Immune and Antioxidant Enzyme Response of Longfin Yellowtail (*Seriola rivoliana*) Juveniles to Ultra-diluted Substances Derived from Phosphorus, Silica and Pathogenic *Vibrio*

José Manuel Mazón-Suástegui[1] Joan Salas-Leiva[1] Andressa Teles[1] Dariel Tovar-Ramírez[1]

[1] Centro de Investigaciones Biológicas del Noroeste S.C. (CIBNOR), La Paz, Baja California Sur, México

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Address for correspondence Dariel Tovar-Ramírez, PhD, Centro de Investigaciones Biológicas del Noroeste S.C. (CIBNOR), La Paz, Baja California Sur, México (e-mail: dtovar04@cibnor.mx).

Abstract

Background This research aimed to observe the effect of homeopathic treatments prepared from *Vibrio parahaemolyticus* and *V. alginolyticus* (H1) and commercial homeopathic medication *Phosphoricum acidum* and *Silicea terra* (H2) on the immune and antioxidant response in *Seriola rivoliana* juveniles under usual culture conditions and challenged with *V. parahaemolyticus*.

Materials and Methods Quantitative polymerase chain reaction analysis was used to study changes in the expression of key genes related to immune response, cytokines (interleukin-1β [IL-1β]), adapter protein for cytokine release (MyD88) and piscidin and spectrophotometric techniques to analyze the activity of antioxidant superoxide dismutase (SOD) and catalase (CAT) enzymes in *Seriola rivoliana* juveniles at 30 (weaning stage [WS]) and 60 (early juveniles [EJ]) days post-hatching.

Results The H1 treatment led to over-expression of the IL-1β and MyD88 genes in fish at WS and EJ with respect to control, contrary to the H2 treatment that led to under-expression of the IL-1β, MyD88 and piscidin genes at the EJ stage. In fish challenged with *V. parahaemolyticus*, both H1 and H2 led to over-expression of IL-1β and MyD88; H2 caused an over-expression of piscidin. The SOD activity was higher in H1 with respect to H2 and the control group. CAT remained relatively stable with both H1 and H2 treatments.

Conclusions The results suggest that the overall effect of H1 was due to the presence of unknown antigens in low concentrations, while the response to H2—specifically during challenge—may have been due to a stimulating effect of nano-structures, prevailing from mother tincture after sequential dilution/succussion, in a pathway similar to that attributed to nano-vaccines.

Keywords
- aquacultural homeopathy
- cytokines
- piscidin
- MyD88
- *Seriola rivoliana*

Introduction

In several countries, the Longfin yellowtail *Seriola rivoliana* (Carangidae) is considered as one of the fish with the greatest potential for intensive aquaculture because of its fast growth, high meat quality and adaptability to intensive farming conditions.[1,2] Studies have been performed in *S. rivoliana* on spawning and larviculture, digestive ontogeny, osseous development, feeding and management of broodstock, and embryo toxicology, among others.[1,3–7] Nonetheless, little information is available related to the immune and antioxidant defense system, which could provide fundamental information for

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improving productivity and developing new cropping technologies. Problems caused by bacteria, viruses and parasites have been reported as limiting factors in culture of several species including *Seriola* spp.[8] Currently, the effort to understand the immune system of these species continues to increase although few characterized genes have been attributed to the immune system for *Seriola* spp.[8,9]

The innate immune system represents the first line of protection against external pathogens, and it is considered a fundamental mechanism to maintain fish homeostasis.[9,10] Innate responses act against invading organisms and foreign substances, including a complex network of cells, cell products and molecules that operate to eliminate and/or inactivate the pathogen. Cells involved in innate responses are mostly leukocytes (i.e., mononuclear phagocytes, polymorphonuclear leukocytes, among others) and molecules that correspond to antibacterial peptides, lysozymes, cytokines, toll-like receptors (TLRs), and reactive oxygen species (ROS), among others.[11]

In aquaculture, super-intensive production generally causes physiological stress which, depending on its intensity, entails several negative effects (i.e. low productive parameters or increased sensitivity to diseases caused by opportunistic organisms).[10] Antibiotics, vaccines and other chemicals have been used to control the negative effects of stress, but they involve drawbacks, including suppression of the aquatic animal immune system, environmental hazards and food safety as human health problems.[12] For this reason, the addition of immune-stimulants to fish diets has been suggested as an alternative for prophylactic treatment against disease outbreaks.[12,13] In this context, homeopathic medicines are offered as a safe alternative for improvement in growth and survival and stimulation of the immune system in fish, molluscs and crustaceans.[14,15] Different studies have suggested that homeopathic preparations, such as Canova (Parana, CU, Brazil), can activate macrophages, stimulate the production of ROS and positively affect some cytokines that play an important role in inflammatory processes.[16–21]

The objective of this research was to evaluate the effect of homeopathic preparations with pathogenic strains of *Vibrio*, *Phosphoricum acidum* and *Silicea terra* on the expression of interleukin-1β (IL-1β), MyD88 and piscidin genes and the antioxidant activities of superoxide dismutase (SOD) and catalase (CAT) of weaning stage (WS—30 days post-hatching [dph]) and early juveniles (EJ—60 dph) of *S. rivoliana*.

