vitamin C on the production of antimicrobial peptides had been previously demonstrated indirectly in young grass carp since vitamin C deficiency in the diet resulted in a decreased transcription of antimicrobial peptides in the liver [11].

In conclusion, we have demonstrated that vitamin C directly modulates many immune functions of rainbow trout. The results obtained with RTS11 monocyte/macrophages and head kidney leukocytes, altogether reveal a positive effect of vitamin C on the antibacterial immune response of rainbow trout, since the positive effects that vitamin C exerted on superoxide production, phagocytic capacities and production of

pro-inflammatory and antimicrobial factors will likely translate into enhanced microbial killing activities. Hence, our results provide new insights on the effects that vitamin C has on the immune system in teleost and might help explain the mechanisms through which vitamin C supplementation enhances survival to bacterial infections in these species.

## Acknowledgements

The authors want to thank Lucia González for technical assistance. This work was supported by the European Commission under the Horizon H2020 research and innovation programme (Grant H2020-634429 ParaFishControl).

## References

- [1] M. Nishikimi, R. Fukuyama, S. Minoshima, N. Shimizu, K. Yagi, Cloning and chromosomal mapping of the human nonfunctional gene for L-gulono-gamma-lactone oxidase, the enzyme for L-ascorbic acid biosynthesis missing in man, *J Biol Chem* 269(18) (1994) 13685-8.
- [2] A. Krasnov, M. Reinisalo, T.I. Pitkanen, M. Nishikimi, H. Molsa, Expression of rat gene for L-gulono-gamma-lactone oxidase, the key enzyme of L-ascorbic acid biosynthesis, in guinea pig cells and in teleost fish rainbow trout (*Oncorhynchus mykiss*), *Biochim Biophys Acta* 1381(2) (1998) 241-8.
- [3] W. Prinz, R. Bortz, B. Bregin, M. Hersch, The effect of ascorbic acid supplementation on some parameters of the human immunological defence system, *Int J Vitam Nutr Res* 47(3) (1977) 248-57.
- [4] Y.J. Jeong, S.W. Hong, J.H. Kim, D.H. Jin, J.S. Kang, W.J. Lee, Y.I. Hwang, Vitamin C-treated murine bone marrow-derived dendritic cells preferentially drive naive T cells into Th1 cells by increased IL-12 secretions, *Cell Immunol* 266(2) (2011) 192-9.
- [5] P. Sharma, S.A. Raghavan, R. Saini, M. Dikshit, Ascorbate-mediated enhancement of reactive oxygen species generation from polymorphonuclear leukocytes: modulatory effect of nitric oxide, *J Leukoc Biol* 75(6) (2004) 1070-8.

- [6] S.M. Bozonet, A.C. Carr, J.M. Pullar, M.C. Vissers, Enhanced human neutrophil vitamin C status, chemotaxis and oxidant generation following dietary supplementation with vitamin C-rich SunGold kiwifruit, *Nutrients* 7(4) (2015) 2574-88.
- [7] A. Tewary, B.C. Patra, Use of vitamin C as an immunostimulant. Effect on growth, nutritional quality, and immune response of *Labeo rohita* (Ham.), *Fish Physiol Biochem* 34(3) (2008) 251-9.
- [8] Z. Roosta, A. Hajimoradloo, R. Ghorbani, S.H. Hoseinifar, The effects of dietary vitamin C on mucosal immune responses and growth performance in Caspian roach (*Rutilus rutilus caspicus*) fry, *Fish Physiol Biochem* 40(5) (2014) 1601-7.
- [9] M.F. Lin, S.Y. Shiau, Dietary L-ascorbic acid affects growth, nonspecific immune responses and disease resistance in juvenile grouper, *Epinephelus malabaricus*, *Aquaculture* 244 (2005) 215-221.
- [10] M.A. Dawood, S. Koshio, M. Ishikawa, S. Yokoyama, Immune responses and stress resistance in red sea bream, *Pagrus major*, after oral administration of heat-killed *Lactobacillus plantarum* and vitamin C, *Fish Shellfish Immunol* 54 (2016) 266-75.
- [11] H.J. Xu, W.D. Jiang, L. Feng, Y. Liu, P. Wu, J. Jiang, S.Y. Kuang, L. Tang, W.N. Tang, Y.A. Zhang, X.Q. Zhou, Dietary vitamin C deficiency depresses the growth, head kidney and spleen immunity and structural integrity by regulating NF-kappaB, TOR, Nrf2, apoptosis and MLCK signaling in young grass carp (*Ctenopharyngodon idella*), *Fish Shellfish Immunol* 52 (2016) 111-38.
- [12] Y. Liu, L. Chui, L. Feng, J. Jiang, W.D. Jiang, K. Hu, S.H. Li, X.Q. Zhou, Effects of graded levels of dietary vitamin C on the growth, digestive capacity and intestinal microflora of juvenile Jian carp (*Cyprinus carpio* var. Jian), *Aquac Res* 42 (2011) 534-548.
- [13] V. Verlhac, A. Obach, J. Gabaudan, W. Schuep, R. Hole, Immunomodulation by dietary vitamin C and glucan in rainbow trout (*Oncorhynchus mykiss*), *Fish Shellfish Immunol* 8(8) (1998) 409-424.
- [14] J. Ortuno, A. Cuesta, M. Angeles Esteban, J. Meseguer, Effect of oral administration of high vitamin C and E dosages on the gilthead seabream (*Sparus aurata* L.) innate immune system, *Vet Immunol Immunopathol* 79(3-4) (2001) 167-80.
- [15] J. Kumari, P.K. Sahoo, High dietary vitamin C affects growth, non-specific immune responses and disease resistance in Asian catfish, *Clarias batrachus*, *Mol Cell Biochem* 280(1-2) (2005) 25-33.
- [16] T. Ren, S. Koshio, M. Ishikawa, S. Yokoyama, F.R. Micheal, O. Uyan, H.T. Tung, Influence of dietary vitamin C and bovine lactoferrin on blood chemistry and non-specific immune responses of Japanese eel, *Anguilla japonica*, *Aquaculture* 267(1) (2007) 31-37.
- [17] M. Rahimi, S. Ahmadvand, S. Eagderi, S. Shamohammadi, Effects of Vitamin C and E administration on leukocyte counts in rainbow trout (*Oncorhynchus mykiss*), *J Nutr Health* 1(2) (2015) 5.
- [18] T. Wahli, W. Meier, K. Pfister, Ascorbic acid induced immune-mediated decrease in mortality in *Ichthyophthirius multifiliis* infected rainbow-trout (*Salmo gairdneri*), *Acta Trop* 43(3) (1986) 287-9.
- [19] J. Kumari, P.K. Sahoo, Dietary immunostimulants influence specific immune response and resistance of healthy and immunocompromised Asian catfish *Clarias batrachus* to *Aeromonas hydrophila* infection, *Dis Aquat Organ* 70(1-2) (2006) 63-70.

