β -glucan administration induces metabolic changes and differential survival rates after bacterial or viral infection in turbot (*Scophthalmus maximus*)

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Abstract

The innate immune response is able to ward off pathogens and remember previous infections using different mechanisms; this kind of immune reaction has been called “trained immunity”. Changes in cellular metabolism (aerobic glycolysis) have been observed during training with some immunostimulants like β -glucans or during viral and bacterial infections. We hypothesize that β -glucans can induce metabolic changes used by the host to fight pathogens. Accordingly, we evaluated changes in metabolic parameters in turbot that could affect their survival after a previous intraperitoneal treatment with β -glucans and subsequent administration of Viral Hemorrhagic Septicemia Virus (VHSV) or bacteria (*Aeromonas salmonicida* subsp. *salmonicida*). The results obtained support that β -glucans, VHSV and *A. salmonicida* induce changes in lactate, glucose and ATP levels in plasma, head kidney and liver and in the mRNA expression of enzymes related to glucose and fatty acid metabolism in head kidney. Additionally, the metabolic changes induced by β -glucans are beneficial for VHSV replication, but they are harmful to *A. salmonicida*, resulting in reduced mortality. β -glucans appear to have great therapeutic potential and can induce trained immunity against bacterial disease but not against viral disease, which seems to take advantage of β -glucan metabolic alterations.

Keywords: turbot, innate immune, metabolism, immunostimulants, β -glucans.

1. Introduction

Aquaculture has increased in an exponential way in the last years becoming one of the main food sectors with a high growth potential. However, this increase has been associated with pathogen-induced diseases, which has diminished the production resulting in one of the major problems on fish culture [1,2,3,4,5,6,7,8,9,10]. Treatments with approved methods such as chemotherapeutics or antibiotics are often neither totally

effective nor consumer/environmentally friendly, and a high level of antibiotic resistance is emerging [11], therefore preventive measures are needed to guarantee the sustainable development of aquaculture.

In recent years, a growing interest has emerged in the use of immunostimulants that are beneficial for improving the immune status in fish and may help to reduce susceptibility to diseases [12]. Immunostimulants can be grouped under chemical agents, animal or plant extracts, bacterial components, polysaccharides, nutritional factors, hormones and cytokines [13]. Moreover, some immunostimulants, called pathogen associated molecular patterns (PAMPs), are recognized by receptors present on cells of the innate immune system such as Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) to trigger an effector response [14,15]. In fish, the PAMPs that are most commonly used as immunostimulants are β -glucans or lipopolysaccharide (LPS) and peptidoglycan (PGN) from bacteria [16,17]. β -glucans are glucose polymers produced by a variety of plants. These polysaccharides are the constituents of the cell walls of cereals, mushrooms and other fungi as well as several bacterial species [12]. Diverse studies have demonstrated that β -glucans are a potential and promising immunostimulant for fish, and their administration via the diet, immersion or injection enhances resistance against bacterial and viral pathogens [18,19,20,21,22,23,24].

The mechanisms by which β -glucans or other molecules are able to activate the innate immune system has not been widely studied in fish to date; however, similar to tumor or proliferative cells, epigenetic reprogramming of the human cells of the innate immune system and changes in cellular metabolism have been observed during priming with these immunostimulants [25]. Some of these metabolic changes involve an increment in the rate of glucose uptake, where even in the presence of oxygen and fully functioning mitochondria, glucose is used for lactate production.

The importance of metabolic pathways in the regulation of immune responses has been studied. In mammals, pathogens, in addition to PAMPs or external stimuli, are also able to modulate metabolic processes. These modulation of the metabolic pathways have an important role in the generation of key products to promote cell survival or growth. Glycolysis has been shown to be involved in several immune processes such as T cell activation. Also, monocytes rely on glycolysis as a main energy source and enhanced glycolysis occurs in LPS-activated macrophages, dendritic cells, and natural

killer cells [26]. Some viruses are able to drastically alter glucose metabolism in infected cells by diverting it from the tricarboxylic acid cycle (TCA) and enhancing its use for the production of fatty acids and lipids, which are needed for the synthesis of the membranes required for virion envelopes, ensuring in this way greater success of the virus infection [27,28,29]. In some processes of inflammation and sepsis produced by LPS, glucose metabolism is altered [30], and a metabolic switch from oxidative phosphorylation to aerobic glycolysis has been also observed in some cases [31].

This phenomenon of “aerobic glycolysis”, also known as the “Warburg effect” [32], is a less efficient mechanism of energy metabolism since only two molecules of ATP are produced from one molecule of glucose, whereas the completion of oxidative phosphorylation results in the production of a total of 36 ATP molecules per molecule of glucose [33,34]. However, the ATP generation is much quicker than that involving glucose catabolism through TCA and posterior oxidative phosphorylation. Several authors have proposed different functions of the Warburg effect during cell growth and survival. It is not clear if this effect is needed for faster production of ATP because the amount of ATP or if the production of lactate and higher levels of glycolytic intermediates are advantageous as a source of biomass [33,35,36].

However, if the consequences of the Warburg effect and the observed metabolic changes are beneficial for the pathogen, or if they are mechanisms used for the host to fight against the infection, have not been widely studied in fish. Therefore, in the present study, we aimed to evaluate whether the effect of previous treatment with β -glucans could induce changes in metabolism that could affect the survival of turbot in experimental infections conducted with a virus Viral Hemorrhagic Septicemia Virus (VHSV) or bacteria (*Aeromonas salmonicida* subsp. *salmonicida*).

2. Materials and methods

2.1 Fish, Viruses, and bacteria

Juvenile turbot ($2.5 \text{ g} \pm 0.3 \text{ g}$) were obtained from a commercial fish farm (Insuiña S.L., Galicia, Spain) and maintained in 500-liter fiberglass tanks with a recirculating saline water system (total salinity of approximately 35 g/L) and a 12L:12D photoperiod at 15 °C. Prior to the experiments, the fish were acclimatized to the

laboratory conditions for 2 weeks. The animals were fed daily to satiety with a commercial dry diet (LARVIVA-BioMar). Fish care and challenge experiments were reviewed and approved by the CSIC National Committee on Bioethics under approval number 151-2014.

Viral Hemorrhagic Septicemia Virus (VHSV) strain UK-860/94 was propagated in the Epithelioma Papulosum Cyprini (EPC) cell line using Eagle's Minimum Essential Medium (MEM) supplemented with 2% fetal bovine serum (FBS; Gibco) and primocin (InvivoGen) at 15 °C. The second passage was stored at -80 °C until use. The viral stock was titrated into 96-well plates according to an established protocol [37], and VHSV aliquots were stored at -80°C until use.

Aeromonas salmonicida subsp. *salmonicida* (strain VT 45.1 WT, kindly donated by Dr. M.L. Lemos; University of Santiago de Compostela, Spain) was cultured in tryptic soy agar (TSA) plates at 22 °C for 24 h before being use. A bacterial suspension was prepared in PBS and its optical density was adjusted to 0.5 (OD₆₂₀) just before its inoculation [38]. Serial dilutions were performed to calculate the number of cfu/ml and colony counts on inoculum suspension using TSA (Tryptic Soy Agar) plates and counting the bacterial colonies produced.

2.2 Chemicals

β -glucans extracted from *Saccharomyces cerevisiae* were obtained from Biotec-Mackzymal. Several washes with 1x phosphate buffered saline (PBS; Thermo Fisher Scientific) were conducted, and a solution at 25 mg/ml were kept at -20°C until use. The working solution was 1mg/ml.