Previous studies have shown that *Phosphoricum acidum* and *Silicea terra* improved survival and SOD activity in *Argopecten ventricosus* juveniles after challenge with *Vibrio parahaemolyticus*.[14] Other studies with molluscs and crustaceans (*Crassostrea virginica*, *C. sikamea*, *Litopenaeus vannamei*) showed that a mixture with *Phosphoricum acidum* and *Silicea terra* enhanced protection and stimulated the immune system.[22] It is important to note that phosphorus is considered an important mineral for immune system function;[23] furthermore, silica nanostructures have an ample role in aspects related to immune functions, mainly with those associated with pro-inflammatory cytokines.[24]

The relevance of these chosen indicators is that Myd88 functions as a key downstream adapter protein for cytokine release;[25,26] IL-1β is a cytokine that regulates a variety of biological processes, associated with inflammation, immune response, leukocyte chemo-attractance;[27,28] and piscidin is a powerful antimicrobial peptide.[29] The activities of SOD and CAT can be classified as indirect indicators of the immune response since they neutralise the ROS that release macrophages in the presence of pathogens or xenobiotics.[30,31]

Materials and Methods

Experimental Design and Culture Conditions

Larviculture of *S. rivoliana* was developed according to Teles et al[1] at Centro de Investigaciones Biológicas del Noroeste (CIBNOR), La Paz, Baja California Sur, Mexico. The experimental design involved two tests.

Test no. 1 used 180 fish at initial weaning stage (WS, i.e., 30 dph) which were initially distributed in four 80-Liter fiberglass tanks (45 fish/tank). The experimental design included two tanks without homeopathic treatment (positive and negative control groups), and two tanks with homeopathic treatment. The positive control tank was named that way at first; however, at this stage the fish from that tank were not infected, until the bacterial challenge. Then, the positive control group was separated, so that density and water quality were not different from the negative control. After 15 days of treatment with homeopathic medication (see next item for details), 21 fish from each tank were transferred to three experimental units (10 L), which allocated seven fish/unit for a bacterial challenge with *V. parahaemolyticus*.

Test no. 2 used fish that were allowed to grow to juvenile stage (EJ, i.e., 60 dph). The fish were distributed in three 80-L fiberglass tanks (30 fish/tank) corresponding to one control and two treatment tanks: ***Treatment H1*** (ViP 7c + ViA 7c) and ***Treatment H2*** (PhA 7c + SiT 7c) (see next section).

During both bioassays, environmental conditions were maintained at 24.0 ± 1°C temperature, 5.0 ± 1.0 mg/L dissolved oxygen, 37.0 ± 1 g/L salinity, 12-hour light/dark photoperiod and water change of ≈ 5.0 L/min. The WS fish were fed *ad libitum* twice daily with a commercial diet of OTOHIME B1 and B2 (Reed Mariculture Inc. Campbell, California, United States; proteins 51.0%, lipids 11.0%, ashes 15.0%, and carbohydrates 3.0%). Once the fish were transferred to the 10-L bottles, they were fed once a day (≤ 1% of the biomass) to avoid excess toxic compounds in the water (NH_3, NO_2) commonly associated with closed systems. The EJ fish were initially fed the diet C2 OTOHIME (Reed Mariculture Inc; proteins 51.0%, lipids 11.0%, ashes 15.0%, carbohydrates 3.5%); then, they were fed with Gemma Diamond 1.2 - 1.5 diet (Skretting, Stavanger, Norway; proteins 57.0%, lipids 15.0%, ashes 10.3%, carbohydrates 0.2%).

Preparation and Application of Homeopathic Treatments

Treatment H1 (ViP 7c + ViA 7c) consisted of the application of centesimal homeopathic dynamizations (CIB-HOM-ViP 6c and CIB-HOM-ViA 6c; CIB, La Paz, B.C.S. Mexico), prepared at Centro de Investigaciones Biológicas del Noroeste (CIBNOR)

from highly pathogenic strains of *V. parahaemolyticus* and *V. alginolyticus* (CAIM 170; www.ciad.mx/caim [ViP and *V. alginolyticus* CAIM 57]; www.ciad.mx/caim [ViA]). Mother tincture (MT) of each bacterial strain was produced from bacterial cultures at 10^8 CFU/mL through application of basic principles from the Mexican homeopathic pharmacopeia, using ethanol as dilution and succussion vehicle, at CIBNOR in a certified microbiology laboratory. Briefly, bacterial cells were harvested by centrifugation (8,000 g, 4°C, 20 minutes) from 15 mL of bacterial culture and washed twice. The corresponding pellets were diluted in 7.5 mL of MilliQ water; inactivated through three freezing-thawing cycles (−80°C and 24°C, respectively); and sonicated eight times for 30 seconds each time to disrupt the cell wall and intracellular organelles. Unbroken cells were removed by centrifugation (3,000 g, 4°C, 20 minutes). The resultant supernatant was diluted (1:1 v/v) in ethanol 87° GL (Similia, Mexico City, Mexico) and vortexed at 3,200 RPM (BenchMixer, Edison, New Jersey, United States) for 2 minutes to obtain 15 mL of MT from each bacterial strain. Finally, ViP 7c and ViA 7c were obtained through dynamization (6c) with distilled water as a final dilution and succussion vehicle.