- [20] M.M. Barros, D.R. Falcon, O. Orsi Rde, L.E. Pezzato, A.C. Fernandes, Jr., I.G. Guimaraes, A. Fernandes, Jr., C.R. Padovani, M.M. Sartori, Non-specific immune parameters and physiological response of Nile tilapia fed beta-glucan and vitamin C for different periods and submitted to stress and bacterial challenge, *Fish Shellfish Immunol* 39(2) (2014) 188-95.
- [21] K. Dabrowski, A. Ciereszko, Ascorbic acid protects against male infertility in a teleost fish, *Experientia* 52(2) (1996) 97-100.
- [22] A. Ciereszko, L. Liu, K. Dabrowski, Effects of season and dietary ascorbic acid on some biochemical characteristics of rainbow trout (*Oncorhynchus mykiss*) semen, *Fish Physiol Biochem* 15(1) (1996) 1-10.
- [23] R.C. Ganassin, N.C. Bols, Development of a monocyte/macrophage-like cell line, RTS11, from rainbow trout spleen., *Fish Shellfish Immunol* 8 (1998) 457-76.
- [24] C. Aquilino, A.G. Granja, R. Castro, T. Wang, B. Abos, D. Parra, C.J. Secombes, C. Tafalla, Rainbow trout CK9, a CCL25-like ancient chemokine that attracts and regulates B cells and macrophages, the main antigen presenting cells in fish, *Oncotarget* 7(14) (2016) 17547-64.
- [25] A.G. Granja, E. Leal, J. Pignatelli, R. Castro, B. Abos, G. Kato, U. Fischer, C. Tafalla, Identification of teleost skin CD8alpha+ dendritic-like cells, representing a potential common ancestor for mammalian cross-presenting dendritic cells, *J Immunol* 195(4) (2015) 1825-37.
- [26] A. Cuesta, J. Meseguer, M.A. Esteban, The antimicrobial peptide hepcidin exerts an important role in the innate immunity against bacteria in the bony fish gilthead seabream, *Mol Immunol* 45(8) (2008) 2333-42.
- [27] T. Wang, S. Bird, A. Koussounadis, J.W. Holland, A. Carrington, J. Zou, C.J. Secombes, Identification of a novel IL-1 cytokine family member in teleost fish, *J Immunol* 183(2) (2009) 962-74.
- [28] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, *Clin Chem* 55(4) (2009) 611-22.
- [29] T. Korytar, H. Dang Thi, F. Takizawa, B. Kollner, A multicolour flow cytometry identifying defined leukocyte subsets of rainbow trout (*Oncorhynchus mykiss*), *Fish Shellfish Immunol* 35(6) (2013) 2017-9.
- [30] G.R. Buettner, The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate, *Arch Biochem Biophys* 300(2) (1993) 535-43.
- [31] A. Parker, S.L. Cuddihy, T.G. Son, M.C. Vissers, C.C. Winterbourn, Roles of superoxide and myeloperoxidase in ascorbate oxidation in stimulated neutrophils and H<sub>2</sub>O<sub>2</sub>-treated HL60 cells, *Free Radic Biol Med* 51(7) (2011) 1399-405.
- [32] I. Salinas, S.A. Anderson, J. Wright, V.L. Webb, In vivo innate immune responses of groper (*Polyprion oxygeneios*) against *Miamiensis avidus* infection and lack of protection following dietary vitamin C administration, *Fish Shellfish Immunol* 32(1) (2012) 8-15.
- [33] V. Mulero, M.A. Esteban, J. Meseguer, Effects of in vitro addition of exogenous vitamins C and E on gilthead seabream (*Sparus aurata* L.) phagocytes, *Vet Immunol Immunopathol* 66(2) (1998) 185-99.