Zymosan A from *Saccharomyces cerevisiae* (glucans with repeating glucose units connected by a β -1,3-glycosidic linkage) was obtained from Sigma-Aldrich (Z-4250). Prior to its use, this immunostimulant was boiled at 100°C for 30 min, washed twice in PBS and finally resuspended in fresh 1x PBS for obtain a final concentration of 20 mg/ml. The Zymosan working solution was diluted in PBS at 1 mg/ml.

The 2-deoxy-d-glucose (2-DG) is an analogue derivative of glucose that inhibits glycolysis. It was obtained from Sigma-Aldrich (D-8375) and diluted in PBS to a final concentration of 500 mg/ml.

2.3 Experimental design

2.3.1 Sampling procedures

Following the two-week acclimation period, fish were randomly assigned to two 60-liter experimental tanks. Fish (n=26 fish per treatment) were anaesthetized with MS-222 (50 mg/l) and intraperitoneally injected (i.p.) with a volume of 50 μ l of β -glucans or the same volume of PBS for the control group. One week later, each group of 26 fish was divided into two groups of 13 individuals each, in which a solution of 50 μ l of VHSV (5×10^4 TCID₅₀/ml) or an equivalent volume of MEM for the controls was i.p. injected into each turbot. Four 30-liter experimental tanks were used (Control, β -glucans, VHSV and β -glucans+VHSV). The same experimental design was also applied in bacterial infection: one week after the β -glucans treatment, 13 fish from each group were i.p. injected (50 μ l) with PBS (controls) or with *A. salmonicida* (2×10^7 CFU/ml) (Control, β -glucans, *A. salmonicida* and *A. salmonicida*+VHSV).

One day after the viral and bacterial infections, the fish were lightly anesthetized with MS-222 immediately prior to the sampling collection. Blood was collected via a caudal puncture with heparinized syringes. Plasma samples were obtained after blood centrifugation at 400 xg for 10 minutes, immediately deproteinized (using 0.6 M perchloric acid) and neutralized (using 1 M potassium bicarbonate) before freezing and storage at -80°C until further use. Fish were humanely sacrificed with MS-222 overdose, and liver and head kidney were collected and snap-frozen in dry ice. Samples from a total of 8 fish were stored at -80 °C and used to assess metabolite and ATP levels, whereas the remaining 5 fish were stored in a homogenization solution with thioglycerol from Maxwell 16 LEV simplyRNA Tissue Kit (Promega) at -80 °C and they were used for the assessment of mRNA levels by qRT-PCR.

2.3.2 Survival experiments

In parallel, survival experiments were carried out. Following a two-week acclimation period, 80 fish were randomly divided into 8 tanks with 10 turbot each. Turbot were then i.p. injected with MEM or PBS (controls), with β -glucans +/- VHSV or *A. salmonicida* in the same way as described in the previous section. Mortality was registered during the next days post-infection (dpi).

For the bacterial challenges, four more additional survival assays were carried out to corroborate the protective effect of β -glucans after *A. salmonicida* infection:

Priming by immersion: Forty fish were randomly divided into 2 tanks with 20 turbot each. One batch of fish was immersed in saline water, and the other batch was immersed in saline water with β -glucans (100 μ g/ml). One week later, 10 fish from the saline water group and 10 fish from the β -glucan-stimulated group were i.p. injected (50 μ l) with PBS (control group), and the remained 10 fish per treatment were i.p. injected with 50 μ l of an *A. salmonicida* solution using a dose of 2.7×10^7 CFU/ml per fish.

Dose response: Sixty fish were randomly divided into 6 experimental tanks with 10 turbot each. Each group was inoculated with a volume of 50 μ l of different concentrations of β -glucans (1 mg/ml, 5 mg/ml and 10 mg/ml) or Zymosan (1 mg/ml and 5 mg/ml). The remaining batch was inoculated with 50 μ l of PBS as a control. One week later, all fish were i.p. injected with an *A. salmonicida* solution using a dose of 2.3×10^7 CFU/ml per fish.

Longer priming: Forty fish were randomly divided into 4 tanks with 10 turbot each. Two batches of fish received an i.p. injection (50 μ l) of β -glucans (1 mg/mL) or the same volume of PBS (control group). Twenty-five days later, 10 fish from the control group and 10 from the β -glucan-stimulated group were i.p. injected (50 μ l) with PBS, and 10 fish from the control group and 10 from the β -glucan-stimulated group were IP injected (50 μ l) with *A. salmonicida* using a dose of 2.7×10^7 CFU/ml per fish.

Glucans or 2-deoxy-d-glucose (2-DG) priming: Eighty fish were subdivided into 8 tanks with 10 turbot each and subjected to several stimulations. A total of 20 fish (from 2 tanks) were i.p. inoculated with a volume of 50 μ l of β -glucans (1 mg/ml); two tanks were inoculated with 2-DG (500 mg/ml), two tanks with a combination of both (β -glucans+2-DG) and the remaining fish from the last 2 tanks acted as a control inoculated with the same volume of PBS. One week later, one batch of PBS, one of β -glucans, one of 2-DG and one of β -glucans+2-DG were i.p. injected with PBS, and the four remaining groups were i.p. injected with *A. salmonicida* using a dose of 1.7×10^7 CFU/ml per fish.

Survival assays were repeated three times in parallel to minimize the influence of tank position, and the results are shown as the mean + SEM of the data obtained in

three different tanks per treatment. Mortality was recorded during the subsequent dpi in all survival assays.

2.4 Assessment of metabolite and ATP levels

Lactate and glucose levels were determined enzymatically using commercial kits (Spinreact, Barcelona, Spain) adapted to a microplate format. Tissue samples used to assess metabolite levels were homogenized immediately by mechanical disruption using a potter linked to a motor pestle in 7.5 vol of ice-cold 6% PCA and neutralized (using 1 mol/l potassium bicarbonate). The homogenate was centrifuged at 16000 xg for 4 minutes, and the supernatant was used to assay the tissue metabolites.

Blood cell and tissue ATP levels were determined by luminescence using a commercial kit (Promega).

2.5 RNA extraction, cDNA synthesis and quantitative PCR analysis

Total RNA was extracted from head kidney using the Maxwell 16 LEV simplyRNA Tissue Kit (Promega) with the automated Maxwell 16 Instrument in accordance with the instructions provided by the manufacturer. cDNA synthesis was performed with the NZY First-Strand cDNA Synthesis Kit (NZYtech) using 500 ng of RNA. Quantitative analysis of gene expression was determined using the 7300 Real Time PCR System (Applied Biosystems). cDNA amplification was performed using specific primers designed with Primer 3 software [39], the efficiency of which had been previously tested according to the protocol described by Schmittgen and Livak [40]. Analyses were performed in 1 µl of diluted cDNA using SYBR GREEN PCR Master Mix (Applied Biosystems) in a total PCR reaction volume of 25 µl containing 10 µM of each primer. Specific qPCR primers for turbot genes were designed based on sequences obtained from the turbot genome [41] using the Primer 3 program [39]. The amplification efficiency was calculated using seven serial five-fold dilutions of head kidney cDNA from unstimulated turbot with the threshold Cycle (C_T) slope method [42], and the identity of the amplicons was confirmed by sequencing. Sequences of the forward and reverse primers used for each gene are shown in Table 1.

Thermal cycling was initiated with a denaturation step (95 °C, 10 min), followed by 40 cycles of a denaturation step (95 °C, 15 s) and one hybridization-elongation step

(60 °C, 1 min); mRNA levels of the target genes were normalized to the reference gene *eukaryotic translation elongation factor 1 alpha* (EF-1 α), which was stably expressed in this experiment. Expression levels were calculated following the Pfaffl method [42].

2.6 Statistics

Comparisons among groups were carried out using the Student's t test (paired comparisons) or one-way ANOVA (multiple comparisons) followed by a Student-Newman-Keuls test, and differences were considered statistically significant at $P < 0.05$.