Treatment H2 (PhA 7c + SiT 7c), on the other hand, was made of centesimal homeopathic dynamizations from the commercial homeopathic drugs *Phosphoricum acid* 6c and *Silicea terra* 6c (Similia ethanolic dynamization, National Homeopathic Pharmacy, Mexico). Dynamization (6c) was performed with distilled water as a final dilution and succussion vehicle.

The homeopathic preparations (6c and 7c) were stored at room temperature and protected from direct sunlight. In both tests (WS and EJ), 7c in-water dynamizations were applied daily directly to seawater of the experimental units for 15 days. Thus, in the case of H1, 25 µL of ViP 7c/L was added daily in the morning and 25 µL of ViA 7c/L in the afternoon, and 25 µL of PhA 7c/L and 25 µL of SiT 7c/L in the morning and in the afternoon, respectively. The negative control group (no homeopathic treatment) did not receive any treatment, only distilled water. The homeopathic dynamizations 7c and control samples were succussed in vortex at 3,200 RPM (BenchMixer, Edison) for 3 minutes before being added directly into seawater of the experimental units just before feeding. In previous works,[32–35] vortexing was used as succussion mechanism. We respect the homeopathic pharmacopeia, but it is necessary to have a reproducible succussion mechanism that can be used in any laboratory/experiment to obtain comparable results. The water flow was then closed for 1 hour, and aeration was increased to homogenize the liquid medium in the experimental units. After that time, water–air flow was restored.

Vibrio parahaemolyticus and Challenge

The strain of *V. parahaemolyticus* was obtained from CIBNOR bacterial collection (strain code A01; isolated from the digestive tract of *Lutjanus guttatus* with vibriosis symptoms). This bacterium was grown in TBS liquid medium (tryptic soy broth, BD catalog, 211825, Becton, Dickinson and Company, Franklin Lakes, New Jersey, United States) supplemented with 2.5% NaCl and incubated at 35°C for 24 hours. The bacteria were harvested in final exponential phase (retardation) by centrifugation at 8,000 g at 4°C for 20 minutes and then washed with sterile PBS. The supernatant was removed, and the pellet was resuspended in sterile seawater. Subsequently, the bacterial suspension was adjusted using a spectrophotometer (Jenway 6505, Felsted, United Kingdom) at an optical density from 1 nm to 600 nm ($OD_{600} = 1$) equivalent to 10^7 CFU/mL. For the bacterial challenge, the equivalent of 10^5 CFU/mL was added to the experimental units of 10 L and conducted for 3 days with a positive control that consisted of challenging fish without homeopathic medication.

Collection of Fish Samples

Animal Research Ethics

The experiment complied with the Guidelines of the European Union Council (2010/63/EU) and the Mexican Government (NOM-062—ZOO-1999) for the production, care, and use of experimental animals, and with the *ARRIVE* guidelines. All the experimental protocols and procedures were carefully revised and approved by an internal committee at CIBNOR (►**Supplementary File 1** [available in online version only]). A completed *ARRIVE* Guidelines Checklist is provided as ►**Supplementary File 2** (available in online version only).

The Longfin yellowtails (*Seriola rivoliana*) were maintained, by trained staff, under standard conditions in terms of temperature, salinity, oxygen supply, and appropriate photoperiod according to the age for this species. Water-quality parameters were checked and controlled early every morning before food administration; the resulting water quality measurements complied with the standard requirements for this species.

In accordance with the EU Directive, our research was developed following the principles of the '3Rs' (Replacement, Reduction and Refinement). This investigation required us to use live fish to know the effects on growth, immune and antioxidant response in whole animals, so they could not be replaced. The lowest number of experimental animals was used based on the need to obtain information of a given amount and precision, considering the minimum numbers of individuals to perform robust statistical analysis and thus obtain meaningful results. The state-of-the-art nature of our laboratory analytical methods ensured the technical precision needed to ensure minimum sample size. Regarding refinement, any distress or physical harm to the animals was contained to an absolute minimum by our use of efficient euthanasia at the time of tissue sampling at the experimental end-point: each sampled fish was euthanized with an overdose of 2-phenoxyethanol (56753 Sigma-Aldrich, Missouri, United States). It was then dissected on a frozen glass to avoid wastage through enzyme denaturation, and to separate and weigh the organs of our interest. Anticipating these experimental methods, it was judged prospectively that these minimized harmful effects on the living animals would be outweighed by significant scientific discovery likely to result from the work.

Sample Collection

At the end of the 15-day bioassay with homeopathic and control treatments (before challenge), 18 fish were randomly taken from each experimental unit to perform the analyses contemplated in this study. After removal of intestines, total ribonucleic acid (RNA) was isolated and gene expression was performed from the IL-1β, MyD88 and piscidin genes ($n = 9$); in addition, SOD and CT activities ($n = 9$) were also performed. Subsequently, after the challenge (3 days), three fish were taken per replicate ($n = 9$) to observe the expression of the mentioned genes. In addition, intestine and liver samples were collected to determine SOD and CAT activities. The intestine was used not only because it is the part where the digestive functions take place but it also acts as a microbial barrier and synthesizes immune-related molecules.[8] The liver was selected as the main metabolic organ where antioxidant reactions take place, such as those performed by the SOD, CAT and glutathione peroxidase enzymes and others. Finally, in test no. 2, the sample collection was performed in a similar way as before the challenge. Samples for gene expression were immersed in Eppendorf tubes with RNAlater (Thermo Fisher Scientific, Waltham, Massachusetts, United States) solution and placed at 4°C for 24 hours and then stored at −80°C. For enzymatic activity, the samples were immersed in a buffer solution (Tris-HCl 50 mmol, and $CaCl_2$ 20 mmol pH 7.5) in a 1:5 (w:v) proportion and stored at −80°C until processing.

