



Effects of Vitamin D₂ (Ergocalciferol) and D₃ (Cholecalciferol) on Atlantic Salmon (*Salmo salar*) Primary Macrophage Immune Response to *Aeromonas salmonicida* subsp. *salmonicida* Infection

Manuel Soto-Dávila¹, Katherine Valderrama¹, Sabrina M. Inkpen², Jennifer R. Hall³, Matthew L. Rise² and Javier Santander^{1*}

¹ Marine Microbial Pathogenesis and Vaccinology Lab, Department of Ocean Sciences, Memorial University of Newfoundland, St. John's, NL, Canada, ² Department of Ocean Sciences, Memorial University of Newfoundland, Ocean Science Centre, St. John's, NL, Canada, ³ Aquatic Research Cluster, CREAT Network, Ocean Sciences Centre, Memorial University of Newfoundland, St. John's, NL, Canada

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*Correspondence:

Javier Santander
jsantander@mun.ca

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Vitamin D₂ (ergocalciferol) and vitamin D₃ (cholecalciferol) are fat-soluble secosteroid hormones obtained from plant and animal sources, respectively. Fish incorporates vitamin D₂ and D₃ through the diet. In mammals, vitamin D forms are involved in mineral metabolism, cell growth, tissue differentiation, and antibacterial immune response. Vitamin D is an essential nutrient in aquafeeds for finfish. However, the influence of vitamin D on fish cell immunity has not yet been explored. Here, we examined the effects of vitamin D₂ and vitamin D₃ on *Salmo salar* primary macrophage immune response to *A. salmonicida* subspecies *salmonicida* infection under *in vitro* conditions. We determined that high concentrations of vitamin D₂ (100,000 ng/ml) and D₃ (10,000 ng/ml) affect the growth of *A. salmonicida* and decrease the viability of *S. salar* primary macrophages. In addition, we determined that primary macrophages pre-treated with a biologically relevant concentration of vitamin D₃ for 24 h showed a decrease of *A. salmonicida* infection. In contrast, vitamin D₂ did not influence the antibacterial activity of the *S. salar* macrophages infected with *A. salmonicida*. Vitamin D₂ and D₃ did not influence the expression of canonical genes related to innate immune response. On the other hand, we found that *A. salmonicida* up-regulated the expression of several canonical genes and suppressed the expression of *leukocyte-derived chemotaxin 2 (lect-2)* gene, involved in neutrophil recruitment. Primary macrophages pre-treated for 24 h with vitamin D₃ counteracted this immune suppression and up-regulated the transcription of *lect-2*. Our results suggest that vitamin D₃ affects *A. salmonicida* attachment to the *S. salar* primary macrophages, and as a consequence, the *A. salmonicida* invasion decreased. Moreover, our study shows that the positive effects of vitamin D₃ on fish cell immunity seem to be related to the *lect-2* innate immunity mechanisms. We did not identify positive effects of

vitamin D₂ on fish cell immunity. In conclusion, we determined that the inactive form of vitamin D₃, cholecalciferol, induced anti-bacterial innate immunity pathways in Atlantic salmon primary macrophages, suggesting that its utilization as a component of a healthy aquafeed diet in Atlantic salmon could enhance the immune response against *A. salmonicida*.

Keywords: Atlantic salmon, vitamin D₃, vitamin D₂, innate immunity, primary macrophages, *Aeromonas salmonicida*, Gram-negative

INTRODUCTION

Vitamin D is a fat-soluble secosteroid hormone that plays a crucial role in calcium and phosphorus homeostasis, cardiovascular physiology, cell proliferation and differentiation, among other functions (1–6). In fish, vitamin D is involved in the endocrine control of calcium and phosphorus homeostasis, similar to mammals (4). Also, it has been shown that vitamin D can act as an immunomodulatory agent in mammals (7, 8).

In contrast to terrestrial vertebrates, fish are not able to obtain vitamin D through the photochemical pathway, thus fish must ingest vitamin D from dietary sources (9). In freshwater and marine environments, the main dietary sources of vitamin D are phytoplankton and zooplankton (9). Phytoplankton provide fish with vitamin D₂ (ergocalciferol), while zooplankton provide them with vitamin D₃ (cholecalciferol) (10).

The beneficial stimulatory effects of vitamins D₂ and D₃ on innate immunity have been described in humans and other mammals (5, 8, 11–14). The positive effects of vitamins in fish are well-established, and currently vitamins D₂ and D₃ are essential components of aquafeed diets (4). Additionally, vitamin D is utilized as an adjuvant in aqua-vaccine preparations (15). However, the role of vitamin D in fish physiology is still enigmatic, and the immune stimulant mechanisms against infectious diseases are unknown.

Atlantic salmon (*Salmo salar*) is a high-value cultured finfish species, and the main species cultured in Canada, Chile, UK, and Norway (16–18). Infectious diseases caused by bacterial pathogens such as *Renibacterium salmoninarum*, *Piscirickettsia salmonis*, *Vibrio anguillarum*, and *Aeromonas salmonicida* subsp. *salmonicida* (19–24), have affected this industry since its origin (25, 26). Currently, several measures are utilized to prevent infectious diseases in the Atlantic salmon aquaculture industry, including a healthy diet that includes immunostimulants (27–29).

Functional constituents of healthy aquafeed diets like essential nutrients (such as vitamins, probiotics, prebiotics, and immunostimulants) are currently being considered to improve not only fish growth and stress tolerance, but also the resistance to diseases by enhancing non-specific defense mechanisms (30, 31). These essential nutrients are capable of directly activating immune mechanisms, such as phagocytic activity (e.g., macrophages and neutrophils), complement system, lysozyme activity, and others (32–34). Phagocytosis is an active host defense mechanism, involving the action of monocytes, dendritic cells, neutrophils, and macrophages (34–36). From these phagocytic leukocytes, the macrophages play an important

role linking the innate and adaptive immune responses, and previous studies have shown that immunostimulants (i.e., fructooligosaccharides, mannanoligosaccharides) can successfully enhance the phagocytic activity in rainbow trout and European sea bass (34, 37, 38).

Immunostimulants are natural compounds that have been shown to be safe and effective for fish (30, 33, 39). In humans, vitamin D plays an important role in the suppression of pro-inflammatory cytokines such as *il-17*, *il-1b*, and *tnf-α* in individuals affected by type 2 diabetes and autoimmune diseases, preventing chronic inflammation (40, 41). Additionally, vitamin D significantly reduces *Staphylococcus aureus* infection in pre-treated bovine epithelial cells and modulates the expression of innate immune related genes (8, 13, 14). These studies suggest that vitamin D could trigger similar protective effects in fish. Here, we evaluate the effects of vitamins D₂ and D₃ on the innate immune responses of Atlantic salmon primary macrophages to *A. salmonicida* infection.

MATERIALS AND METHODS

Aeromonas salmonicida Growth Conditions

A. salmonicida was grown in accordance to the protocol used by Soto-Dávila et al. (42). Briefly, a single colony of *A. salmonicida* J223 (24) was incubated in 3 ml of Trypticase Soy Broth (TSB, Difco, Franklin Lakes, NJ) at 15°C in a 16 mm diameter glass tube and placed in a roller for 24 h. After growth, 300 μl of the overnight culture was added to 30 ml of TSB media using a 250 ml flask and incubated for 24 h at 15°C with aeration (180 rpm). After bacteria reached an O.D. 600 nm ~0.7 (1 × 10⁸ CFU ml⁻¹), the bacterial culture was centrifuged at 6,000 rpm at room temperature for 10 min. The pellet was washed twice with phosphate buffered saline [PBS; 136 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄ (pH 7.2)] and centrifuged at 6,000 rpm at room temperature for 5 min, and finally resuspended in 300 μl of PBS (~5 × 10¹⁰ CFU ml⁻¹). The concentrated bacterial inoculum was serially diluted and quantified by plating onto TSA supplemented with Congo red (50 μg ml⁻¹) for 4 days.