In survival experiments, Kaplan-Meier cumulative survival curves [43] were analyzed for statistical significance with the log-rank (Mantel-Cox) test [44], and significant differences are displayed if the p-value is less than $*(p \leq 0.05)$, $**(p \leq 0.01)$ or $*** (p \leq 0.001)$.

3. Results

3.1 β -glucan treatment enhances survival in turbot against *A. salmonicida* but not against VHSV

We evaluated the capacity of β -glucans to protect against VHSV or *A. salmonicida* infections (Fig. 1). The treatment with β -glucans did not protect against the viral infection, as fish treated with β -glucans during one week and infected with VHSV reached 100% cumulative mortality at 11 dpi (Fig. 1A). Fish infected with VHSV reached the same mortality rate at 13 dpi and the survival at the end of the experiment in the control and β -glucan group was 100%. There were significant differences between the controls and infected groups ($p \leq 0.001$), but not between the VHSV and β -glucans+VHSV groups. However, in fish infected with *A. salmonicida* (Fig. 1B), the treatment with β -glucans during one week prolonged survival after *A. salmonicida* infection (40%) while mortalities of infected fish reached 100% cumulative mortality at the end of the experiment ($p \leq 0.01$).

3.2 β -glucans, VHSV and *A. salmonicida* induce changes in metabolite and ATP levels of turbot

To investigate the effects in the metabolism of β -glucans, VHSV and *A. salmonicida*, we evaluated the changes in the levels of some metabolites of glycolysis pathway and fatty acid metabolism which are related to the Warburg effect (Fig. 2 and Fig. 3). Levels of plasma, liver and head kidney metabolites and ATP in the set of fish infected with VHSV are shown in Fig. 2. Glucose levels in plasma (Fig. 2A) increased with the viral infection compared with immunostimulated fish or immunostimulated/infected fish, however, the liver levels decreased after treatment with β -glucans when compared with those in the other groups (Fig. 2C). There were no significant differences in glucose levels in head kidney (Fig. 2B). Lactate levels increased in plasma in all groups (Fig. 2D). In head kidney, lactate levels were higher after β -glucan treatment and VHSV infection than those observed in the other groups (Fig. 2E) and higher in liver after VHSV infection compared with those in the controls and after treatment with β -glucans (Fig. 2F). ATP levels decreased in blood cells after VHSV infection and after β -glucan treatment and VHSV infection (Fig. 2G). In liver, ATP levels decreased in the VHSV group compared with those in the control group (Fig. 2I). Finally, no significant changes were noted in ATP levels in head kidney (Fig. 2H).

In the set of fish infected with *A. salmonicida* (Fig. 3), glucose levels decreased in plasma in the *A. salmonicida* group (Fig. 3A) and in liver in all groups compared with those in the controls (Fig. 3C). There were no significant differences in glucose levels in head kidney (Fig. 3B). Lactate levels (Fig. 3D) increased in plasma compared with those in the controls after treatment with β -glucans and β -glucans+ *A. salmonicida*. In head kidney (Fig. 3E), lactate levels decreased after β -glucan treatment and *A. salmonicida* infection compared with the *A. salmonicida* group and in liver were lower after treatment with β -glucans (Fig. 3F). Compared with the controls and β -glucan treatment, ATP levels in blood cells (Fig. 3G) decreased after *A. salmonicida* infection. No significant changes were noted for ATP levels in head kidney (Fig. 3H). In liver, ATP levels (Fig. 3I) increased after *A. salmonicida* infection and after β -glucan treatment and *A. salmonicida* infection.

3.3 Differentially regulated metabolic genes in VHSV or *A. salmonicida*-infected turbot after β -glucan administration

The modulation of the gene expression after the previous treatment with β -glucans during one week and the subsequent infection with VHSV or *A. salmonicida* was also studied (Fig. 4. and Fig. 5). Concerning viral infections in head kidney, β -glucan treatment produced an up-regulation of mRNA levels of LDH-A (Fig. 4A) and FAT/CD36 (Fig. 4C) and the infection with VHSV (with or without previous treatment with β -glucans) induced an increase in the expression levels of LDH-A (Fig. 4A), PK (Fig. 4B), FAT/CD36 (Fig. 4C) and FAS (Fig. 4D) compared with controls.

Changes in mRNA levels assessed in head kidney in the group of fish infected with *A. salmonicida* are shown in Fig. 5. *A. salmonicida* infection compared with controls up-regulated mRNA levels of LDH-A (Fig. 5A) and PK (Fig. 5B). FAT/CD36 mRNA levels (Fig. 5C) were elevated in the β -glucans and *A. salmonicida* groups compared with those in the control and β -glucans+*A. salmonicida* groups. No significant differences were noted in the mRNA levels of FAS (Fig. 5D).

3.4 Protective effect of β -glucans in turbot in different experimental *A. salmonicida* infections

In order to further investigate the protective effect of β -glucans after an *A. salmonicida* infection, four more survival assays were carried out (Fig. 6). First, the protective effect of β -glucans by immersion against *A. salmonicida* was confirmed (Fig. 6A). Fish infected with *A. salmonicida* did not survive but fish previously treated with β -glucans presented a 30% survival at the end of the experiment. Significant differences ($p < 0.001$) were noted between both groups and compared with the controls.

Also, we evaluated if different concentrations of β -glucans or Zymosan produce also a protective effect against the bacterial infection (Fig. 6B). Both, β -glucans and Zymosan increased significantly the survival rates at the end of the experiment. Higher protection was observed with higher concentrations of Zymosan.

We also wanted to determine if longer priming (25 days) with β -glucans, could affect the survival after *A. salmonicida* infection (Fig. 6C). Fish infected with *A. salmonicida* reached 100% cumulative mortality at 11 dpi, while survival in the β -glucans+*A. salmonicida* group was 40% at the end of the experiment.

Finally, in order to confirm if the effect of the β -glucans against the *A. salmonicida* infection was indeed associated to the Warburg effect, we repeated the

stimulation with 2-DG, a derivative of glucose that inhibits the glycolysis pathway (Fig. 6D). The protective effect of β -glucans was observed at the end of the experiment in β -glucans+A. salmonicida group with a survival rate of 30%. Significant differences ($p < 0.001$) were noted in all control groups (control, β -glucans, 2-DG and β -glucans+2-DG) compared with the infected groups and between the β -glucans+A. salmonicida, A. salmonicida, 2-DG+A. salmonicida and β -glucans+2-DG+A. salmonicida groups ($p < 0.05$).

4. Discussion

It is known that β -glucans can induce metabolic changes in mammals that have been associated to an increased innate immune response [25,45]. In cultured fish, immunostimulant have been used enhancing the resistance and survival against bacterial and viral infections [46,47,48,49] and regulating the innate response during pathogen infection [22,23,50,51,52,53,54,55]. In the present study, we assessed whether a previous treatment with β -glucans was able to alter metabolism as well as provide protection during bacterial or viral infection in turbot. Changes observed in most parameters were in general compatible with the metabolic alterations provoked by the Warburg effect. The decreased glucose levels in liver, increased lactate levels in plasma and increased lactate dehydrogenase (LDH-A) mRNA levels in head kidney after β -glucan treatment suggest that these components stimulate glucose metabolism to produce lactate. These changes are similar to the increased glucose consumption and lactate production reported in β -glucan-trained human monocytes [25]. Glucose consumption is increased in developing cells and is converted into lactate even in the presence of oxygen [32]. Moreover, glucose is converted via multiple biosynthetic pathways into pyruvate, most of which is converted to lactate in the cytoplasm by the action of LDH-A, resulting in increased lactate production [56]. The alteration in glucose metabolism induces changes in lipid and fatty acid (FA) biosynthetic pathways, such as an increase in the cholesterol synthesis pathway induced in β -glucan-trained macrophages [57]. In our results, fatty acid translocase (FAT/CD36), that is a key protein involved in regulating the uptake of FA [58], showed mRNA levels up-regulated after β -glucan treatment in head kidney, suggesting that β -glucans could also contribute to alterations in fatty acid metabolism.