- [34] J. Li, D.R. Barreda, Y.A. Zhang, H. Boshra, A.E. Gelman, S. Lapatra, L. Tort, J.O. Sunyer, B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities, *Nat Immunol* 7(10) (2006) 1116-24.
- [35] M. Bergman, H. Salman, M. Djaldetti, L. Fish, I. Punsky, H. Bessler, *In vitro* immune response of human peripheral blood cells to vitamins C and E, *J Nutr Biochem* 15(1) (2004) 45-50.
- [36] J. Schwager, A. Bompard, P. Weber, D. Raederstorff, Ascorbic acid modulates cell migration in differentiated HL-60 cells and peripheral blood leukocytes, *Mol Nutr Food Res* 59(8) (2015) 1513-23.

## Figure legends

**Fig. 1. Effect of vitamin C on the respiratory burst activity.** RTS11 cells (A) and head kidney leukocyte (B) cultures were incubated with NBT (1 mg/ ml) in the presence of different concentrations of vitamin C (0, 0.1  $\mu$ M, 0.5  $\mu$ M and 1  $\mu$ M). SOD (300 units/ ml) was included in parallel wells in triplicate to verify the specificity of the reaction. After 1 h at room temperature, NBT reduction was measured as described in Materials and Methods. Data are shown as mean optical densities at 620 nm (n= 8, mean + SD). Asterisks denote statistical differences between control cells and those exposed to vitamin C (\* p < 0.05 and \*\*\* p < 0.005) whereas an “a” indicates significant decrease of NBT reduction in respective samples treated with SOD.

**Fig. 2. Effect of vitamin C on the phagocytic capacities of RTS11 (A) and head kidney leukocytes (B).** Cells were incubated with Crimson Red fluorescent beads at a ratio of 1:10 (cell:beads) with different concentrations of vitamin C (0, 0.1  $\mu$ M, 0.5  $\mu$ M and 1  $\mu$ M) (co-incubation). In other experiments, cells were pre-incubated with different concentrations of vitamin C (0, 0.1  $\mu$ M, 0.5  $\mu$ M and 1  $\mu$ M) 24 h before the addition of the beads (pre-incubation). In both cases, once the beads were added to the cultures, cells were incubated for 3 h at 20°C and then analyzed by flow cytometry to measure the fluorescence of internalized beads. Results are shown as mean percentages of cells with internalized beads + SD (n=9 for RTS11 and n=6 for HK leukocytes). Asterisks denote statistical differences between control cells and those exposed to vitamin C (\* p < 0.05, \*\* p < 0.01 and \*\*\* p < 0.005)

**Fig. 3. Effect of vitamin C on the phagocytic capacities of different head kidney leukocyte subpopulations.** Head kidney cells were incubated with Crimson Red fluorescent beads at a ratio of 1:10 (cell:beads) with different concentrations of vitamin C (0, 0.1  $\mu$ M, 0.5  $\mu$ M and 1  $\mu$ M ) (co-incubation). In other experiments, cells were pre-incubated with different concentrations of vitamin C (0, 0.1  $\mu$ M, 0.5  $\mu$ M and 1  $\mu$ M) for 24 h before the addition of the beads (pre-incubation). In both cases, once the beads were added to the cultures, cells were incubated for 3 h at 20°C and then analyzed by flow

cytometry to measure the fluorescence of internalized beads in IgM<sup>+</sup> B cells (A) or cells that fall into the myeloid gate according to their FSC/SSC profile (B). Results are shown as mean percentages of cells with internalized beads + SD (n=9). Asterisks denote statistical differences between control cells and those exposed to vitamin C (\* p < 0.05, \*\* p < 0.01 and \*\*\* p < 0.005).

**Fig. 4. Effect of vitamin C on the levels of surface MHC II expression.** RTS11 cells (A) or head kidney leukocytes (B) were incubated at 20°C with different doses of vitamin C (0, 0.1 µM, 0.5 µM and 1 µM) during 24 h and then stained with anti-MHC II antibody and analyzed by flow cytometry. Results are shown as mean fluorescence intensity (MFI) + SD (n=8).