Vitamin D₂ and D₃ Inhibitory Effects on *A. salmonicida* Growth

To determine whether vitamin D₂ or vitamin D₃ have inhibitory effect on *A. salmonicida* growth, 30 μl of the overnight growth bacteria were placed in 3 ml of TBS containing different

concentrations of vitamin D₂ (10; 100; 1,000; 10,000; and 100,000 ng ml⁻¹; Sigma-Aldrich) or vitamin D₃ (10; 100; 1,000; and 10,000 ng ml⁻¹; Sigma-Aldrich). Bacterial growth was measured by O.D. 600 nm until 48 h. The effect of each vitamin concentration on *A. salmonicida* growth was measured in triplicate, and TSB media containing the respective vitamin D concentration was utilized as a blank.

Fish Holding

Adult specimens of Atlantic salmon 4.0 ± 0.1 kg (mean ± SE) were obtained from the Dr. Joe Brown Aquatic Research Building (JBARB) at the Department of Ocean Sciences, Memorial University of Newfoundland, Canada. The animals were kept in 37 m³ tanks, with flow-through (100 l min⁻¹) seawater (6.5°C) and ambient photoperiod. The individuals were fed twice per day with commercial salmonid dry pellets (Skretting Optiline Microbalance 3000 EP, 12.0 mm pellets: 38% protein, 33% fat, 1.6% calcium, 1.5% fiber, 1% phosphorus) with a ration of 0.5% of body weight per day. The experiment was performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by Memorial University of Newfoundland's Institutional Animal Care Committee (protocols #17-01-JS; #17-02-JS).

Macrophage Isolation

Primary macrophages were isolated from Atlantic salmon head kidney. Tissues from 6 fish were aseptically removed and individually minced through 100 μm nylon sterile cell strainers (Fisher Scientific, Thermo Fisher Scientific, Waltham, MA, USA) in isolation media [(Leibovitz-15 (Gibco®), Gran Island, NY, USA) supplemented with 250 μg ml⁻¹ heparin, 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, and 0.1% Fetal Bovine Serum (FBS)]. After this period, 4 ml of cell suspension were centrifuged (1,000 × g at 4°C) for 30 min in a 34/51% Percoll gradient (GE Healthcare, Uppsala, Sweden). Macrophages collected from the macrophage-enriched interface were washed with PBS twice and the number and viable cells were determined using the Countess™ cell counter (Invitrogen), and trypan blue stain (Invitrogen). After determining the cell concentration (number of cells per ml⁻¹) of each sample, the primary macrophages were seeded in 22 mm 12-well or 35 mm 6-well cell-culture multidishes (Thermo Scientific, Roskilde, Denmark) at a concentration of 1 × 10⁷ cells ml⁻¹. The plates were incubated at 15°C for 24 h in isolation media. After this period the cells were washed with PBS and incubated at 15°C for an additional 4 days in 1 ml of culture media (Leibovitz-15 (Gibco®), supplemented with 0.1% 2-Mercaptoethanol, 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, and 5% FBS) to allow cell attachment until the assays were performed.

Vitamin D₂ and D₃ Toxicity in Atlantic Salmon Primary Macrophages

Atlantic salmon primary macrophages were seeded in 12-well cell-culture multidishes at a concentration of 1 × 10⁷ cells ml⁻¹. After 4 days of incubation the culture media was removed, cells washed with PBS, and 1 ml of culture media containing different concentrations of vitamin D₂ (10; 100; 1,000; 10,000; and 100,000

ng ml⁻¹) or vitamin D₃ (10; 100; 1,000; and 10,000 ng ml⁻¹) was added. Twenty-four hours and 48 h post-vitamin treatment, cells were treated with 500 μl of trypsin-EDTA (0.5%; Gibco) for 10 min, and then trypsin was inactivated with 500 μl of culture media. The cells were stained with trypan blue (0.4%; Invitrogen) in a ratio of 1:1 (10 μl: 10 μl) and quantified using Countess™ Cell Counting Chamber Slides (Invitrogen) and Countess® Automated Cell Counter (Invitrogen) according to the manufacturer's instructions. Viability of cells was determined for each vitamin D₂ and D₃ concentration and the control group. All samples were taken from 6 individual fish.

Gentamicin Exclusion Assay

Infections of primary macrophages with *A. salmonicida* were performed according to the protocol used by Soto-Dávila et al. (42) with modifications. Briefly, after 4 days, the primary macrophages were washed with 1 ml of PBS and inoculated with 1 ml of culture media without antibiotics containing either 100 ng ml⁻¹ of vitamin D₂ or D₃ for 24 h. After this period, media was removed, cells washed with 1 ml of PBS, and pre-treated primary macrophage monolayers were infected with 10 μl of bacterial suspension [~1 × 10⁷ cells ml⁻¹; Multiplicity of Infection (MOI) 1:1 (bacteria:macrophage)] and incubated at 15°C. For attachment, after 1 h of infection, the infected primary macrophage monolayers were washed twice with 1 ml of PBS and then lysed using 400 μl of Triton X100 (0.01%; Sigma) for 10 min (43). After this 600 μl of PBS was added to complete 1 ml of lysed macrophage suspension. Then, the lysed macrophage suspensions were serially diluted (1:10) and plate/counted on TSA plates supplemented with Congo red to determine the number of viable *A. salmonicida* per monolayer. The plates were incubated at 15°C for 5 days to determine the CFU per well.

For invasion, the primary macrophages were infected for 1 h, washed twice with 1 ml of PBS, and 1 ml of fresh culture media supplemented with gentamicin (10 μg ml⁻¹, a higher concentration than the minimal inhibitory concentration) (24) was added. Gentamicin treatment was applied to kill remaining extracellular bacteria. After 2, 3, and 4 h of infection, the infected primary macrophage monolayers were washed twice with PBS and then lysed using 400 μl of Triton X100 (0.01%) for 10 min (43). After this, 600 μl of PBS was added to complete 1 ml of lysed macrophage suspension. Then, the lysed macrophage suspensions were serially diluted (1:10) and plate/counted on TSA plates supplemented with Congo red to determine the number of viable intracellular *A. salmonicida* per monolayer. The plates were incubated at 15°C for 5 days to determine the CFU per well. All samples were taken from 6 individual fish.

Vitamin D₂ and D₃ Pre-treated Primary Macrophage Viability After *A. salmonicida* Infection

To determine the viability of infected primary macrophages, the cells were seeded in 12 wells plates, pre-treated with 100 ng ml⁻¹ of vitamin D₂ or vitamin D₃, infected with *A. salmonicida*, and processed following the method used during the gentamicin exclusion assay. Cells were washed with 1 ml of PBS and then

treated with 500 μ l of trypsin-EDTA (0.5%; Gibco) for 10 min. After this period, the trypsin was inactivated with 500 μ l of culture media. The primary macrophages were stained using trypan blue (0.4%; Invitrogen) in a ratio of 1:1 (10 μ l: 10 μ l) and quantified using Countess™ Cell Counting Chamber Slides (Invitrogen) and the Countess® Automated Cell Counter (Invitrogen) according to the manufacturer's instructions. The numbers of alive and dead cells were determined at each time point post-infection. All the primary macrophages were isolated from 6 individual fish and technical triplicates were utilized.

RNA Extraction

RNA samples were obtained from head kidney primary macrophages inoculated with either PBS; live *A. salmonicida* (J223); formalin-killed *A. salmonicida* (FK J223); 100 ng ml⁻¹ vitamin D₂ or D₃; 1,000 ng ml⁻¹ vitamin D₂ or D₃, or 100 ng ml⁻¹ vitamin D₂ or D₃ + live *A. salmonicida* (J223). The treatments that included vitamin D (D₂ or D₃) were pre-treated with the respective concentration 24 h before the challenge, meanwhile treatments without vitamin D were pre-treated only with the control vehicle 24 h before the challenge. Each sample was obtained 3 h post-inoculation.