Some viruses are known to cause the Warburg effect by, for example, shifting to glycolysis and decreasing cellular respiration. Studies have shown that glucose metabolism is highly altered in human cytomegalovirus-infected cells [28,29] and after white spot syndrome virus infection impairs glycolysis resulting in increased glucose utilization and lactate accumulation in shrimp hemocytes [59,60]. After VHSV infection in the present study, a metabolic switch from oxidative phosphorylation to aerobic glycolysis resulted in increased lactate production, as illustrated by the increased lactate levels in plasma and liver and the increased LDH-A mRNA levels in head kidney. With this mechanism of “aerobic glycolysis”, only two molecules of ATP are produced from one molecule of glucose, whereas oxidative phosphorylation would have resulted in the production of 36 ATP molecules per molecule of glucose [33,34]. Our results after VHSV infection are in agreement with this finding, as supported by decreased ATP levels in blood cells and liver. In addition to LDH-A, a critical enzyme that plays an important role in the Warburg effect is pyruvate kinase (PK) [61], which catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate as the last step of glycolysis. Additionally, pyruvate is converted into lactic acid by LDH-A at low oxygen levels [62]. PK was highly expressed in the present study after viral infection, and no comparable references were found for fish; however, it is known to be required for an optimized inflammatory response in various pathological contexts in mammals [63,64] and is highly expressed in cancer cells [65,66]. Additionally, glucose is used for production of the fatty acids and lipids needed for the success of virus infection [28,29], which requires the activation of some lipogenic enzymes such as fatty acid synthetase (FAS) [67]. FAT/CD36 has been reported to facilitate Hepatitis C virus entry in human Cytomegalovirus [68], and several studies have suggested that it contributes to virion release in human immunodeficiency virus-infected macrophages [69]. Our results are again in agreement with these studies in humans, as VHSV infection in fish provoked an increase in the mRNA levels of both FAS and FAT/CD36. These metabolic changes suggest that the virus can induce alterations related to the Warburg effect.

In mammals, the Warburg effect appears to participate in the metabolic regulation of proinflammatory cytokines [64] and has been described to play a role in inflammation and sepsis [31]. Bacterial sepsis affects the ability of target tissues to utilize glucose via glycolysis and alternative fuel sources, such as ketone bodies and

free fatty acids, via oxidative phosphorylation [70]. The parameters assessed after *A. salmonicida* infection in the present study showed similar results to those obtained after VHSV infection. The increase in LDH-A, PK, and FAT/CD36 mRNA levels in head kidney and the decrease in ATP levels in blood cells suggested that *A. salmonicida* also caused metabolic changes in fish related to the Warburg effect.

A decrease in glucose levels in plasma and liver was observed after bacterial infection. These results are consistent with other studies in turbot in which i.p. injection of sublethal doses of *A. salmonicida* was shown to decrease glycogen levels in liver and glucose levels in plasma [71]. After viral infection, these changes were reversed. These differences can be attributed to the requirement for glucose for adaptation and survival during the stress of antiviral inflammation, despite its prevention of adaptation to the stress of bacterial inflammation [30].

On the other hand, changes in metabolic parameters after previous β -glucan treatment and VHSV infection were similar to those observed after viral infection, with increases in lactate levels in plasma, decreases in ATP levels in blood cells and increases in PK, FAS and FAT/CD36 mRNA levels in head kidney. β -glucans even potentiate the effects of VHSV in some cases, such as the lactate and LDH-A mRNA levels in head kidney. Our results agree with the observed changes in survival rates because mortality in the group of fish infected with VHSV reached 100% 2 days later than the group previously treated with β -glucans and then infected. Accordingly, the apparently enhanced mortality observed with β -glucans after viral infection in the present study could be associated with the observed metabolic changes. β -glucans and virus induce similar metabolic responses, and data suggest that VHSV seems to take advantage of β -glucan-induced metabolic alterations. Strikingly, our mortality results after β -glucan treatment and viral infection do not agree with the findings of other studies, in which rainbow trout i.p. injected with or fed a diet supplemented with β -glucans appeared to be less susceptible to Infectious Hematopoietic Necrosis Virus (IHNV) [22,23]. However, our experiments are not exactly comparable to those observed by LaPatra et al. [22] and Sealey et al. [23] because different fish species, virus and administration route were used.

The changes observed in the metabolic parameters after previous β -glucan treatment and *A. salmonicida* infection were similar to those observed after *A. salmonicida* infection alone in terms of glucose levels in liver and ATP levels in liver.

In contrast, the changes observed in lactate levels in head kidney, ATP levels in blood cells and LDH-A, PK and FAT/CD36 mRNA levels in head kidney after previous β -glucan treatment and *A. salmonicida* infection differed from those observed after *A. salmonicida* infection. The presence of β -glucans was able to counteract the action of *A. salmonicida* in turbot, resulting in levels of some metabolites and in expression of metabolic enzymes similar to those of controls. Thus, these results agree with the changes obtained in survival rates. Accordingly, we observed that the survival rate of turbot infected by *A. salmonicida* was significantly higher in the β -glucan-treated groups, either by i.p. injection or by immersion and using different concentrations and times. Improved protection against *A. salmonicida* was observed after Zymosan (5 mg/mL) i.p. treatment for one week. Zymosan is a glucan with repeating glucose units connected by β -1,3-glycosidic linkages and has been utilized in numerous experiments owing to its ability to activate macrophages [72,73,74]. Furthermore, the changes in survival observed in the different assays carried out in this study after β -glucan administration through various routes, including immersion or injection, and the subsequent bacterial infection are generally in agreement with other studies in fish [47,55,75,76,77,78,79,80]. In the present study, the lack of protection observed when glycolysis was inhibited by 2-DG after the β -glucan treatment and the *A. salmonicida* infection suggests that the metabolic changes produced by aerobic glycolysis appeared to be beneficial for the effects of β -glucans on bacteria. The shift of glucose metabolism from oxidative phosphorylation to aerobic glycolysis could be involved in the protection against the bacteria and it would mean a trained response induced by glucans as has been reported in other species [47,81,82,83,84].

The results obtained herein suggest different behaviors of both infections after prior β -glucan treatment. The metabolic changes observed after VHSV and *A. salmonicida* were similar and could induce alterations related to the Warburg effect; however, after β -glucan administration, the opposite changes were observed. We may hypothesize that these phenomena could be due to differences in infection mechanisms, with each condition creating a distinct challenge and potentially requiring different management strategies. Viruses are intracellular pathogens that depend on the metabolic machinery of the host cell to supply the energy and macromolecules necessary for successful replication. During viral infection, there is a demand for nutrients and energy [67], and successful virus replication requires increased metabolism and oxygen

utilization; for example, fatty acids are needed for membranes and enlargement of the nucleus of infected cells [27]. Furthermore, whereas glucose utilization is required for survival in models of viral inflammation, it is lethal in models of bacterial inflammation [30]. Concordantly, we found that metabolic changes induced by β -glucans were beneficial for VHSV replication, but they were harmful to *A. salmonicida*, resulting in reduced mortality. It seems possible that the differences in susceptibility of turbot to both infections after β -glucan treatment resides in differences in the ability of the viruses to attach, enter and replicate in different host cells.