**Fig. 5. Effect of vitamin C on the migratory capacity of RTS11 (A) or head kidney leukocytes (B) towards CK9.** For the chemotaxis assays, 10 ng/ml of CK9 were introduced in the bottom wells of transwell chambers, whereas RTS11 cells or head kidney leukocytes previously incubated for 24 h with different doses of vitamin C (0, 0.1 µM, 0.5 µM and 1 µM ) were dispensed into the upper wells. After 2 h of incubation at 20°C, the number of cells that had migrated to the bottom of the wells was quantified by flow cytometry. Negative controls with no CK9 were also included. Average numbers of migrating cells are shown (n=9 for RTS11 and n=7 for HK leukocytes). Asterisks denote statistical differences between control cells and those exposed to vitamin C (\* p ≤ 0.05 and \*\* p ≤ 0.01).

**Fig. 6. Effect of vitamin C on the transcriptomic response of RTS11 cells to *E. coli*.** RTS11 cells were incubated for 24 h with *E. coli* (cell:bacteria ratio 1:10) in the presence or absence of 0.5 µM vitamin C. Controls with vitamin C alone or media alone were also included as controls. Thereafter, RNA was extracted from cells and the levels of transcription of different immune genes analyzed by real time PCR. Data are shown as the mean relative gene expression normalized to the transcription of the house-keeping gene EF-1α ± SD (n = 4). Asterisks denote statistical differences between cells treated with *E.*

*coli* alone and cells exposed to *E. coli* in the presence of vitamin C (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.005$ ).

**Fig. 7. Effect of vitamin C on the transcriptomic response of head kidney leukocytes to *E. coli*.** Head kidney leukocytes were incubated for 24 h with *E. coli* (cell:bacteria ratio 1:10) in the presence or absence of 0.5  $\mu\text{M}$  vitamin C. Controls with vitamin C alone or media alone were also included as controls. Thereafter, RNA was extracted from cells and the levels of transcription of different immune genes analyzed by real time PCR. Data are shown as the mean relative gene expression normalized to the transcription of the house-keeping gene EF-1 $\alpha$   $\pm$  SD (n = 4). Asterisks denote statistical differences between cells treated with *E. coli* alone and cells exposed to *E. coli* in the presence of vitamin C (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.005$ ).

## Supplementary Material

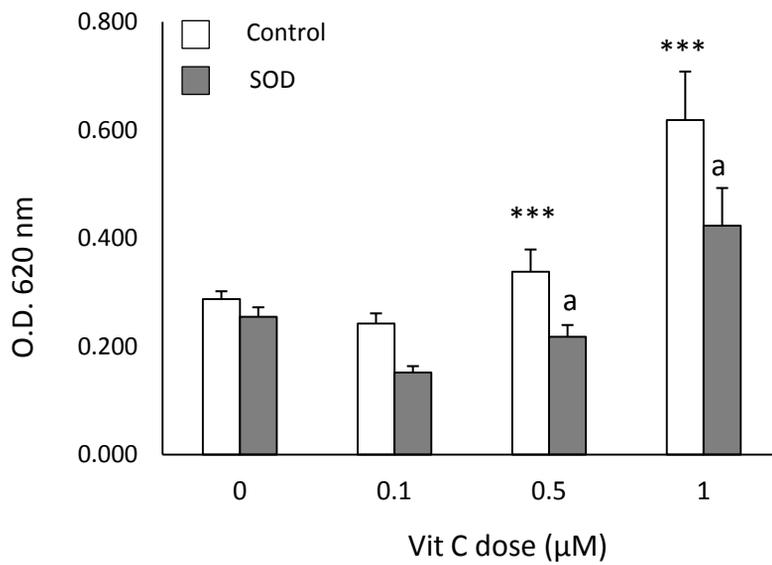
**Fig. S1. Viability of RTS11 cells upon exposure to vitamin C.** RTS11 cells were incubated at 20°C with different vitamin C doses (0.1, 0.5, 1, 5 and 10  $\mu$ M). After 3, 24, 48 or 72 h, cell viability was checked by Propidium Iodide (PI) staining on a FACSCalibur flow cytometer. A. Representative dot plots obtained after 72 h. B. Representative plots showing percentages of dead (PI<sup>+</sup>) cells.

Table 1. List of primers used in this study to evaluate gene transcription

Gene	Forward primer	Reverse primer
EF-1 $\alpha$	CAAGGATATCCGTCGTGGCA	ACAGCGAAACGACCAAGAGG
IL-1 $\beta$ 1	CCTGGAGCATCATGGCGTG	GCTGGAGAGTGCTGTGGAAGAACATATAG
IL-8	TCCTGACCATTACTGAGGGGATGA	AGCGCTGACATCCAGACAAATCTC
TNF- $\alpha$	CCACACACTGGGCTCTTCTT	GTCCGAATAGCGCAAATAA
COX-2B	CCAGTATCAGAACCGCATCTCGT	GACCTCCAGCAACCCGTCC
CATH2	ACATGGAGGCAGAAGTTCAGAAGA	GAGCCAAACCCAGGACGAGA
Hepcidin	GCTGTTCTTTCTCCGAGGTGC	GTGACAGCAGTTGCAGCACCA