Total RNA from Atlantic salmon primary macrophages was extracted using 1 ml of TRIzol Reagent (Invitrogen), and purified using the RNeasy® Mini Kit (QIAGEN) following the manufacturer's instructions (44). RNA samples were treated with 2 U of TURBO DNase (TURBO DNA-free™ Kit, Invitrogen) following the manufacturer's instructions to degrade any residual genomic DNA. Briefly, samples were incubated at 37°C for 30 min, 2.5 μ l of DNase Inactivation Reagent was added, and samples incubated 5 min at room temperature. Then, samples were centrifuged at 10,000 x g for 1.5 min and the supernatant containing the RNA carefully transferred to a new tube. Purified RNA samples were quantified and evaluated for purity (A260/280 and A260/230 ratios) using a Nano-quant spectrophotometer (Genway, UK), and evaluated for integrity using 1% agarose gel electrophoresis (45). Column purified RNA samples had A260/280 ratios between 1.9 and 2.1 and A260/230 ratios between 1.9 and 2.2. A PCR test was conducted using the reference genes' primers [*60S ribosomal protein L32* (*rpl32*) and *β -actin*] and the RNA as template to rule out the presence of DNA. All RNA samples did not show presence of DNA.

First-strand cDNA templates for qPCR were synthesized from 500 ng of DNaseI-treated, column-purified total RNA using SuperScript™ IV VILO™ Master Mix (Invitrogen) following the manufacturer's instructions. Each sample was incubated at 25°C for 10 min, at 50°C for 10 min, and at 85°C for 5 min.

Gene Parologue Discovery and qPCR

All qPCR reactions were performed in a 20 μ l reaction, containing 1 \times PowerUp SYBR Green Master Mix (Applied BioSystems, Foster City, CA, USA), 500 nM (final concentration) of both the forward and reverse primer and the indicated cDNA quantity. All samples were amplified and detected using the QuantStudio 3 Real Time PCR System (Applied BioSystems). The

reaction mixtures were incubated for 2 min at 50°C, then 2 min at 95°C, followed by 40 cycles of 1 s at 95°C, 30 s at 60°C, and finally 15 s at 95°C, 1 min at 60°C, and 15 s at 95°C.

The primer sequences of *interleukin 1 beta* (*il-1b*), *interleukin 8* (*il-8*), *tumor necrosis factor alpha* (*tnf- α*), *soluble toll-like receptor 5* (*stlr5*), and *leukocyte-derived chemotaxin 2* (*lect-2*) are listed in **Table 1**. Gene paralogue discovery, qPCR primer design and initial quality testing were performed as described in Caballero-Solares et al. (49). Since the reagents, cycling conditions and samples were different in the current study, primer efficiencies (**Table 1**) were reassessed. Briefly, a 7-point 1:3 dilution series starting with cDNA representing 40 ng of input total RNA was generated, and efficiencies then calculated using the formula $E=10^{(-1/\text{slope})}$ (50).

Transcripts levels of the genes of interest (*il-1b*, *il-8*, *tnf- α* , *stlr5*, and *lect-2*) were normalized to transcript levels of two endogenous control genes. Levels of five candidate normalizers (*60S ribosomal protein 32*; *β -actin*, 18S, *elongation factor 1 alpha*, and *hypoxanthine phosphoribosyl transferase 1*) were assessed in 50% of the samples (i.e., in 3 random samples per treatment) using cDNA representing 40 ng of input total RNA. Reference gene stability was then analyzed using both geNorm and BestKeeper (**Supplementary Table 1**). Both analyses identified *β -actin* (geNorm M = 0.592; BestKeeper value = 0.263) and *60S ribosomal protein L32* (geNorm M = 0.592; BestKeeper value = 0.364) and as the most stably expressed genes (**Supplementary Table 1**).

After normalizer testing was completed, transcript levels of the genes of interest were analyzed in the individual study samples, with normalization to both *β -actin* and *60S ribosomal protein L32*. In all cases, levels were assessed (in triplicate) in six individuals per treatment using cDNA representing 40 ng of input total RNA. On each gene a no RT control was included. Gene expression was determined using the comparative 2^{- $\Delta\Delta$ Ct} method (51).

Phagocytosis Assay

The phagocytosis assay was performed following the protocol used by Smith et al. (39) with modifications. Cells were incubated for 3 days and inoculated with vehicle control (2 μ l of ethanol in 1 ml of culture media without antibiotics), 100; 1,000; or 10,000 ng ml⁻¹ of either vitamin D₂ or D₃ for 24 h. After this time, cells were washed twice with PBS, and inoculated with 1 μ m of Fluoresbrite YG microspheres at a ratio of ~1:30 macrophage:microsphere (Polysciences, Warrington, PA, USA) (39, 52). Twenty-four hours after microsphere addition, primary macrophages were washed with PBS and posteriorly cells treated with trypsin-EDTA (0.5%; Gibco) for 10 min. Then, cells were resuspended in 500 μ l of FACS buffer (PBS + 1% FBS). Fluorescence was detected from 10,000 cells using a BD FACS Aria II flow cytometer and analyzed using BD FACS Diva v7.0 software (BD Biosciences, San Jose, CA, USA). The control pre-treated macrophages were used to compare with the FITC positive cells in vitamin D pre-treated cells. Percentages of FITC positive cells were determined for each condition. The experiments were conducted using macrophages isolated from 3 independent fish.

TABLE 1 | Primers used in qPCR studies.

Gene name (symbol) (GenBank acc. no.)	Nucleotide sequence (5'-3')	°Efficiency (%)	Amplicon size (bp)	References
interleukin 1 beta (<i>il-1b</i>) (AY617117)	F: GTATCCCATCACCCATCAC R: TTGAGCAGGTCCTTGCTCTT	99.7	119	This study
interleukin 8 (<i>il-8</i>) (BT046706)	F: GAAAGCAGACGAATTGGTAGAC R: GCTGTTGCTCAGAGTTGCAAT	100.7	99	This study
tumor necrosis factor alpha (<i>tnf-α</i>) (AY929386)	F: GGATGGAATGGAGCATCAGC R: TGCACGGTGTAGCGGTAAG	106.4	141	(39)
leukocyte cell derived chemotaxin 2 (<i>lect-2</i>) (BT059281)	F: CAGATGGGGACAAGGACACT R: GCCTTCTTCGGGTCTGTGTA	101.1	150	(39)
toll-like receptor 5 (soluble) (<i>stlr5</i>) (AY628755)	F: ATCGCCCTGCAGATTTTATG R: GAGCCCTCAGCGAGTTAAAG	94.1	103	(39)
^a β-actin (<i>actb</i>) (BG933897)	F: CCAAAGCCAACAGGGAGAAG R: AGGGACAACACTGCCTGGAT	104.4	91	(46)
^a 60S ribosomal protein L32 (<i>rpl32</i>) (BT043656)	F: AGGCGGTTTAAAGGTCAGAT R: TCGAGCTCCTTGATGTTGTG	100.7	119	(46)
^b 18S (<i>18S</i>)	F: GTCCGGGAAACCAAAGTC R: TTGAGTCAAATTAAGCCGCA	91.0	Not provided	(47)
^b elongation factor 1 alpha (<i>EF-1α</i>) (AF321836)	F: TGGCACTTTCACTGCTCAAG R: CAACAATAGCAGCGTCTCCA	96.3	197	(39)
^b hypoxanthine phosphoribosyl transferase 1 (<i>HPRT1</i>) (EG866745)	F: CCGCCTCAAGAGCTACTGTAAT R: GTCTGGAACCTCAAACCTATG	94.7	255	(48)

^aNormalizers used in experimental qPCR analyses.

^bCandidate normalizer genes.

^cAmplification efficiencies were calculated using a 7-point 1:3 dilution series starting with cDNA representing 40 ng of input total RNA. See methods for details.