RNA Extraction, cDNA Synthesis, and qPCR

For the extraction of total RNA from the intestines, TRIzol RNA Isolation Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and a tissue homogeniser (FastPrep-24 5G, M.P. Biomedicals, Santa Ana, California, United States) were used according to the manufacturer's instructions. Subsequently, a DNase treatment with RQ1 RNase-Free DNase kit (Promega, Madison, United States) was performed. The concentration and purity of the RNA were determined with NanoDrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and RNA quality was observed on agarose gel (1.5%) with Sybr Safe DNA Gel Stain (Invitrogen, Paisley, United Kingdom). Finally, the Improm II kit (Promega, Madison, Wisconsin, United States) was used for cDNA synthesis following the manufacturer's instructions. The reaction was performed with 2.5 µg of total RNA in iCycler thermocycler (Bio-Rad, Berkeley, California, United States). The primers used for IL-1β, MyD88, and piscidin, which were specific for *S. rivoliana*, were obtained from the GeneBank database. ►**Table 1** details the sequences of the primers used for qPCR analyses.

The qPCR was performed using a Bio-Rad CFX 96 thermocycler (BioRad Laboratories, Inc., Hercules, California, United States) to analyze the expression of IL-1β, MyD88, piscidin, and 18S genes. The latter was taken as the reference gene. A standard curve was used to choose the working dilution according to the highest efficiency (% E). The qPCR assay was performed in triplicate for each sample, taking a total volume of 10 µL containing 2-µL cDNA, 0.20 µL primers (50 µM), 5-µL SsoFast EvaGreen Supermix (BioRad Laboratories, Hercules, California, United States) and 2.8 µL of sterile water. A negative control (NTC – no template control) was included for each of the reaction sets on each plate (96-well plate). The reactions were incubated using the following conditions: one cycle at 95°C for 10 minutes; 40 cycles at 95°C for 15 seconds; 60°C for 30 seconds. To perform the analysis, the normalized relative expression to the control group was taken into account.

Biochemical Analyses

Samples (intestine and liver) for this test were processed into their preservation buffer using a tissue homogenizer (FastPrep-24 5G, MP Biomedicals, Santa Ana, California, United States), which were then centrifuged (Eppendorf 5430R, Hamburg, Germany) at 13,000 g at 4°C for 10 minutes; the supernatant was used for the following biochemical assays:

Superoxide Dismutase and Catalase Activities

Superoxide dismutase activity was measured using colorimetric kit catalog 19160 (SOD determination kit, Sigma-Aldrich, Missouri, United States) according to the manufacturer's instructions. Expressing the SOD activity in U mg of protein (U SOD/mg protein), the concentration of soluble microplate proteins was determined previously using the Bradford[36] method and bovine serum albumin as standard in Varioskan (Thermo Scientific, Waltham, Massachusetts, United States) similar to that proposed by Eyckmans et al.[37] The CT activity was studied with the Clairborne[38] method, where the decrease in H_2O_2 absorbance was measured at 240 nm. A unit of enzyme activity was defined as the amount

Table 1 Primers used for the qPCR analysis of the target genes of *Seriola rivoliana*

Target genes	Accession no.	Primer sequence (5′-3′)	Melting temperature (°C)	Amplicon (bp)
IL-1β	KY860519	AGCCAGCAGAGACACTTAG	60	124
IL-1β		TGGGTAAAGGTGGCAAGTAG		
MyD88	KY860521	ATGAAGCGACGAAAAACCCC	60	135
MyD88		AAGACTGAAGATCCTCCACAATGTC		
Piscidin	KY860523	TCGTCCTGTTTCTTGTGTTGTC	60	151
Piscidin		TGCTGTAGGTCTGTCATGCC		
18S		CTGAACTGGGGCCATGATTAAGAG	60	165
18S		GGTATCTGATCGTCGTCGAACCTC		

Abbreviations: IL, interleukin; qPCR, quantitative polymerase chain reaction.

of enzyme required to degrade 1 nmol of H_2O_2 in 1 minute per mL, and it was expressed as nmol/min/mL per mg of protein.

Statistical Analysis

All tests corresponded to a completely randomized design. Before and after challenge, 18 individuals were selected from each treatment to take measurements, gene expression, and antioxidant activity. RNA extractions and homogenates were performed for this effect by pooling three individuals, and expression and activity data were presented as mean ± standard error (SE). For the challenge, from each replicate, three individuals were taken to make the analyses. All gene expression analyses were performed using the Cq method with the Bio-Rad CFX Manager 3.1 Gene Study software (Bio-Rad, Berkeley, California, United States) using the efficiency-corrected values of Cq.[39] The qPCR data were normalized to the geometric mean of the expression efficiency-corrected data for 18S. The expression difference between treated and control fish was tested by significant differences in Bio-Rad CFX Manager 3.1 (Bio-Rad, Berkeley, California, United States), using a two-tailed, unpaired *t*-test.[40] Differences were considered statistically significant if $p < 0.05$.