Figure  
Fig. 1

### A. RTS11



### B. HK leukocytes

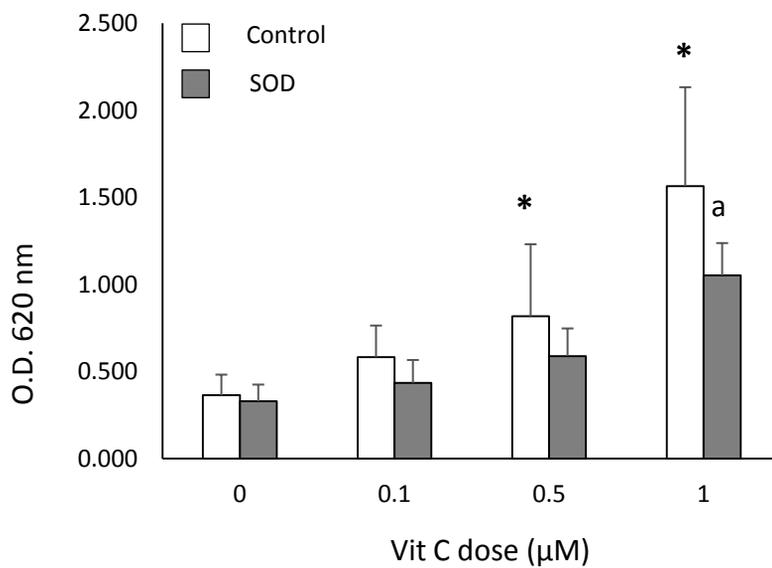
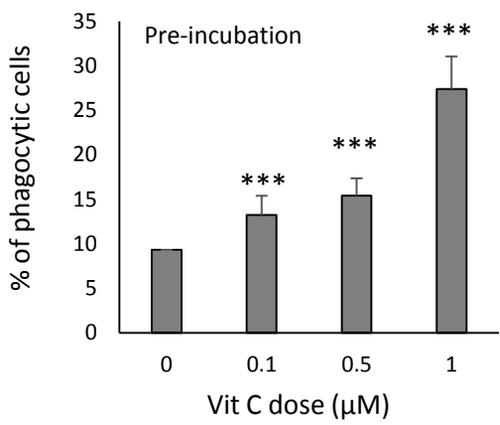
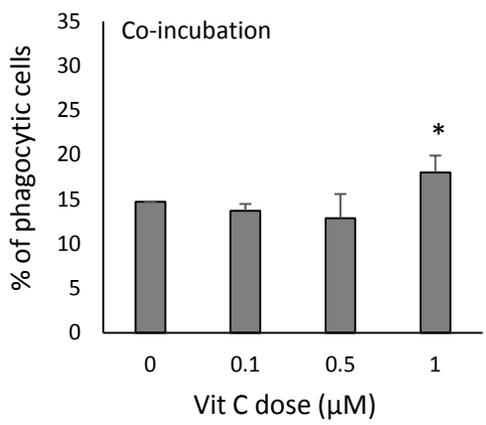


Figure  
Fig. 2

**A. RTS11**



**B. HK leukocytes**

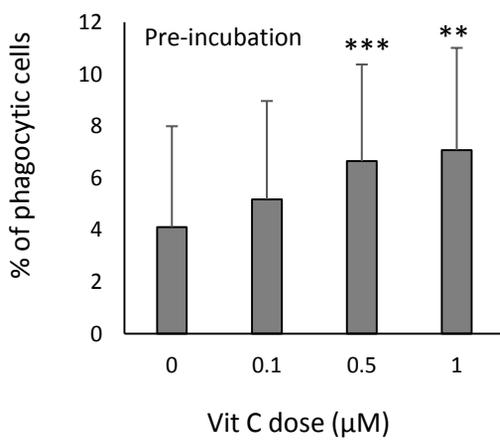
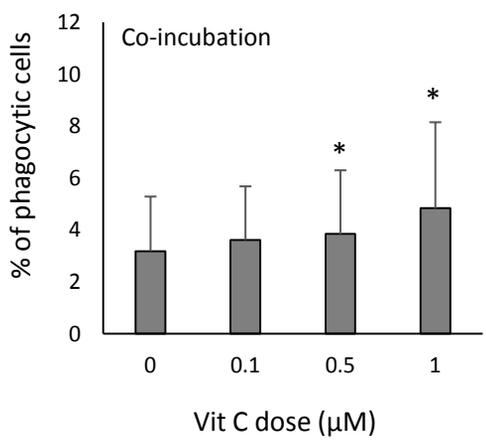
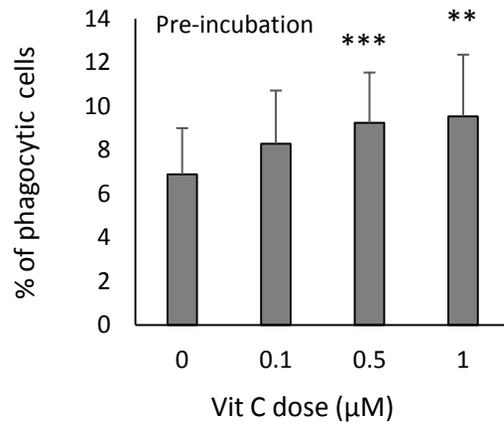
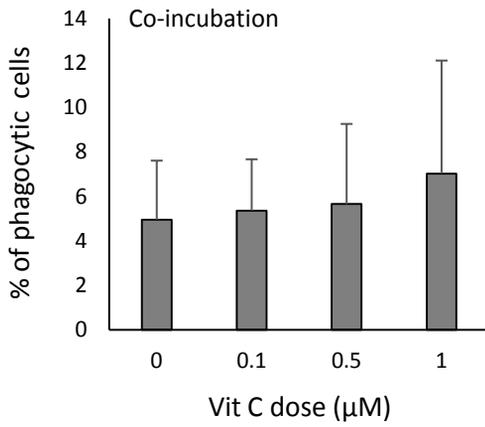