Statistical Analysis

All data are shown as the mean ± standard error (SE). Assumptions of normality and homoscedasticity were tested for the detected variances. A Kruskal-Wallis non-parametric test was performed for *A. salmonicida* growth curve and gene expression results. Macrophages viability, gentamicin exclusion assay, and phagocytosis assay data were analyzed using a repeated measures two-way ANOVA test, followed by Sidak multiple comparisons *post hoc* test to identify significant differences of each treatment in different times or concentrations and between treatments in the same time point. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla California USA, www.graphpad.com).

RESULTS

Inhibitory Effects of Vitamin D₂ and D₃ on *A. salmonicida* Growth

Growth of *A. salmonicida* at different concentrations of vitamin D₂ and D₃ was determined by O.D. 600 nm at different time points until 48 h. Bacteria growth was not affected in concentrations of 10, 100, 1,000, and 10,000 ng ml⁻¹ of vitamin D₂ (vitamin D₂) (Figure 1A). In contrast, *A. salmonicida* growth was significantly reduced in the presence of 100,000 ng ml⁻¹ of vitamin D₂ (Figure 1A). *A. salmonicida* growth was not affected by 10 and 100 ng ml⁻¹ of vitamin D₃ (vitamin D₃) (Figure 1B).

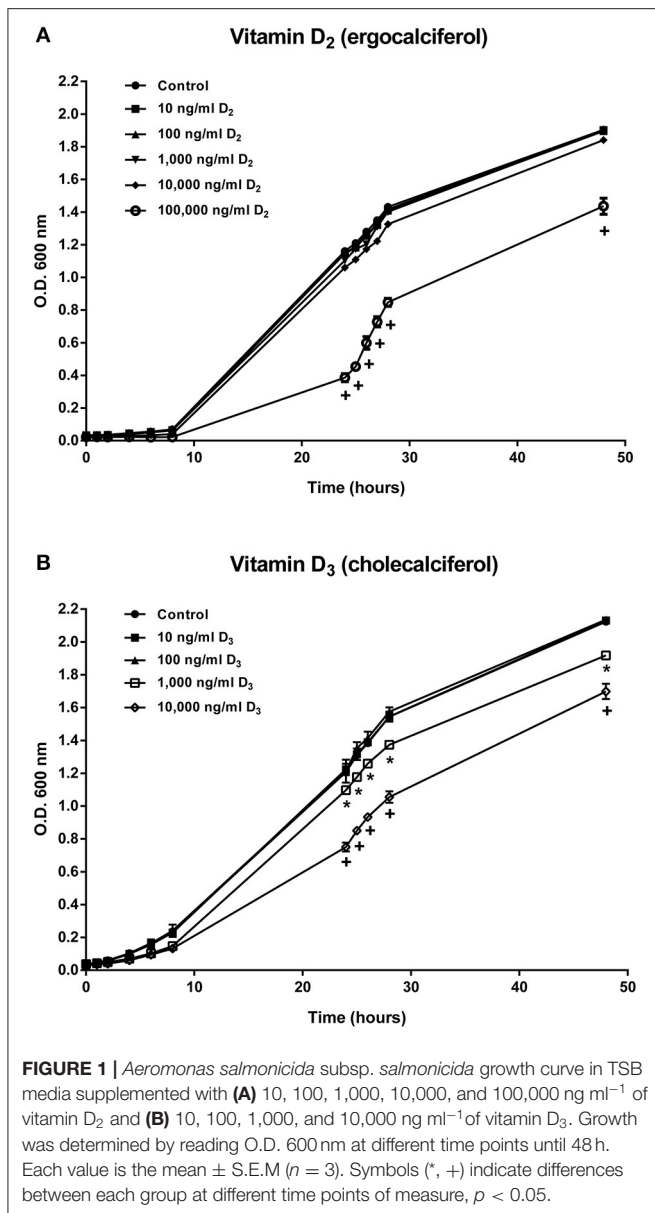
However, *A. salmonicida* growth was significantly affected by 1,000 and 10,000 ng ml⁻¹ of the vitamin D₃ (Figure 1B).

Evaluation of the Toxicity of Vitamin D₂ and D₃ in Atlantic Salmon Primary Macrophages

Atlantic salmon primary macrophage viability was determined after 24 and 48 h of exposure to concentrations of 0, 10, 100, 1,000, 10,000, and 100,000 ng ml⁻¹ of vitamin D₂. Results obtained did not show significant differences in viability between the control group, compared with the cells treated with 10, 100, 1,000, and 10,000 ng ml⁻¹ of vitamin D₂. However, a significant difference was observed in cells inoculated with the media containing a concentration of 100,000 ng ml⁻¹ of vitamin D₂ after 24 h ($1.67 \times 10^4 \pm 6.67 \times 10^3$) and 48 h ($6.67 \times 10^3 \pm 6.67 \times 10^3$) (Figure 2A).

The percentage of viability did not show significant differences between the control group and the primary macrophages treated with 10, 100, 1,000, and 10,000 ng ml⁻¹ of vitamin D₂. Nevertheless, a highly significant decrease in macrophage viability was observed at a concentration of 100,000 ng ml⁻¹ of vitamin D₂ after 24 h ($13.33 \pm 4.48\%$) and 48 h ($5.67 \pm 5.67\%$) (Figure 2B).

The viability of Atlantic salmon primary macrophages was also determined at 24 and 48 h post-treatment with vitamin D₃ in concentrations of 10, 100, 1,000, and 10,000 ng ml⁻¹. The number of live cells per ml did not show significant differences



in primary macrophages treated with 10, 100, and 1,000 ng ml⁻¹ (Figure 2C). Moreover, no significant differences were observed in the viability of primary macrophages treated with 10,000 ng ml⁻¹ of vitamin D₃ after 24 h compared with the control (Figure 2C). Nevertheless, in cells exposed to 10,000 ng ml⁻¹ of vitamin D₃, a significant decrease in the viability was observed after 48 h of treatment ($2.85 \times 10^5 \pm 1.44 \times 10^4$) (Figure 2C).

The percentage of viability in vitamin D₃ treated cells did not show significant differences after 24 h of exposure to concentrations of 10, 100, 1,000, and 10,000 ng ml⁻¹ compared with the control group (Figure 2D). Also, no significant differences were observed in Atlantic salmon macrophages treated during 48 h with vitamin D₃ in a concentration of 10, 100, and 1,000 ng ml⁻¹ compared with the control (Figure 2D). However, a significant decrease was observed in cells incubated

with a concentration of 10,000 ng ml⁻¹ of vitamin D₃ compared with the control and all the lower doses of vitamin D₃ at the 48 h time point ($59.00 \pm 1.15\%$) (Figure 2D). Moreover, in the group incubated at a concentration of 1,000 ng ml⁻¹ of vitamin D₃, a significant higher percentage of viability was observed in cells treated for 48 h compared with the 24 h group (Figure 2D). Also, in the primary macrophages treated with 10,000 ng ml⁻¹ of vitamin D₃, a significant difference was observed at different times, showing a decrease in the viability of cells treated for 48 h with vitamin D₃ compared with the cells incubated for only 24 h (Figure 2D).