For the analysis of antioxidant enzymes, the sampling procedure was similar to that described previously in this section. Antioxidant activity data were presented for SOD and CAT as mean ± SD of U SOD mg/protein and nmol/min/mL, respectively. For the challenge, three fish were also taken per replicate. The photometric readings for each sample were done in triplicate. All comparisons were made between individuals of the same stage, using a one-way ANOVA, followed by Tukey's multiple range analysis if significant differences were found. Statistical analyses were performed with a significance level of 5% ($\alpha = 0.05$) using the software Statistica 10 (StatSoft, Tulsa, Oklahoma, United States).

Results

Effect of ViP + ViA (H1) and PhA + SiT (H2) on Weaning Stage and Early Juvenile Fish

Gene Expression

Gene expression in intestine of WS fish treated with H1 varied from 0.78 to 3.79, in piscidin and MyD88, respectively (►**Fig. 1A**). Under this treatment, an over-expression was

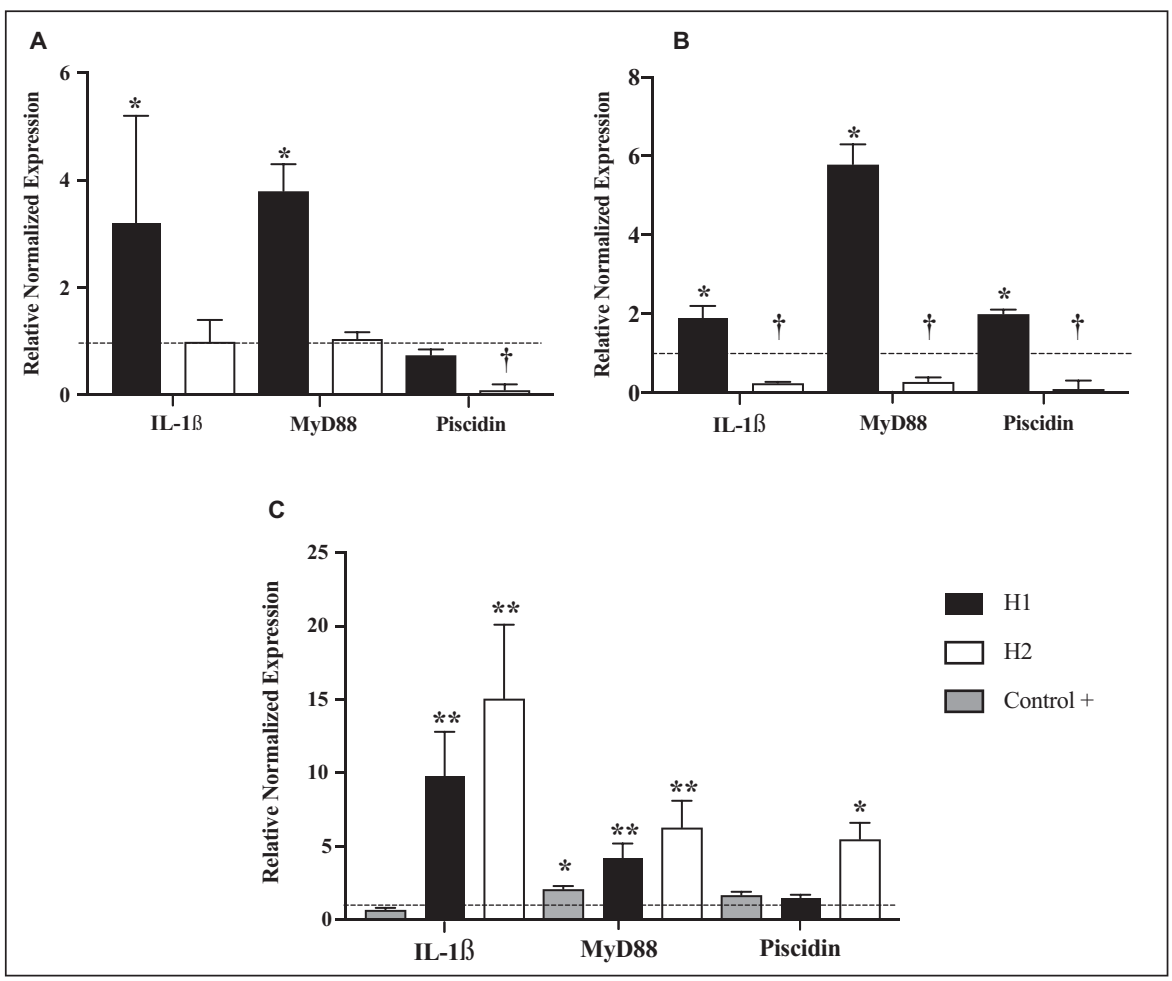

Fig. 1 Relative gene expression in interleukin-1β (IL-1β), MyD88 and piscidin normalized to the 18S gene in *Seriola rivoliana* intestine. Fish control (dotted line, relative expression = 1). Treatment H1 (ViP 7c + ViA 7c) and H2 (PhA 7c + SiT 7c). (**A**) Weaning stage fish (WS). (**B**) Fish in early juvenile (EJ) stage. (**C**) Fish challenged with *Vibrio parahaemolyticus*; infected without treatment [Control +]. Mean ± standard error (*over-expressed and †sub-expressed with respect to control; *, †$p < 0.05$; **$p < 0.01$).