In summary, in the present study, we provide information supporting the abilities of β -glucans, VHSV and *A. salmonicida* to induce metabolic changes in turbot that could be related to the Warburg effect. However, the observed changes were different between viral and bacterial infections after previous β -glucan treatment. Therefore, β -glucan administration could be used as a potential therapeutic in the bacterial disease but not in the viral disease that seems to take advantage of β -glucan metabolic alterations. We suggest that VHSV mechanisms are integrated to manipulate cellular responses to alter cellular metabolism in ways that promote efficient viral infection, while β -glucans appear to induce trained immunity after *A. salmonicida* infection in turbot. Further studies are necessary to characterize the underlying mechanisms as well as the impact of β -glucans on the metabolism and immune system in fish.

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References

- 498 [1] Birkbeck, T.H., Feist, S.W., Verner-Jeffreys, D.W. Francisella infections in fish
499 and shellfish. *Journal of Fish Diseases*, 34 (2011), pp. 173-187.
- 500 [2] Dixon, P.F. VHSV came from the marine environment: clues from the literature, or
501 just red herrings? *Bull. Eur. Ass. Fish Pathol.*, 19 (1999), pp. 60-65.
- 502 [3] Frans, I., Michiels, C.W., Bossier, P., Willems, K.A., Lievens, B., Rediers, H.
503 *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention.
504 *Journal of Fish Diseases*, 34 (2011), pp. 643-661.
- 505 [4] Jacobs, J.M., Stine, C.B., Baya, A.M., Kent, M.L. A review of mycobacteriosis in
506 marine fish. *Journal of Fish Diseases*, 32 (2009), pp. 119-130.
- 507 [5] Kämpfer, P., Lodders, N., Martin, K., Avendaño-Herrera, R. *Flavobacterium*
508 *chilense* sp. nov. and *Flavobacterium araucanum* sp. nov., isolated from farmed
509 salmonid fish. *Int. J. Syst. Evol. Microbiol.*, 62 (2012), pp. 1402-1408.
- 510 [6] Khoo, L. Fungal diseases in fish. *Seminars in Avian and Exotic Pet Medicine*, 9
511 (2000), pp. 102-111.
- 512 [7] Langdon, J.S., Humphrey, J.D., Williams, L.M., Hyatt, A.D., Westbu, H.A. First
513 virus isolation from Australian fish: an iridovirus-like pathogen from redfin
514 perch, *Perca fluviatilis* L. *Journal of fish diseases*, 9 (1986), pp. 263- 268.
- 515 [8] Schlotfeldt, H.J., Ahne, W., Vestergard-Jorgensen, P.E., Glende, W. Occurrence of
516 viral haemorrhagic septicaemia in turbot (*Scophthalmus maximus*) - A natural
517 outbreak. *Bull Eur. Assoc. Fish. Pathol.*, 11 (1991), pp. 105-107.
- 518 [9] Smail, D.A. Viral haemorrhagic septicaemia. *Fish diseases and disorders: Viral,*
519 *bacterial and fungal infections*, 3 (1999), pp.123-147.
- 520 [10] Wang, W. Bacterial diseases of crabs: a review. *Journal of Invertebrate Pathology*,
521 106 (2001), pp. 18-26.
- 522 [11] Romero-Ormazábal, J.M., Feijoó, C.G., Navarrete Wallace, P.A. Antibiotics in
523 aquaculture — use, abuse and alternatives. In: Carvalho, E.D., David, J.S., Silva,
524 R.J. (Eds.), *Health and Environment in Aquaculture*, 159 (2012).
- 525 [12] Meena, D.K. Das, P., Kumar, S., Mandal, S.C., Prusty A.K., Singh, S.K., Akhtar,
526 M.S., Behera, B.K., Kumar, K., Pal, A.K., Mukherjee, S.C. Beta-glucan: an ideal
527 immunostimulant in aquaculture (a review). *Fish. Physiol. Biochem.*, 39 (2013), pp.
528 431-457.
- 529 [13] Sakai, M., 1999 .Current research status of fish immunostimulants. *Aquaculture*,
530 172 (1999), pp. 63-92.
- 531 [14] Akira, S., Uematsu, S., Takeuchi, O. Pathogen recognition and innate immunity.

Cell, 124 (2006), pp. 783-801.

- [15] Medzhitov, R., 2007. Recognition of microorganisms and activation of the immune response. *Nature*, 449 (2007), pp. 819-826.
- [16] Dalmo, R.A., Bøgwa, J. β -glucans as conductors of immune symphonies. *Fish Shellfish Immunol.*, 25 (2008), pp. 384-396.
- [17] Vallejos-Vidal, E., Reyes-López, F., Teles, M., MacKenzie, S.. The response of fish to immunostimulant diets. *Fish Shellfish Immunol.*, 56 (2016), pp. 34-69.
- [18] Ai, Q., Mai, K., Zhang, L., Tan, B., Zhang, W., Xu, W., Li, H. Effects of dietary β -1,3 glucan on innate immune response of large yellow croaker, *Pseudosciaena crocea*. *Fish Shellfish Immunol.* 22 (2007), pp. 394-402.
- [19] Bagni, M., Romano, N., Finola, M.G., Abelli, L., Scapigliati, G., Tiscar, P.G., Sarti, M., Marino, G. Short- and long-term effects of a dietary yeast beta-glucan (Macrogard) and alginic acid (Ergosan) preparation on immune response in sea bass (*Dicentrarchus labrax*). *Fish Shellfish Immunol.*, 18 (2005), pp. 311-325.
- [20] Beaulaurier, J., Bickford, N., Gregg, J.L., Grady, C.A., Gannam, A.L., Winton, J.R., Hershberger, P.K. Susceptibility of pacific herring to viral hemorrhagic septicemia is induced by diet. *J. Aquat. Anim. Health*, 24 (2012), pp. 43-48.
- [21] Figueras, A., Santarem, M.M., Novoa, B. Influence of the sequence of administration of β -glucans and a *Vibrio damsela* vaccine on the immune response of turbot (*Scophthalmus maximus* L.). *Vet. Immunol. Immunopathol.*, 64 (1998), pp. 59-68.
- [22] LaPatra, S.E., Lauda, K.A., Jones, G.R., Shewmaker, W.S., Bayne, C.J. Resistance to IHN virus infection in rainbow trout is increased by glucan while subsequent production of serum neutralizing activity is decreased. *Fish Shellfish Immunol.*, 8(6) (1998), pp. 435-446.
- [23] Sealey, W.M., Barrows, F.T., Hang A., Johansen, K.A., Overturf, K., LaPatra, S.E. Hardy, R.W. Evaluation of the ability of barley genotypes containing different amounts of β -glucan to alter growth and disease resistance of rainbow trout *Oncorhynchus mykiss*. *Animal Feed Science and Technology* 141 (2208), pp. 115-128.
- [24] Whittington, R., Lim, C., Klesius, P.H. Effect of dietary β -glucan levels on the growth response and efficacy of *Streptococcus iniae* vaccine in Nile tilapia, *Oreochromis niloticus*. *Aquaculture*, 248 (2005), pp. 217-225.