Gentamicin Exclusion Assay in Vitamin D₂ and D₃ Pre-treated Cells Infected With *A. salmonicida*

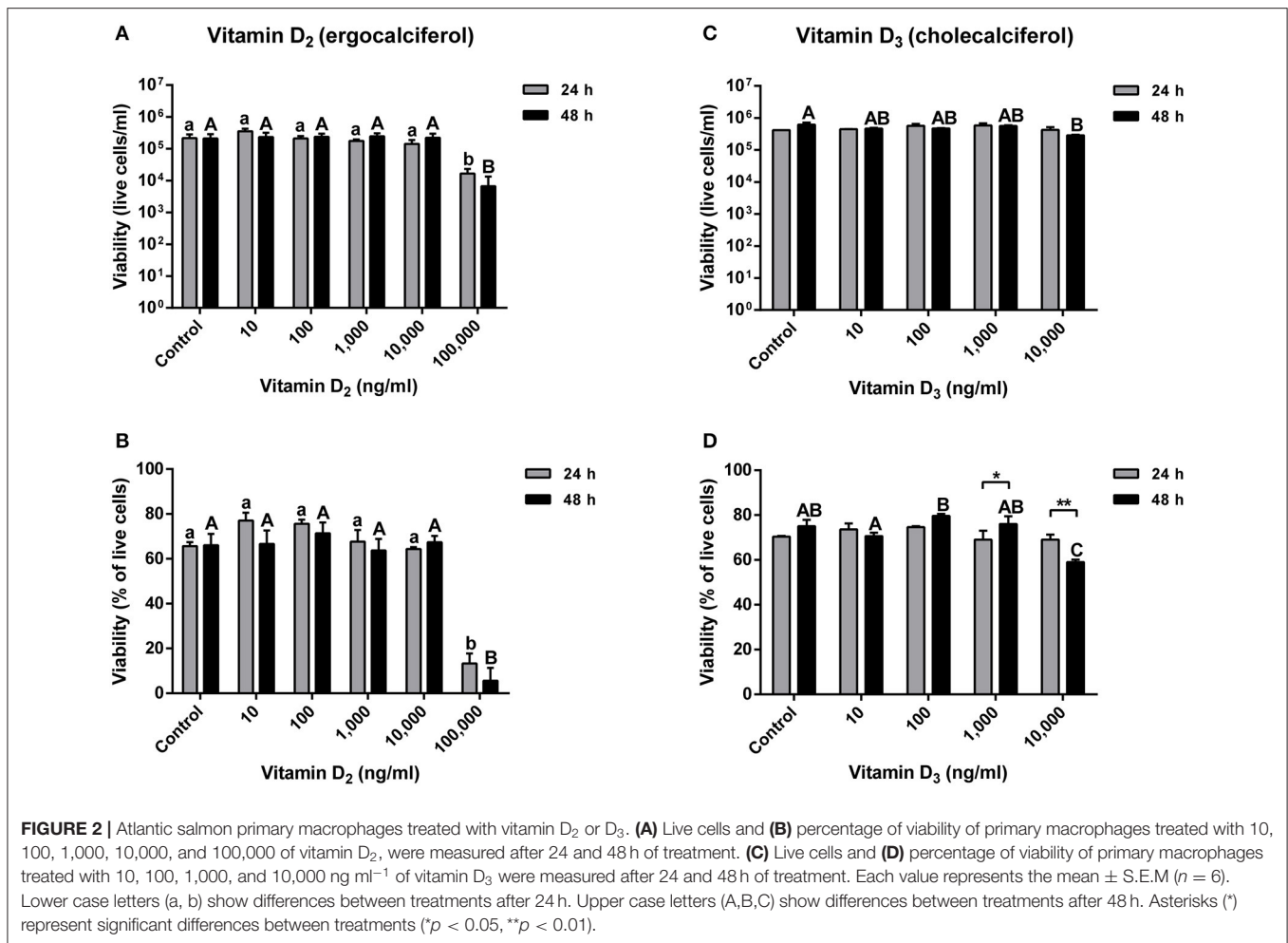
The effects of vitamins D₂ and D₃ on the growth of *A. salmonicida* (Figure 1) and the effects on the viability of primary macrophages (Figure 2) were used to determine the concentration to be utilized in the gentamicin exclusion assays. Based on these results, the primary macrophages were pre-treated with 100 ng ml⁻¹ (vitamin D₂ or D₃) for the gentamicin exclusion assay.

Cells pre-treated for 24 h with 100 ng ml⁻¹ of vitamin D₂ and then infected with *A. salmonicida*, did not show significant differences in cell numbers at 1, 2, 3, and 4 h post-infection compared with the control group (Figure 3A). Also, no significant differences were observed in the viability of primary macrophages pre-treated and then infected with *A. salmonicida* at 1, 2, 3, and 4 h post-infection compared with the control group (Figure 3B).

The primary macrophages were infected with a total of 4.3×10^6 CFU per ml at a MOI of 1. The percentage of *A. salmonicida* attached was significantly higher in primary macrophages pre-treated with vitamin D₂ ($49.09 \pm 2.76\%$) compared with the control group ($34.39 \pm 2.12\%$) (Figure 3D). At invasion time-points, after 2 h of infection no significant differences were observed in cells pre-treated with vitamin D₂ compared with the control group at the same time point (Figure 3D). Moreover, no significant differences were observed in the control group and the vitamin D₂ pre-treated primary macrophages after 3 h of infection with *A. salmonicida* (Figure 3D). No significant differences were also found between the control and the vitamin D₂ treatment 4 h post-infection. However, a significant decrease in bacterial invasion was observed between 2 and 4 h in the primary macrophages pre-treated with vitamin D₂ (Figure 3D).

A similar response was obtained in cells pre-treated for 24 h with vitamin D₃ and then infected with *A. salmonicida*. For instance, no significant differences were found in the percentage of viability between the control group after 1, 2, 3, and 4 h of infection and the vitamin D₃ pre-treated macrophages at 1, 2, 3, and 4 h (Figure 3F).

Atlantic salmon primary macrophages pre-treated with vitamin D₃ were infected with a total of 2.56×10^6 bacterial cells per ml⁻¹ (Figure 3G). The percentage of attachment (1 h post-infection) was significantly lower in the primary macrophages pre-treated for 24 h with vitamin D₃ ($10.61 \pm 0.97\%$) compared



with the control group ($51.56 \pm 17.12\%$) (Figure 3H). In contrast, even when a tendency of lower invasion is observed, no significant differences were observed between the control group after 2, 3, and 4 h of infection compared with the fish cells pre-treated with vitamin D₃ (Figure 3H).

Atlantic Salmon Primary Macrophages Gene Expression

Transcript levels of innate immune response-related genes were evaluated by qPCR in Atlantic salmon primary macrophages after 3 h of the aforementioned treatments.

In the experiments conducted for both vitamin D₂ and D₃, a significant increase in the transcript expression of *il-1b* (Figures 4A,F), *il-8* (Figures 4B,G), *tnf-α* (Figures 4C,H), and *stlr5* (Figures 4E,J) was observed in cells inoculated with live *A. salmonicida*, formalin-killed *A. salmonicida*, and in cells pre-treated with either vitamin D₂ or D₃ and then infected, compared to the PBS inoculated primary macrophages. In contrast, no differences in the expression of *il-1b*, *il-8*, *tnf-α*, and *stlr5* were observed in Atlantic salmon cells inoculated only with 100 ng ml⁻¹ or 1,000 ng ml⁻¹ of each vitamin D form compared with the control (Figures 4A–C,E–H,J).

A different pattern was observed in the expression of *lect-2* transcript between both treatments. For instance, in cells pre-treated with vitamin D₂, no significant differences were observed in the expression of *lect-2* in any of the treatments compared with the PBS inoculated primary macrophages (Figure 4D). In contrast, *lect-2* was significantly up-regulated compared with the control in primary macrophages pre-treated with vitamin D₃ and then challenged with *A. salmonicida* (Figure 4I). The primary macrophages treated with live *A. salmonicida*, formalin-killed *A. salmonicida*, 100 ng ml⁻¹ of vitamin D₃, and 1,000 ng ml⁻¹ of vitamin D₃ did not show significant differences in the expression of *lect-2* compared with the control (Figure 4I).