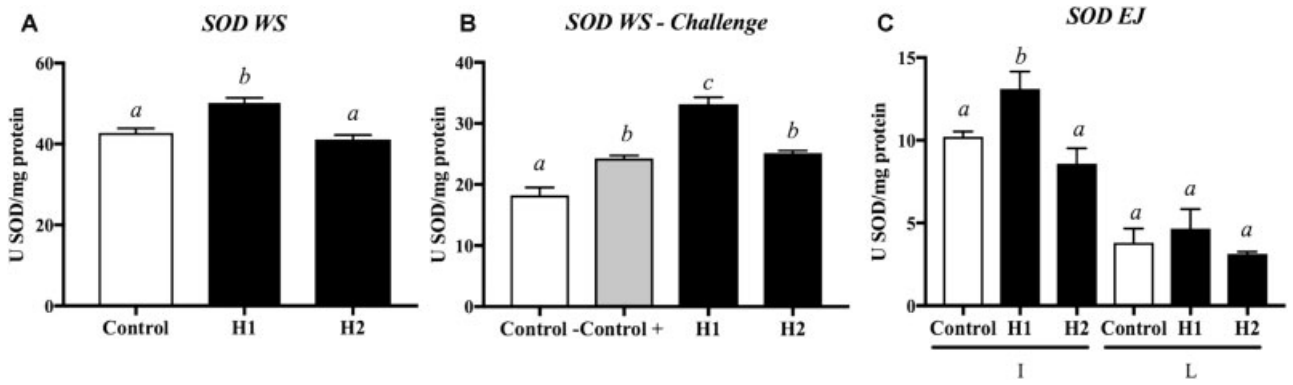

Fig. 2 Superoxide dismutase (SOD) activities in *Seriola rivoliana*: (**A**) weaning stage (WS) fish, (**B**) with fish challenged with *Vibrio parahaemolyticus*, and (**C**) early juvenile (EJ) fish. Treatment H1 (ViP 7c + ViA 7c) and H2 (PhA 7c + SiT 7c). Control +: Fish challenged with *Vibrio parahaemolyticus* without treatment. In EJ, I: Intestine; L: Liver. Average ± standard deviation. Different letters on the bars indicate significant inter-group differences ($p < 0.05$).

observed in IL-1β and MyD88 WS fish, whereas piscidin had an expression similar to that of the control (►**Fig. 1A**). In H2-treated fish, the IL-1β and MyD88 genes showed similar expression to that of the control, whereas the piscidin gene showed under-expression. In EJ fish, the H1 treatment led to over-expression of the IL-1β, MyD88, and piscidin genes with respect to control, contrary to the H2 treatment that led to under-expression of all evaluated genes (►**Fig. 1B**). In WS and EJ fish treated with H1, a common pattern was observed, which was a greater expression of IL-1β and MyD88 with respect to the control (►**Fig. 1** A, B).

Antioxidant Activities of Superoxide dismutase and Catalase

Superoxide dismutase activity before challenge in WS fish can be observed in ►**Fig. 2A**; in all cases, the SOD activity in intestine was higher than 40 U SOD/mg of protein. The SOD activity was higher in H1 relative to control and in H2 (►**Fig. 2A**). In EJ fish, SOD mean activity from intestine fluctuated from 8.58 to 13.10 U SOD/mg of protein, lower than that observed in weaned larvae (►**Fig. 2C**). Once more, SOD activity was higher with H1 relative to H2 and control. The activity pattern of this enzyme in this organ was similar to that obtained in WS fish. Furthermore, no differences in SOD activity were observed in the liver of these fish (►**Fig. 2C**). Catalase activity in intestine in WS fish ranged from 4.33 to 6.52 nmol/min/mL. The highest average CAT activity was that of the control, and it was significantly different from that of H2 (►**Fig. 3A**). The H1 treatment showed an intermediate CAT activity between the two groups (control and H2). In the EJ intestine, no significant differences were observed for this enzyme (►**Fig. 3C**). In the liver of the fish treated with H1, CAT activity was greater than that of the control group, and H2 had an intermediate activity between the control and H1 (►**Fig. 3C**).

Effect of ViP + ViA (H1) and PhA + SiT (H2) on Fish Challenged with *Vibrio parahaemolyticus*

Gene Expression

At the end of the challenge with *V. parahaemolyticus*, in the positive control group (control +), the genes IL-1β and piscidin showed a similar expression to that of the negative control, whereas MyD88 was greater than the negative control (►**Fig. 1C**)]. On the other hand, H1 and H2 induced a greater relative gene expression than the control groups (positive and negative) in IL-1β and MyD88; additionally, H2 produced a greater expression in piscidin with respect to the positive and negative control groups.