Figure 3

### A. IgM<sup>+</sup> B cells



### B. HK myeloid cells

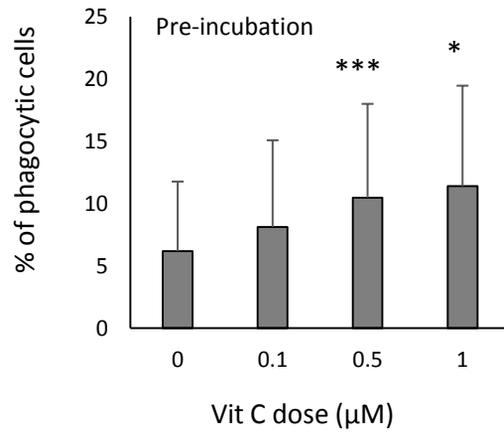
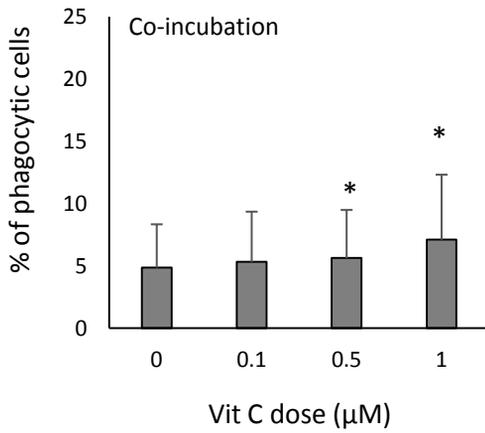
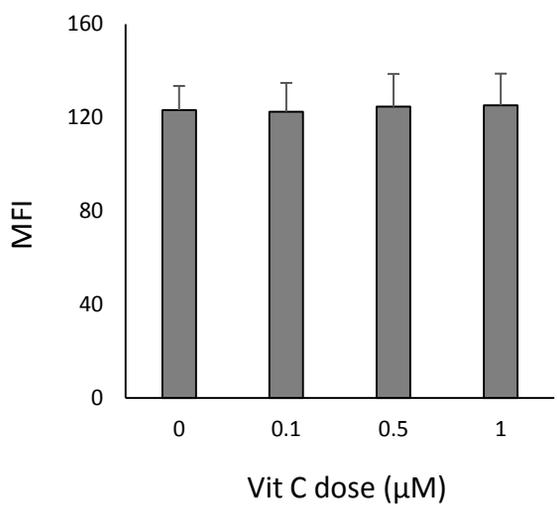


Figure 4

**A. RTS11**



**B. HK leukocytes**

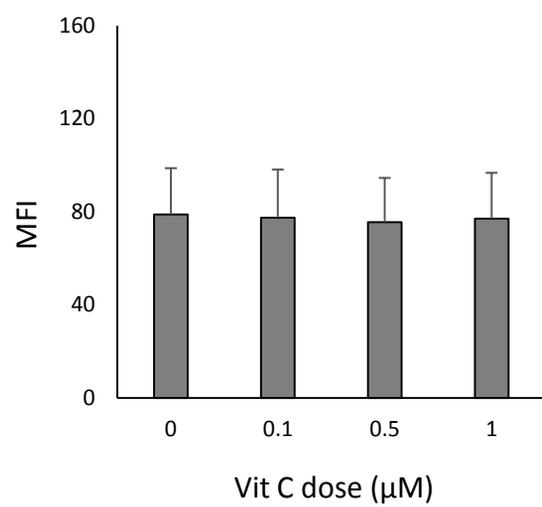
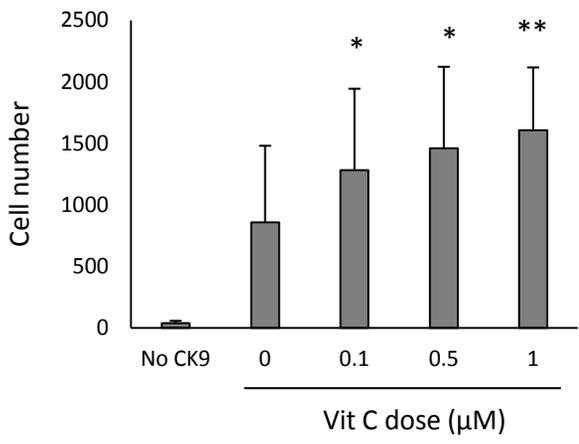
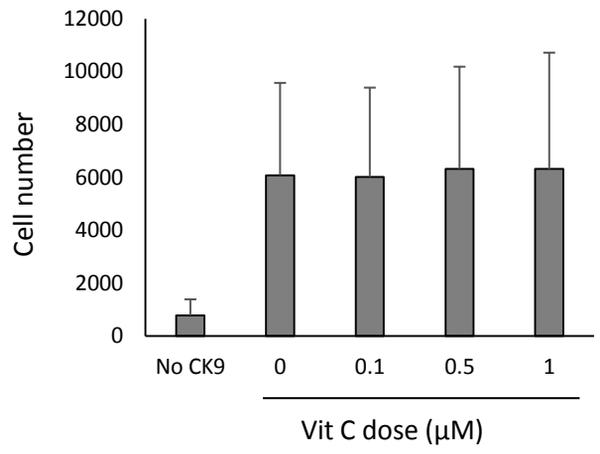