Phagocytosis Assay

Atlantic salmon primary macrophages did not show significant differences in phagocytosis after 24 h of treatment with 100 ng ml⁻¹ of vitamin D₂ ($4.80 \pm 1.62\%$) and vitamin D₃ ($4.93 \pm 1.56\%$), 1,000 ng ml⁻¹ of vitamin D₂ ($3.33 \pm 0.67\%$) and vitamin D₃ ($2.67 \pm 0.41\%$), and 10,000 ng ml⁻¹ of vitamin D₂ ($0.97 \pm 0.20\%$) and vitamin D₃ ($0.87 \pm 0.09\%$) compared with the control cells ($4.80 \pm 1.08\%$) (Supplementary Figure 1).

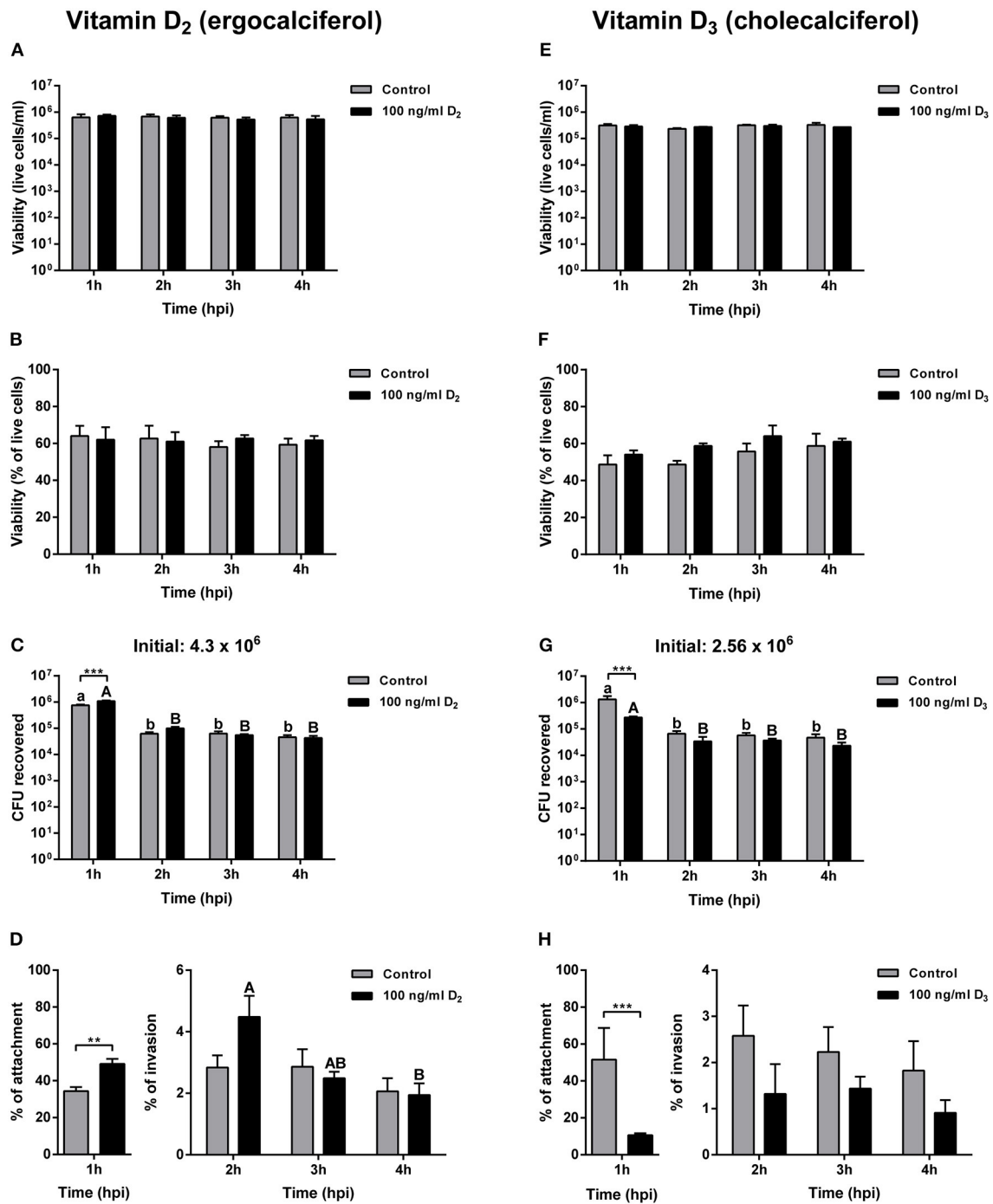


FIGURE 3 | Gentamicin exclusion assay in Atlantic salmon primary macrophages pre-treated for 24 h with either control or 100 ng ml⁻¹ of vitamin D₂ or vitamin D₃, and then infected with *Aeromonas salmonicida* subsp. *salmonicida*. Live cells of primary macrophages pre-treated with vitamin D₂ (A), or vitamin D₃ (E); percentage of viability of primary macrophages pre-treated with vitamin D₂ (B), or vitamin D₃ (F), Colony forming unit (CFU) of *A. salmonicida* in Atlantic salmon primary macrophages pre-treated with vitamin D₂ (C), or vitamin D₃ (G); and percentage of attachment and invasion of *A. salmonicida* in Atlantic salmon primary macrophages pre-treated with vitamin D₂ (D), or vitamin D₃ (H), were measured 1, 2, 3, and 4 h post-infection. Initial *A. salmonicida* inoculum calculated in TSA Congo red plates are shown above the CFU figures. Each value represents the mean ± S.E.M (n = 6). Asterisks (*) represent significant differences between treatments on each time-point (**p < 0.01, ***p < 0.001). Lower case letters (a, b) show differences in the control at different time points post-infection. Upper case letters (A, B) show differences in vitamin D₂ or D₃ pre-treated cells in different time points post-infection.

Vitamin D₂ (ergocalciferol)

Vitamin D₃ (cholecalciferol)