- [25] Cheng, S.C., Quintin, J., Cramer, R.A., Shephardson, K.M., Saeed, S., Kumar, V., Giamarellos-Bourboulis, E.J., Martens, J.H., Rao, N.A., Aghajani-Refah, A., Manjeri, G.R., Li, Y., Ifrim, D.C., Arts, R.J., van der Veer, B.M., Deen, P.M., Logie, C., O'Neill, L.A., Willems, P., van de Veerdonk, F.L., van der Meer, J.W., Ng, A., Joosten, L.A., Wijmenga, C., Stunnenberg, H.G., Xavier, R.J., Netea, M.G. mTOR- and HIF-1 α -mediated aerobic glycolysis as metabolic basis for trained immunity. *Science* (2014), 345-1250684. doi:10.1126/science.1250684.
- [26] O'Neill, L.A.J., Kishton, R.J., Rathmell, J. A guide to immunometabolism for immunologists. *Nat. Rev. Immunol.*, 16 (2016), pp. 553-565.
- [27] Buchkovich, N.J., Maguire, T.G., Alwine, J.C. Role of the endoplasmic reticulum chaperone Bi.p., SUN domain proteins, and dynein in altering nuclear morphology during human cytomegalovirus infection. *J. Virol.*, 84 (14) (2013), pp. 7005-7017.
- [28] Munger, J., Bennett, B.D., Parikh, A., Feng, X.J., McArdle, J., Rabitz, H.A.T.S., Rabinowitz, J.D. Systems-level metabolic flux profiling identifies fatty acid synthesis as a target for antiviral therapy. *Nat. Biotechnol.*, 26 (10) (2008), pp. 1179-1186.
- [29] Munger, J., Bajad, S.U., Collier, H.A., Shenk, T., Rabinowitz, J.D. Dynamics of the cellular metabolome during human cytomegalovirus infection. *PLoS Pathog.*, 2 (12) (2006), pp. 1165-1175.
- [30] Wang, A., Huen, S.C., Luan, H.H., Yu, S., Zhang, C., Gallezot, J.D., Booth C.J., Medzhitov, R. Opposing Effects of Fasting Metabolism on Tissue Tolerance in Bacterial and Viral Inflammation. *Cell*, 166(6) (2016), pp. 1512-1525.
- [31] Bar-Or, D., Carrick, M., Tanner, A., Lieser, M.J., Rael, L.T., Brody, E. Overcoming the Warburg Effect: Is it the key to survival in sepsis? *J. Crit. Care.*, 43 (2018), pp. 197-201.
- [32] Warburg, O. The metabolism of carcinoma cells. *The Journal of Cancer Research.*, 9(1) (1925), pp. 148-163
- [33] Locasale, J.W. and Cantley, L.C. Metabolic flux and the regulation of mammalian cell growth. *Cell Metab.*, 14 (2011), pp. 443-451.
- [34] Vander Heiden, M.G., Cantley, L.C., Thompson, C.B. Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science*, 324 (2009), pp. 1029-1033.
- [35] Liberti, M.V. and Locasale, J.W. The Warburg effect: how does it benefit cancer cells? *Trends Biochem. Sci.*, 41 (2016), pp. 211-218.

- [36] Lunt, S.Y. and Vander Heiden, M.G. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu. Rev. Cell Dev. Biol.*, 27 (2011), pp. 441-464.
- [37] Reed, L. J. and Muench, H. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.*, 27 (1983), pp. 493-497.
- [38] Costa, M.M., Pereiro, P., Wang, T., Secombes, C.J., Figueras, A., Novoa, B. Characterization and gene expression analysis of the two main Th17 cytokines (IL-17A/F and IL-22) in turbot, *Scophthalmus maximus*. *Dev. Comp. Immunol.*, 38(4) (2012), pp. 505-516.
- [39] Rozen, S. and H.J. Skaletsky, H.J. Primer3 on the WWW for general users and for biologist programmers. *MIMB*, 132 (2000), pp. 365-386.
- [40] Schmittgen, T.D. and Livak, K.J. Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.*, 3 (6) (2008), pp. 1101-1108.
- [41] Figueras, A., Robledo, D., Corvelo, A., Hermida, M., Pereiro, P., Rubiolo, J.A. Gómez-Garrido, J., Carreté, L., Bello, X., Gut, M., Gut, I.G., Marcet-Houben, M., Forn-Cuní, G., Galán, B., García, J.L., Abal-Fabeiro, J.L., Pardo, B.G., Taboada, X., Fernández, C., Vlasova, A., Hermoso-Pulido, A., Guigó, R., Álvarez-Dios, J.A., Gómez-Tato, A., Viñas, A., Maside, X., Gabaldón, T., Novoa, B., Bouza, C., Alioto, T., Martínez, P. Whole genome sequencing of turbot (*Scophthalmus maximus*; Pleuronectiformes): a fish adapted to demersal life. *DNA Res.*, 23 (2016), pp. 181-192.
- [42] Pfaffl, M.W. A new mathematical model for relative quantification in real time RT-PCR. *Nucleic Acids Res.*, 29 (2001), p. e45.
- [43] Kaplan, E. and Meier, P. Nonparametric estimation from incomplete observations. *J. Amer. Statist. Assoc.*, 53 (1958), pp. 457-481.
- [44] Mantel, N. Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemotherapy Rep.*, 50 (1966), pp. 163-170.
- [45] Arts, R.J.W., Novakovic, B., Horst, R., Carvalho, A., Bekkering, S., Lachmandas, E., Rodrigues, F., Silvestre, R., Cheng, S.C., Wang, S.Y., Habibi, E., Gonçalves, L.G., Mesquita, I., Cunha, C., van Laarhoven, A., van de Veerdonk, F.L., Williams, D.L., van der Meer, J.W., Logie, C., O'Neill, L.A., Dinarello, C.A., Riksen, N.P., van Crevel, R., Clish, C., Notebaart, R.A., Joosten, L.A., Stunnenberg, H.G., Xavier, R.J., Netea, M.G. Glutaminolysis and Fumarate

Accumulation Integrate Immunometabolic and Epigenetic Programs in Trained Immunity. *Cell Metabolism.*, 24(6) (2016), pp. 807-819.

[46]Guselle, N.J., Markham, R.J., Speare, D.J. Intraperitoneal administration of β -1, 3/1, 6-glucan to rainbow trout, *Oncorhynchus mykiss* (Walbaum), protects against *Loma salmonae*. *J. Fish. Dis.*, 29 (2006), pp. 375-381.

[47]Rodríguez, I., Chamorro, R., Novoa, B., Figueras, A. β -glucan administration enhances disease resistance and some innate immune responses in zebrafish (*Danio rerio*). *Fish Shellfish Immunol.*, 27(2) (2009), pp. 369-373.

[48]Selvaraj, V., Sampath, K., Sekar, V. Adjuvant and immunostimulatory effects of β -glucan administration in combination with lipopolysaccharide enhances survival and immune parameters in carp challenged with *Aeromonas hydrophila*. *Vet. Immunol. Immunopathol.*, 114 (2006), pp. 15-24.

[49]Yu-sin, K., Fei, K., Qi-Ya, Z. Effect of β -glucan on activity of antioxidant enzymes and Mx gene expression in virus infected grass carp. *Fish Shellfish Immunol.*, 27(2) (2009), pp. 336-340.

[50]Brogden, G., von Kockritz-Blickwede, M., Adamek, M., Reuner, F., Jung-Schroers, V., Naim, H.Y., Steinhagen, D. β -Glucan protects neutrophil extracellular traps against degradation by *Aeromonas hydrophila* in carp (*Cyprinus carpio*). *Fish Shellfish Immunol.*, 33 (2012), pp. 1060-1064.

[51]Falco, A., Frost, P., Miest, J., Pionnier, N., Irnazarow, I., Hoole, D. Reduced inflammatory response to *Aeromonas salmonicida* infection in common carp (*Cypinus carpio* L.) fed with β -glucan supplements. *Fish Shellfish Immunol.*, 32 (2012), pp. 1051-1057.

[52]Jorgensen, J.B., Sharp, G.J., Secombes, C.J., Robertsen, B. Effects of a yeast-cell-wall glucan on the bactericidal activity of rainbow trout macrophages. *Fish Shellfish Immunol.*, 3 (1993), pp. 267-277.

[53]Lokesh, J., Fernandes, J.M., Korsner, K., Bergh, O., Brinchmann, M.F., Kiron, V. 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), pp. 626-631.