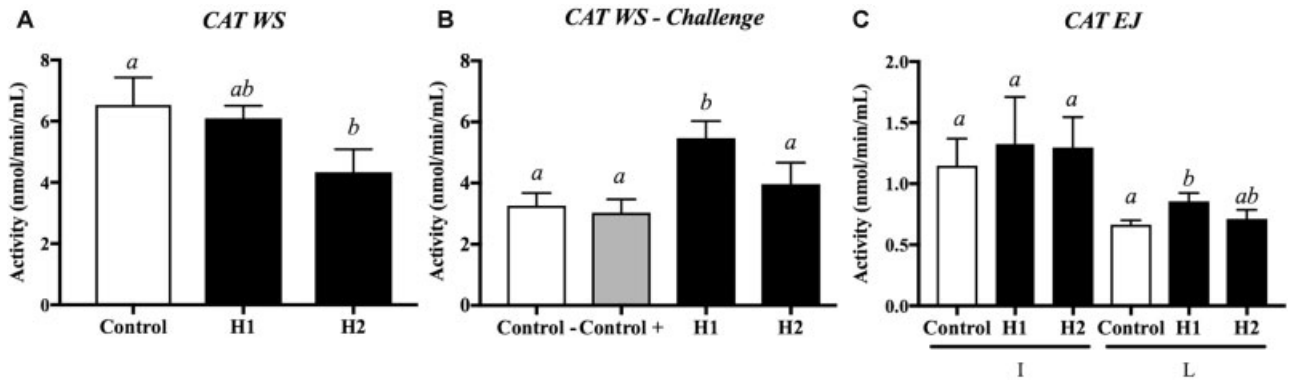

Fig. 3 Catalase (CT) activities in *Seriola rivoliana*: (**A**) weaning stage (WS), (**B**) weaned challenges, and (**C**) early juvenile (EJ) fish. Treatment H1 (ViP 7c + ViA 7c) and H2 (PhA 7c + SiT 7c). Control +: Fish challenged with *Vibrio parahaemolyticus* without treatment. In EJ, I: Intestine; L: Liver. Average ± standard deviation. Different letters on the bars indicate significant inter-group differences ($p < 0.05$).

Antioxidant Activity of Superoxide dismutase and Catalase

The SOD activity in challenged fish ranged from 18.89 to 33.16 U SOD/mg protein. In all treatments, the activity of this enzyme was significantly higher than that of the negative control treatment (►**Fig. 2B**). The positive control and H2 showed similar SOD activity with 24.29 and 25.13 U SOD/mg of protein, respectively. The highest activity of SOD was recorded with the H1 treatment (►**Fig. 2B**). Catalase activity in challenged fish ranged from 3.02 to 5.46 nmol/min/mL (►**Fig. 3B**). The average activity of the control groups (negative, positive) and H2 was statistically similar and lower than that of H1 (►**Fig. 2B**).

Discussion

In intensive aquaculture, factors such as high organic content, low dissolved oxygen and high planting density, among others, can cause physiological alterations that contribute to the increase in susceptibility of fish to stress and infections. Infectious diseases of bacterial origin are perhaps the most significant factors affecting aquaculture.[30,41] One of the major diseases affecting a wide variety of fish, molluscs, and crustaceans is vibriosis produced by *V. parahaemolyticus*.[42] Recently, researchers have made efforts to develop alternatives to increase resistance to vibriosis by strengthening the immune response (i.e., immunotherapy, probiotics).[42,43]

Our study has explored the effect of two homeopathic treatments on the innate and antioxidant immune response, mainly in the intestine of juveniles of *S. rivoliana* since the intestinal tissue constitutes the most extensive and complex part of the immune system of teleost fish;[44] thus, the challenges were conducted with *V. parahaemolyticus* that affects this organ mainly.[45,46] Our results indicated that the application of H1 led to increased expressions in the MyD88 and IL-1β genes in WS fish. In EJ, additional expression of these two genes was also detected, as well as over-expression of the piscidin gene.

Attenuated pathogenic bacteria or microorganisms or their diluted and succussed components, in accordance with homeopathic pharmacopeia, have been used to stimulate immune responses. The preparation of homeopathic bacteria, fungi, viruses or parasites (i.e., nosodes)[47] has shown to have bacteriostatic,[48] anti-cancer,[49] and anti-parasitic activities[50] and modulation of the inflammatory response.[51,52] In a recent study, Reyes-Becerril et al[43] evaluated the effect of *V. parahaemolyticus* lysates (i.e., lysate-vp) on the immune response in Pacific red snapper (*Lutjanus peru*) exposed to infection with the same live bacterium. In the particular case of the IL-1β gene, they observed over-expression in four tissues (including intestine) relative to the control group when the fish were stimulated with lysate-vp. Subsequently, Reyes-Becerril et al[44] found that lysate-vp stimulated the expression of the TLR5 gene in intestine and also regulated the expression of the genes involved in protection against bacteria, such as IL-6, IL-8, IL-10, IL-12, and IL-17.