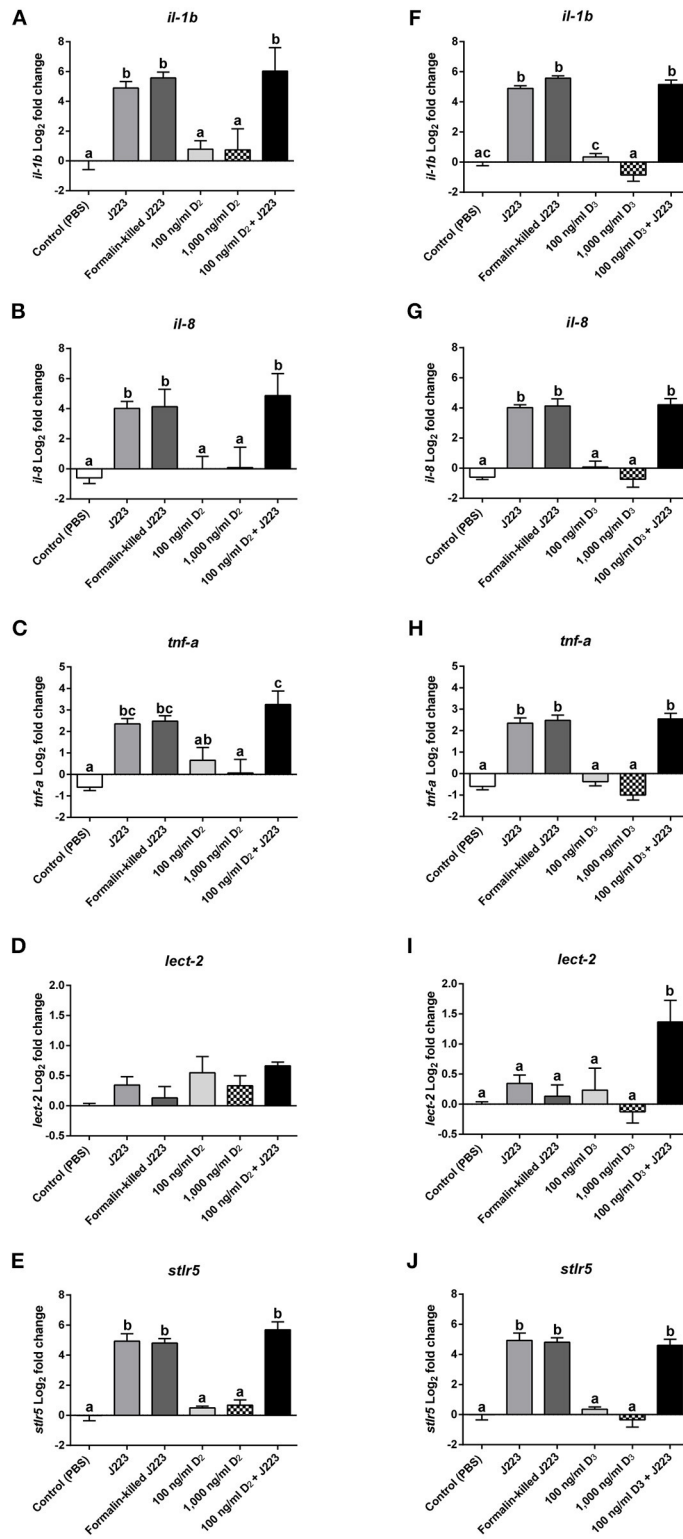


FIGURE 4 | Gene expression of **(A,F)** *Interleukin 1b (il-1b)*, **(B,G)** *Interleukin 8 (il-8)*, **(C,H)** *Tumor necrosis factor alpha (tnf-α)*, **(D,I)** *Leukocyte-derived chemotaxin 2 (lect-2)*, and **(E,J)** *soluble toll-like receptor 5 (stlr5)* in Atlantic salmon primary macrophages isolated from head kidney pre-treated 24 h with either the control, vitamin D₂ or D₃ (100 or 1,000 ng/ml) and J223. (Continued)

FIGURE 4 | D₂ (100 and 1,000 ng ml⁻¹) or vitamin D₃ (100 and 1,000 ng ml⁻¹), and then inoculated with PBS (control) or infected with live (J223) or exposed to formalin-killed *A. salmonicida* for 3 h. Relative expression was calculated using the 2^(-ΔΔC_t) method and Log₂ converted using *β-actin* and *60S ribosomal protein L32 (p/32)* as internal reference genes. Each value is the mean ± S.E.M (n = 6). Different letters represent significant differences between treatments, p < 0.05.

DISCUSSION

Vitamin D is involved in important processes including mineral metabolism, cell growth, and cardiovascular physiology, among others (1–6). Moreover, it has been observed that vitamin D can stimulate the antibacterial immune response in mammals (7, 8). However, these effects and mechanisms have not been explored in fish cells. The two major sources of vitamin D in natural environments are vitamin D₂ and D₃, being obtained in fish after the ingestion of phytoplankton and zooplankton, respectively (9, 10). Different from terrestrial vertebrates that rely on the conversion of 7-dehydrocholesterol to vitamin D by using solar ultraviolet light (wavelength 380–500 nm) through the skin, fish seems to have alternative mechanisms to obtaining vitamin D (4). For instance, it has been hypothesized that bony scales that are part of the fish skin are able to focus and convert the photons obtained from the blue light to breakdown the 7-dehydrocholesterol into vitamin D (53). This does not mean that fish lack of the mechanisms for photosynthesis of vitamin D through the skin. For instance, has been reported that rainbow trout (*Oncorhynchus mykiss*) and Mozambique tilapia (*Tilapia mossambicus*) have the mechanisms to convert 7-dehydrocholesterol into vitamin D, however, authors concluded that in natural environments photosynthesis of vitamin D does not play a significant role (4, 9, 54).

Initially it was thought that both vitamin D forms had the same impact on physiology (55), however, several studies have shown that vitamin D₃ is much more potent compared with vitamin D₂ (55, 56). This evidence suggests a differential modulation on the physiology of fish (i.e., innate immune system) in the presence of the specific vitamin D forms. Also, the effect that vitamin D forms can have on the growth of *A. salmonicida* has not yet been described.

To evaluate if *A. salmonicida* is able to grow in the presence of vitamin D₂ and D₃, a growth curve experiment was conducted in the presence of different concentrations of vitamin D₂ and vitamin D₃ for 48 h. Our results showed that only high concentrations of vitamin D₂ and D₃ reduced the growth of *A. salmonicida* after 48 h (Figures 1A,B). Normally, *A. salmonicida* is able to reach the stationary growth phase in ~36 h (24, 57, 58). We observed a similar pattern of growth previously observed in *A. salmonicida* J223 strain (24) in culture media containing low concentrations of vitamin D₃ (100 and 1,000 ng ml⁻¹). Nevertheless, *A. salmonicida* seemed to tolerate higher concentrations of vitamin D₂ since only the highest concentration (100,000 ng ml⁻¹) reduced its growth rate. A previous study showed that high doses of vitamin C (128, 512, and 2048 mg/ml) can inhibit the growth of *Helicobacter pylori*, a risk factor for gastric carcinoma in mammals, during *in vitro* and *in vivo* experiments (59). Additionally, it has been reported that high concentrations of vitamin C (90 μM) can also

inhibit the growth of *S. aureus* in *in vitro* conditions (60). High concentrations of vitamin D₂ and D₃ decreased the growth rate of *S. aureus* strain A1 after 24 h (14, 61). However, no significant differences were observed in the growth rate of *S. aureus* subsp. *aureus* (ATCC 27543) in the presence of different concentrations of vitamin D₃ after 48 h (8, 62). These results indicate that in the bacterial strains studied, vitamin D can inhibit growth when it is utilized in concentrations over 1,000 ng ml⁻¹, affecting both Gram negative and Gram positive bacteria.

The primary Atlantic salmon macrophage viability decreased after 24 and 48 h of exposure to 100,000 ng ml⁻¹ of vitamin D₂, and a lower viability compared with the control was observed after 48 h with 10,000 ng ml⁻¹ of vitamin D₃ (Figure 2). Similar to our results, it has been observed that low concentrations of vitamin D₃ (1, 10, and 50 nM) did not affect the viability of bovine mammary epithelial cells at 24 h (8). However, it has been reported that a decrease in the viability of bovine mammary epithelial cells in the presence of high concentrations of vitamin D₂ (6,000, 8,000, 10,000, 12,000, and 14,000 ng ml⁻¹) and D₃ (8,000, 10,000, 12,000, and 14,000 ng ml⁻¹) occurred after 24 h of exposure, suggesting that vitamin D can induce cell cycle arrest, apoptosis, or both (14, 63).

In Atlantic salmon, the estimated concentration of vitamin D in flesh varies between 2.9 μg to 18.5 μg per 100 g⁻¹ (64). In our studies, the vitamin D concentrations utilized were similar to other studies conducted in mammalian cells used as a reference (8, 13, 14). The values obtained in the *A. salmonicida* growth curve and Atlantic salmon primary macrophages exposed to different concentrations of vitamin D₂ and D₃ (Figures 1, 2), were utilized to determine the final non-toxic vitamin concentration (100 ng ml⁻¹) for further infection assays. The number of live cells and percentage of viability of Atlantic salmon primary macrophages after 1, 2, 3, and 4 h of infection did not show significant differences between the control and the vitamin D₂ or D₃ pre-treated cells (Figures 2A,B,E,F). Our findings agree with previous infection assays using *A. salmonicida* J223 in Atlantic cod primary macrophages, where no significant differences were observed in macrophage viability after 6 h of infection (42). This indicated that Atlantic salmon and Atlantic cod primary macrophages were not killed during this period by *A. salmonicida* J223. We previously suggested (42) that *A. salmonicida* controls the macrophages machinery to prevent cell apoptosis. As mentioned previously, vitamin D₃ in high concentration (8,000, 10,000, 12,000, and 14,000 ng ml⁻¹) could induce apoptosis (14, 63), perhaps in opposition to this suggested prevention of apoptosis produced by *A. salmonicida* (42). These results agree with the lower attachment and infection rates of *A. salmonicida* in cells pre-treated with D₃, where vitamin D₃ might interfere with the infection.