[54]Olivier, G., Eaton, C.A., Campbell, N. Interaction between *Aeromonas salmonicida* and peritoneal macrophages of brook trout (*Salvelinus fontinalis*). *Vet. Immunol. Immunopathol.*, 12 (1986), pp. 223-234.

- 665 [55] Yano, T., Matsuyama, H., Mangindaan, R.E. Polysaccharide- induced protection of
666 carp, *Cyprinus carpio* L., against bacterial infection. J. Fish Dis.,14 (1991), pp.
667 577-582.
- 668 [56] Jha, A.K., Huang, S.C., Sergushichev, A., Lampropoulou, V., Ivanova, Y.,
669 Loginicheva, E., Chmielewski, K., Stewart, K.M., Ashall, J., Everts, B., Pearce,
670 E.J., Driggers, E.M., Artyomov, M.N. Network integration of parallel metabolic
671 and transcriptional data reveals metabolic modules that regulate macrophage
672 polarization. Immunity, 42 (2015), pp. 419-430.
- 673 [57] Saeed, S., Quintin, J., Kerstens, H.K., Rao, N.A., Aghajani-Refah, A.,
674 Matarese, F., Cheng, S.C., Ratter, J., Berentsen, K., van der Ent, M.A., Sharifi,
675 N., Janssen-Megens, E.M., Ter Huurne, M., Mandoli, A., van Schaik, T., Ng,
676 A., Burden, F., Downes, K., Frontini, M., Kumar, V., Giamarellos-Bourboulis,
677 E.J., Ouwehand, W.H., van der Meer, J.W., Joosten, L.A., Wijmenga, C., Martens,
678 J.H., Xavier, R.J., Logie, C., Netea, M.G., Stunnenberg, H.G. Epigenetic
679 programming of monocyte-to-macrophage differentiation and trained innate
680 immunity. Science, 345 (2014), 1251086.
- 681 [58] Bonen, A., Campbell, S.E., Benton, C.R., Chabowski, A., Coort, S.L., Han,
682 X.X., Koonen, D.P., Glatz, J.F., Luiken, J.J. Regulation of fatty acid transport by
683 fatty acid translocase/CD36. Proc. Nutr. Soc., 63(2) (2004), pp. 245-249.
- 684 [59] Chen, I.T., Aoki, T., Huang Y.T., Hirono, I., Chen, T.C., Huang, J.Y., Chang, G.D.,
685 Lo, C.F., Wang, H.C. White spot syndrome virus induces metabolic changes
686 resembling the warburg effect in shrimp hemocytes in the early stage of infection.
687 J. Virol., 85 (2011), pp. 12919-12928.
- 688 [60] Su, M.A., Huang, Y.T., Chen, I.T., Lee, D.Y., Hsieh, Y.C., Li, C.Y., Ng,
689 T.H., Liang, S.Y., Lin, S.Y., Huang, S.W., Chiang, Y.A., Yu, H.T., Khoo,
690 K.H., Chang, G.D., Lo, C.F., Wang, H.C. An invertebrate Warburg effect: a shrimp
691 virus achieves successful replication by altering the host metabolome via the PI3K-
692 Akt-mTOR pathway. PLoS Pathog., 10 (6) (2014), e1004196.
- 693 [61] Ferguson, E.C. and Rathmell, J.C. New roles for pyruvate kinase M2: working out
694 the Warburg effect. Trends in Biochemical Sciences, 33(8) (2008), pp. 359-362.
- 695 [62] Yeluri, S., Madhok, B., Prasad, K.R., Quirke, P., Jayne, D.G. Cancer's craving for
696 sugar: an opportunity for clinical exploitation. J. Cancer Res. Clin. Oncol., 135
697 (2009), pp. 867-877.

- [63] Palsson-McDermott, E.M., Curtis, A.M., Goel, G., Lauterbach, M.A., Sheedy, F.J., Gleeson, L.E., van den Bosch, M.W., Quinn, S.R., Domingo-Fernandez, R., Johnston, D.G., Jiang, J.K., Israelsen, W.J., Keane, J., Thomas, C., Clish, C., Vander Heiden, M., Xavier, R.J., O'Neill, L.A. Pyruvate kinase M2 regulates Hif-1 α activity and IL-1 β induction and is a critical determinant of the warburg effect in LPS-activated macrophages. *Cell Metab.*, 21(1) (2015), pp. 65-80.
- [64] Yang, L., Xie, M., Yang, M., Yu, Y., Zhu, S., Hou, W., Kang, R., Lotze, M.T., Billiar, T.R., Wang, H., Cao, L., Tang, D. PKM2 regulates the Warburg effect and promotes HMGB1 release in sepsis. *Nat. Commun.*, 5-4436 (2014).
- [65] Christofk, H.R., Vander Heiden, M.G., Harris, M.H., Ramanathan, A., Gerszten, R.E., Wei, R., Fleming, M.D, Schreiber, S.L., Cantley, L.C. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature*, 452 (2008), pp. 230-233.
- [66] Mazurek, S., Boschek, C.B., Hugo, F., Eigenbrodt, E. Pyruvate kinase type M2 and its role in tumor growth and spreading. *Semin. Cancer Biol.*, 15 (2005), pp. 300-308.
- [67] Yu, Y., Clippinger, A.J., Pierciey, F.J. Jr., Alwine, J.C. Viruses and metabolism: alterations of glucose and glutamine metabolism mediated by human cytomegalovirus. *Advances in Virus Research*, 80 (2011), pp. 49-67.
- [68] Cheng, J.J., Li, J.R., Huang, M.H., Ma, L.L., Wu, Z.Y., Jiang, C.C., Li, W.J., Li, Y.H., Han, Y.X., Li, H., Chen, J.H., Wang, Y.X., Song, D.Q., Peng, Z.G., Jiang, J.D. CD36 is a co-receptor for hepatitis C virus E1 protein attachment. *Sci. Rep.*, 6 (2016), p. 21808.
- [69] Berre, S., Gaudin, R., Cunha de Alencar, B., Desdouits, M., Chabaud, M., Naffakh, N., Rabaza-Gairi, M., Gobert, F.X., Jouve, M., Benaroch, P. CD36-specific antibodies block release of HIV-1 from infected primary macrophages and its transmission to T cells. *J. Exp. Med.*, 210 (2013), pp. 2523-2538.
- [70] Agwunobi, A.O., Reid, C., Maycock, P., Little, R.A., Carlson, G.L. Insulin resistance and substrate utilization in human endotoxemia. *J. Clin. Endocrinol. Metab.*, 85 (2000), pp. 3770-3778.
- [71] Rodríguez-Quiroga, J.J., Otero-Rodiño, C., Suárez, P., Nieto, T.P., García Estévez, J.M., San Juan, F., Soengas, J.L. Differential effects of exposure to parasites and