Figure  
Fig. 5

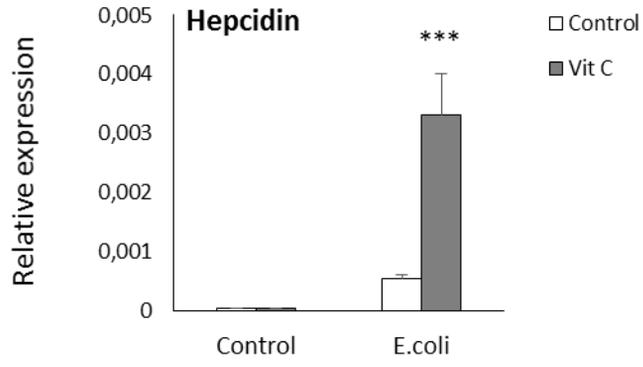
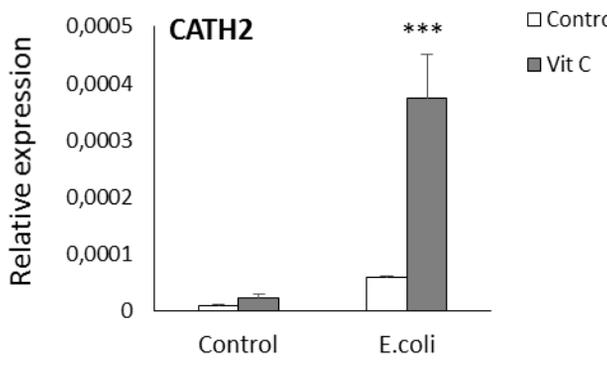
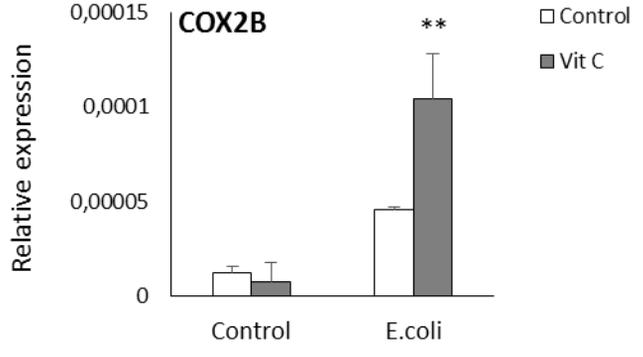
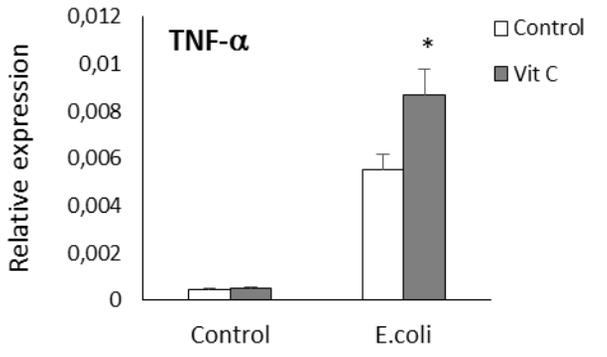
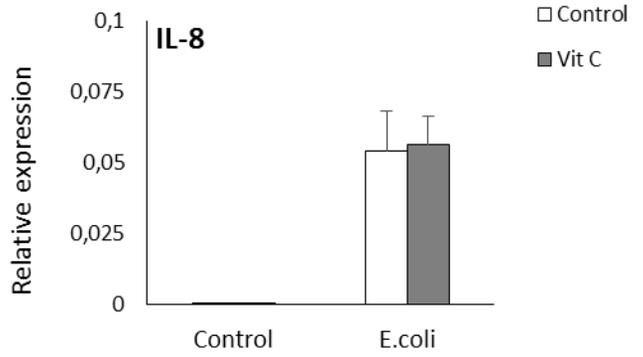
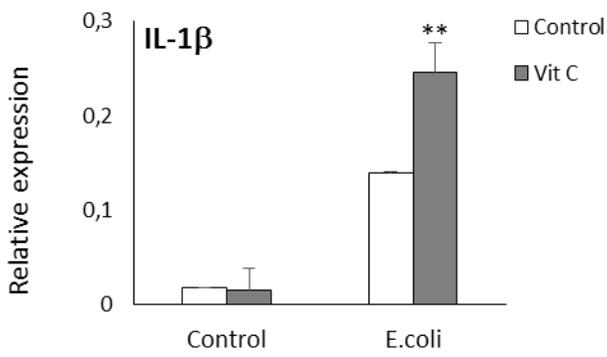
**A. RTS11**