One of the most interesting findings of our results is related to the bacterial attachment and invasion. For instance, a significant

increase in *A. salmonicida* infection was observed at 1 h post-infection in primary macrophages pre-treated with vitamin D₂ compared with the control (Figures 3C,D). In contrast, a significant decrease in *A. salmonicida* attachment at 1 h was observed in cells pre-treated with vitamin D₃ compared with the control (Figures 3G,H). Previous studies indicated that vitamin D₃ has a stronger activity compared to vitamin D₂ in terrestrial mammals (55, 56). This agrees with our results where pre-treatment of primary macrophages with vitamin D₃ decreased *A. salmonicida* infection; while vitamin D₂ in contrast, seems to increase it infection. Our findings agree with the beneficial utilization of vitamin D₃ in aquafeeds (4, 65, 66) and with vitamin D₃ potentially having a broad positive effect in all vertebrates, including fish.

To complement our results described above, we explored part of the immune mechanism behind the beneficial effects of vitamin D by profiling the expression of specific innate immune genes using qPCR. An up-regulation of *il-1b*, *il-8*, *tnf-α*, and *stlr5* occurred in primary macrophages inoculated with either live or formalin-killed *A. salmonicida* (Figures 4A–C,E–H,J). These results were expected, since cytokines and chemokines, such as *il-1b*, *il-8*, *tnf-α*, and the pattern recognition receptor (PRR) *stlr5*, play essential roles controlling both acute and chronic inflammation in fish tissues mediated by macrophages (39). In Atlantic salmon, the evidence shows that this canonical macrophage innate immune response can be triggered rapidly by either a bacterial pathogen-associated molecular pattern (PAMP), such as lipopolysaccharide (LPS), or pathogens like *Yersinia ruckeri*, *Aeromonas salmonicida*, *Pseudomonas aeruginosa*, and *Flavobacterium psychrophilum*, among others (39, 67–70).

Some viruses, bacteria, and parasites can modify the expression of genes related with the host immune response as part of a mechanism of evading its defense mechanisms (71). In humans, three important infectious diseases (e.g., HIV, tuberculosis, and malaria) have developed highly effective mechanisms to subvert the immune response (71), making it difficult to control these diseases and develop effective vaccines. Further examples of this are *Yersinia pseudotuberculosis* and *Y. enterocolitica* which are able to control human macrophage immune response and induce apoptosis after the translocation of effector molecules through the type III secretion system (72–74).

In fish head kidney, a modulation of the expression of *il-1b* and the major histocompatibility complex class 1 (*mhc-I*) has been observed in Atlantic salmon after being infested by the sea louse *Lepeophtheirus salmonis* (75). Moreover, Lewis et al. (76) showed in an Atlantic salmon head kidney (SHK-1) cell line that *L. salmonis* produces substances that modify the expression of genes encoding inflammatory mediators. Here, our results obtained during the infection with live *A. salmonicida* in Atlantic salmon primary macrophages showed no significant differences in the expression of leukocyte-derived chemotaxin 2 (*lect-2*) compared with the control (Figures 4D,I). When the fish cells were pre-treated with vitamin D₂ and then infected with live bacteria, the gene expression also did not increase, suggesting that *lect-2* is not involved in the first line of defense against *A. salmonicida* in Atlantic salmon. However, the expression of *lect-2* in Atlantic salmon macrophages pre-treated with vitamin D₃ and challenged

with live *A. salmonicida* was significantly up-regulated compared to the control (Figure 4I). *lect-2* is a chemotactic factor involved in the recruitment of neutrophils to the site of infection (39, 77). In the study conducted by Smith et al. (39), an up-regulation of *lect-2* was observed only in treatments with LPS, confirming that its role in the presence of external pathogenic agents is active in Atlantic salmon. Comparing the expression of *lect-2* in primary macrophages treated with live *A. salmonicida* and the samples pre-treated with vitamin D₃ and then inoculated with *A. salmonicida*, our results suggest that *A. salmonicida* prevent the transcriptional response of *lect-2*, perhaps to prevent neutrophil recruitment during infection. Our results suggest that pre-treatments with vitamin D₃ can counteract the effect of *A. salmonicida* on this particular gene, and up-regulate the expression of *lect-2* during *A. salmonicida* infection (Figure 4I).

To determine if vitamin D₂ and D₃ can also cause an effect on the phagocytosis of Atlantic salmon primary macrophages, the phagocytic activity was tested using fluorescent latex beads (Supplementary Figure 1). Phagocytosis is used by organisms to eliminate external agents such as bacteria in a highly efficient way (42, 52, 78, 79). The effect of vitamins on macrophages' phagocytic activity has been previously tested in Atlantic salmon, however, no significant variations were observed after treatments with vitamin C or vitamin E (80, 81). We found similar results in Atlantic salmon primary macrophages after 24 h pre-treatments with either vitamin D₂ or D₃, suggesting that, independent of the vitamin D used, the phagocytic activity of Atlantic salmon macrophages is not modulated by its action.

CONCLUSION

In this study we evaluated the effects of vitamin D₂ and D₃ on *A. salmonicida* growth, Atlantic salmon primary macrophage viability, and the fish cells' immune response. We determined that only high concentrations of vitamin D₂ (100,000 ng ml⁻¹) and vitamin D₃ (1,000 and 10,000 ng ml⁻¹) decreased the growth rate of *A. salmonicida*. Moreover, we determined that 100,000 ng ml⁻¹ of vitamin D₂ and 10,000 ng ml⁻¹ of vitamin D₃ decreased the viability of Atlantic salmon primary macrophages after 24 and 48 h. These results suggest that high doses of D₂ and D₃ are toxic for the bacterial and the eukaryotic cells.

Pre-treatment of primary macrophages with 100 ng ml⁻¹ of either vitamin D₂ or D₃ did not have effects on cell viability. Nevertheless, one of the remarkable findings of our study was that pre-treatment with vitamin D₃ reduced *A. salmonicida* attachment, meanwhile, pre-treatment with vitamin D₂ increased attachment, and as a consequence also increased bacterial invasion.

Gene expression of *il-1b*, *il-8*, *tnf-α*, and *stlr5* was up-regulated during *A. salmonicida* infection, agreeing with a canonical innate immune response. However, our results suggested that *A. salmonicida* was able to suppress the expression of *lect-2*, a gene involved in neutrophil recruitment, key in the fight against pathogen clearance. After the addition of vitamin D₂, no variation in the transcriptional expression of this gene was observed. However, cells pre-treated with vitamin D₃ and then

inoculated with live *A. salmonicida*, showed an up-regulation of *lect-2*, suggesting that vitamin D₃ can be useful to counteract the suppression triggered by the pathogen.

Altogether, our results show that vitamin D₃ seems to be a good candidate to be used as an immunostimulant in Atlantic salmon against *A. salmonicida* infection. The mechanisms on how vitamin D₃ modulates the *S. salar* macrophage immunity and its relation to specific receptors, like the vitamin D receptor, deserve future attention. In contrast, vitamin D₂ did not appear to have an effect on the modulation of the immune system of Atlantic salmon, suggesting that vitamin D₂ may not play an important role in the fish innate antibacterial immune response.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Memorial University Institutional Animal Care Committee.

AUTHOR CONTRIBUTIONS

MS-D and JS: conception and design of research, prepared figures, and drafted the manuscript. MS-D: macrophage

isolation. JH: primer design. MS-D, KV, and SI: performed the experiments. MS-D, MR, and JS: interpreted the results of the experiments. MS-D, KV, SI, JH, MR, and JS: edited and revised the manuscript and approved the final version of the manuscript. MR and JS: funding support.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.03011/full#supplementary-material>

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

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