- bacteria on stress response in turbot *Scophthalmus maximus* simultaneously stressed by low water depth. *J. Fish. Biol.*, 91(1) (2017), pp. 242-259.
- [72] Boltaña, S., Doñate, C., Goetz, F.W., MacKenzie, S., Balasch, J.C. Characterization and expression of NADPH oxidase in LPS-, poly(I:C)- and zymosan-stimulated trout (*Oncorhynchus mykiss*W.) macrophages. *Fish Shellfish Immunol.*, 26(4) (2009), pp. 651-661.
- [73] Currie, G.A. Activated macrophages kill tumour cells by releasing arginase. *Nature*, 273 (1978), pp. 758-759.
- [74] Young, S.H., Ye, J., Frazer, D.G., Shi, X., Castranova, V. Molecular mechanism of tumor necrosis factor- α production in 1 $\rightarrow$ 3- β -glucan (zymosan)-activated macrophages. *Journal of Biological Chemistry*, 276 (2001), pp. 20781-20787.
- [75] Burrells, C., Williams, P.D., Forno, P.F. Dietary nucleotides: A novel supplement in fish feeds. I. Effect on resistance to disease in salmonids. *Aquaculture*, 199 (2001), pp. 159-169.
- [76] Kumari, J. and Sahoo, P.K. Non-specific immune response of healthy and immunocompromised Asian catfish (*Clarias batrachus*) to several immunostimulants. *Aquaculture*, 255 (2006), pp. 133-141.
- [77] Nikl, L., Albright, L.J., Evelyn, T.P. Influence of seven immunostimulants on the immune response of coho salmon to *Aeromonas salmonicida*. *Dis. Aquat. Organ.*, 12 (1991), pp. 7-12.
- [78] Robertsen, B., Rorstad, G., Engstad, R., Raa, J. Enhancement of non-specific resistance in Atlantic salmon, *Salmo salar* L., by a glucan from *Saccharomyces cerevisiae* cell walls. *J. Fish. Dis.*, 13 (1990), pp. 391-400.
- [79] Sahoo, P.K. and Mukherjee, S.C. Effect of dietary β -1,3 glucan on immune response and disease resistance of healthy and aflatoxin B1-induced immunocompromised rohu (*Labeo rohita* Hamilton). *Fish Shellfish Immunol.*, 11 (2001), pp. 683-695.
- [80] Selvaraj, V., Sampath, K., Sekar, V. Administration of yeast glucan enhances survival and some non-specific and specific immune parameters in carp (*Cyprinus carpio*) infected with *Aeromonas hydrophila*. *Fish Shellfish Immunol.*, 9 (2005), pp. 293-306.
- [81] Chang, C.S., Huang, S.L., Chen, S., Chen, S.N. Innate immune responses and efficacy of using mushroom beta-glucan mixture (MBG) on orange-spotted

grouper, *Epinephelus coioides*, aquaculture. Fish Shellfish Immunol., 35 (2013), pp. 115-125.

[82] Kawakami, H., Shinohara, N., Sakai, M. The non-specific immunostimulation and adjuvant effects of *Vibrio anguillarum* bacterin, M-glucan, chitin and Freund's complete adjuvant against *Pasteurella piscicida* infection in yellowtail. Fish Pathol., 33 (1998), pp. 287-292.

[83] Paredes, M., Gonzalez, K., Figueroa, J., Montiel-Eulefi, E. Immunomodulatory effect of prolactin on Atlantic salmon (*Salmo salar*) macrophage function. Fish Physiol. Biochem., 39 (2013), pp. 1215-1221.

[84] Verlhac, V., Obach, A., Gabaudan, J., Schuep, W., Hole R. Immunomodulation by dietary vitamin C and glucan in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol., 8 (1998), pp. 409-424.

Figure legends

Fig. 1. Survival rates of fish. Fish were intraperitoneally injected with β -glucans (1 mg/ml) and, after one week, 50 μ L of 5×10^4 TCID₅₀/ml VHSV per fish (A) and with 50 μ L of *A. salmonicida* using a dose of 2×10^7 CFU/ml per fish (B). Kaplan-Meier cumulative survival curves were analyzed for statistical significance with the log-rank (Mantel-Cox) test. Data are presented as the means $\pm$ SEM of three independent experiments, and significant differences are displayed as *($p \leq 0.05$), **($p \leq 0.01$) or *** ($p \leq 0.001$).

Fig. 2. Levels of glucose, lactate and ATP in plasma, blood cells, head kidney and liver of turbot intraperitoneally injected with PBS (control) or PBS containing 1 mg/ml of β -glucans and, after one week, 50 μ L of 5×10^4 TCID₅₀/ml VHSV per fish. Fish were sampled one day after VHSV injection. Each value represents the mean + SEM of $n=8$ fish per treatment. Different letters indicate significant differences ($P<0.05$) among treatments.

Fig. 3. Levels of glucose, lactate and ATP in plasma, blood cells, head kidney and liver of turbot intraperitoneally injected with PBS (control) or PBS containing 1 mg/ml of β -glucans and, after one week, 50 μ L of *A. salmonicida* using a dose of 2×10^7 CFU/ml per fish. Fish were sampled one day after *A. salmonicida* injection. Each value

represents the mean + SEM of n=8 fish per treatment. Different letters indicate significant differences ($P < 0.05$) among treatments.

Fig. 4. Relative mRNA abundance of LDH-A (A), PK (B), FAT/CD36 (C), and FAS (D) in head kidney of turbot 8 days after β -glucan treatment (1 mg/ml) and one day after VHSV (50 μ L of 5×10^4 TCID₅₀/ml) infection. Data represent the mean + SEM of 5 measurements. The results refer to the control group and are normalized to elongation factor 1 α expression. Different letters indicate significant differences ($P < 0.05$) among treatments.

Fig. 5. Relative mRNA abundance of LDH-A (A), PK (B), FAT/CD36 (C), and FAS (D) in head kidney of turbot 8 days after β -glucan (1 mg/ml) treatment and one day after *A. salmonicida* infection (50 μ L 2×10^7 CFU/ml per fish). Data represent the mean + SEM of 5 measurements. The results refer to the control group and are normalized to elongation factor 1 α expression. Different letters indicate significant differences ($P < 0.05$) among treatments.

Fig. 6 Survival rates of fish. Turbot were treated with β -glucans (100 μ g/ml) by immersion and, one week later, intraperitoneally injected with 50 μ L of *A. salmonicida* using a dose of 2.7×10^7 CFU/ml per fish (A). Turbot were treated intraperitoneally with different concentrations of β -glucans (1 mg/ml, 5 mg/ml and 10 mg/ml) and Zymosan (1 mg/ml and 5 mg/ml) and, one week later, intraperitoneally injected with 50 μ L of *A. salmonicida* using a dose of 2.3×10^7 CFU/ml per fish (B). Fish were intraperitoneally injected with β -glucans (1 mg/ml) and, 25 days later, 50 μ L of *A. salmonicida* using a dose of 2.7×10^7 (C). Turbot were intraperitoneally injected with β -glucans (1 mg/ml), 2-DG (500 mg/ml) or β -glucans+2-DG and, one week later, 50 μ L of *A. salmonicida* using a dose of 1.7×10^7 CFU/ml per fish (D). Kaplan-Meier cumulative survival curves were analyzed for statistical significance with the log-rank (Mantel-Cox) test. Data represent the means $\pm$ SEM of three independent experiments, and significant differences are displayed as *($p \leq 0.05$), **($p \leq 0.01$) or *** ($p \leq 0.001$).

Table 1. Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by real time-PCR (RT-qPCR).

Gene	Forward primer	Reverse primer
EF-1 α	GGAGGCCAGCTCAAAGATGG	ACAGTTCCAATACCGCCGATTT
FAS	ATTCTGGGTGTGCGCGATGT	TGTTGATGGTGAGCTGGCGG
FAT/CD36	AGGAGGTTTTGCAGTCCGGC	AGCCAGGTTGAGGGAGGTGA
LDH-A	AAGATCATGGCCGACAAAGACT	CTTGAAAATGTTGACGTTGCGC
PK	GACACGTGGCAGCTTTGAGG	TGATCACACATGCGAGCCCT

827 EF1 α , elongation factor 1 α (house-keeping gene); FAS, fatty acid synthetase;
828 FAT/CD36, fatty acid translocase; LDH- A, lactate dehydrogenase A; PK, pyruvate
829 kinase.

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Highlights

- β -glucans, VHSV and *A. salmonicida* induce metabolic changes in turbot.
- β -glucans induce different changes in viral and bacterial infections.
- Fish treated with β -glucans are not protected against the infection with VHSV
- β -glucans have great therapeutic potential against bacterial disease.