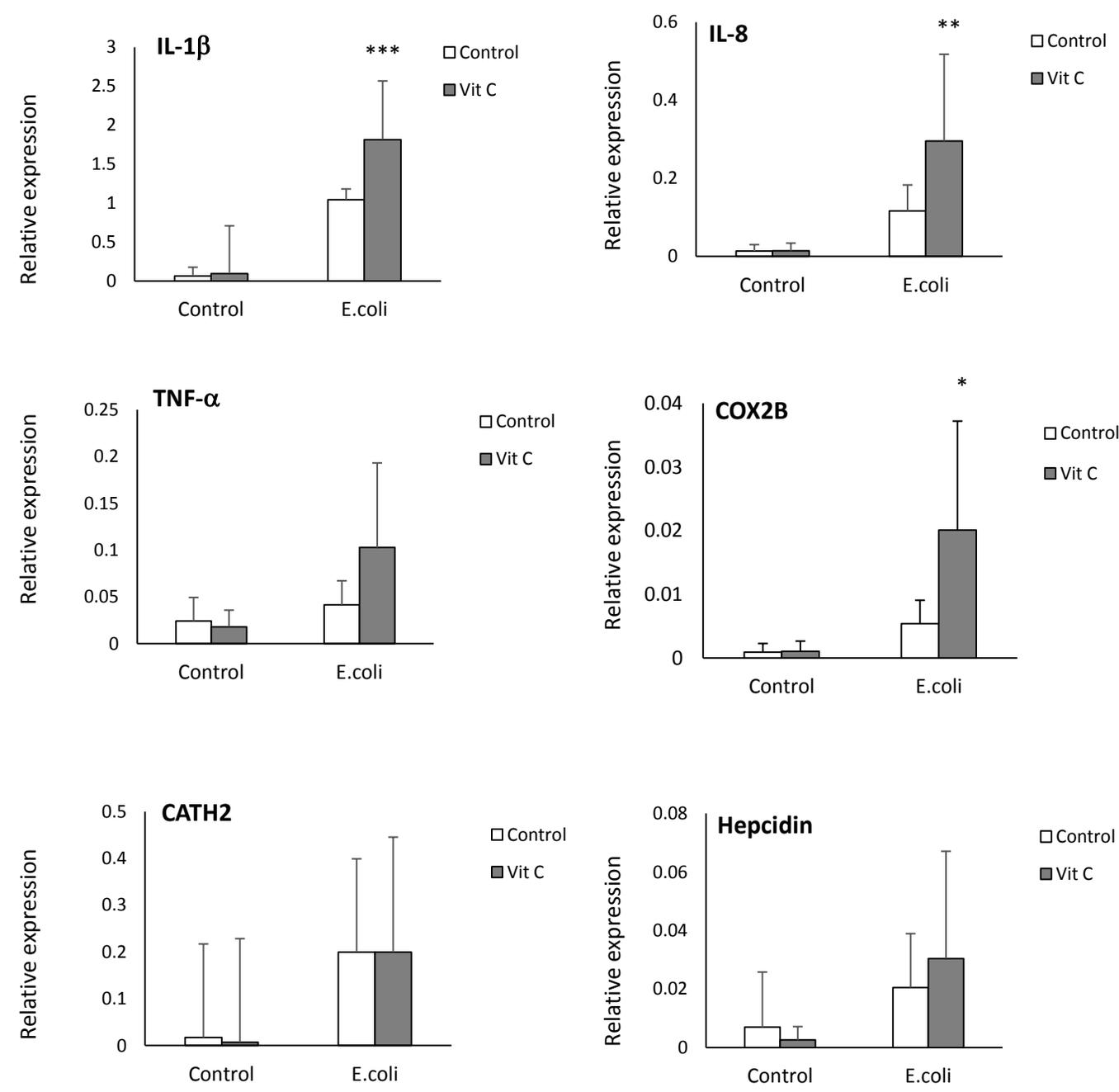
**B. HK leukocytes**



**Figure 6**



**Figure 7**



**Supplementary material for online publication only**

**[Click here to download Supplementary material for online publication only: Supplementary files.pptx](#)**