Though the methods for preparation and administration of lysate-vp and H1 were different, our results were similar to those reported by these researchers. This type of response can be attributed to the fact that lysates have different antigens (flagellin, lipopolysaccharides, exopolysaccharides, and outer membrane proteins, among others) that have been shown to have immunogenic properties. Apparently in our experiment, the antigens (despite being diluted) could transfer some specific signals that were able to promote and modulate the immune response in the host organism.[14,53,54]

Taking into account that the dilution used in this work, which did not exceed the Avogadro limit (< 12c), we considered that the regulation of expression was consistent with the proposal by Khuda-Bukhsh[55] that homeopathic medications carry specific "signals" that can be identified by specific receptors and may activate or deactivate some genes in response to a stimulus. For this reason, the gene expression response under the H1 treatment seemed to indicate the initiation of pathogen-associated molecular pattern (PAMP) recognition by a TLR receptor, which sent activation signals to MyD88 and TRAF6 and translocation to NF-kB to the nucleus of the cells of the intestinal tissue, inducing the expression of pro-inflammatory cytokines such as IL-1β.[26,43] The above was related to the high expression of the MyD88 and IL-1β genes observed. However, a high expression of MyD88 could also have been responsible for the expression of other pro-inflammatory cytokines (i.e., tumour necrosis factor-α [TNFα]).[44,56]

In our study with the H1 treatment, a greater expression of piscidin gene than that of the control group was observed in EJ, but in newly WS fish the expression level was similar to that of the control group. This behavior may have been related to the stimulatory effect of PAMPs on phagocytic cells responsible for producing peptides such as piscidin. The expression of the piscidin gene showed high inter- and intra-species variation, which had been observed to increase with age or degree of development of the organism.[57] Future studies, such as immune-related transcriptomic analyses, are necessary to know the ontogeny of the genes associated with immune response in *S. rivoliana* to avoid under- or over-estimating the real effects of the treatments in both developmental stages and under an immunosuppressor. At present, antimicrobial peptides, such as piscidins, have received more attention at the biotechnological level. They are small peptides (18 to 26 amino acids), highly conserved at the amino terminus, rich in histidine and phenylalanine,[29,58] possessing activity against bacteria, fungi, and some parasites.[59,60] They are distributed in different tissues of teleost fish and show diverse mechanisms of action that have been previously described.[61]

In recent years the uses of molecular tools, such as microarrays and RNAseq, have made it possible to know that some homeopathic medicines generate changes in the expression of different genes. For example, de Oliveira et al[17] observed that using Canova (composed of different homeopathic dilutions of *Aconitum napellus*, *Thuya occidentalis*, *Bryonia alba*, *Arsenicum album*, *Lachesis muta* and less than 1% ethanol in distilled water) induced a decrease in IL-2 and IL-4 expression and a differential expression of 147 genes with respect to the control group in rodent cells. Such genes

appeared to be mainly involved in transcription/translation, immune response, cytoprotection, and various enzymatic processes. At transcriptome level, the authors indicated that Canova generated alterations in the profile of gene expression, related to the activation of the macrophages. For example, Bigagli et al[62] observed that using *Apis mellifica* 3c, 5c, 7c caused modulation of several genes in the RWPE-1 cell line, many of them related to inflammatory processes and oxidative stress. Similar studies with RNA microarrays have been reported by Bellavite et al[63] and Saha et al,[53] indicating that certain homeopathic medications can modulate gene expression in various cell types, making regulatory changes in multiple variables at once and activating biological signaling cascades. In addition, other studies have documented the ability of homeopathic treatments to modulate heat shock protein patterns and release cytokines, macrophages and lymphocyte activation.[64]

In regard to antioxidant activity, the H1 treatment caused greater SOD activity in the intestine of WS and EJ. CAT activity remained similar to that of the control in H1, although such activity was lower in H2-treated WS fish. Similar results have been reported with the administration of some probiotics in fish.[65] In vertebrates, phagocytosis has been associated with the production of ROS, such as superoxide anion, hydrogen peroxide, and hydroxyl radical, which are highly toxic to bacteria.[30] The SOD is the first line of defense to remove superoxide anion, while CAT removes hydrogen peroxide (H_2O_2) from the previous reaction.[37,66] The recording of SOD and CAT activities can be classified as an indirect measurement of the action of leukocyte effector molecules (ROS) to eliminate microorganisms.[30] The increased activity of SOD with H1 may have been directly related to IL-1β gene expression under the same treatment. Pro-inflammatory cytokines, such as IL-1β, TNF-α and interferon-γ, have been shown to induce ROS production via the NADPH oxidase pathway in different cell types.[67] de Oliveira et al[16] observed that the use of Canova stimulated the production of ROS and NO (nitric oxide) in macrophages, increasing NADPH oxidase activity and inducible NO synthase respectively.

Despite increased SOD activity in H1-treated fish, CAT activity was not higher than that of the control (except for liver in EJ). Higher SOD activity may suggest higher catalyzing superoxide anion to H_2O_2, which could also mean more substrate action for CAT (to transform H_2O_2 into water and molecular oxygen). However, different investigations have not reported such reciprocal behavior (direct relationship). For example, in several experiments with xenobiotics in fish, it has been observed that certain conditions have given rise to high SOD and low CAT activities, and vice versa.[68,69] Guzmán-Villanueva et al[70] observed an increase in SOD activity with *Lutjanus peru* fed diets supplemented with 0.1% β-1,3/1,6-glucan, whereas no significant changes of CAT activity were observed; something similar to that was observed in the intestine of WS and EJ fish treated with H1. In the same species, Reyes-Becerril et al[43] observed that fish immunized with *V. parahaemolyticus* toxin ToxA-Vp exhibited higher CAT activity than the control group without direct proportional changes in SOD activity. Therefore, no single activity pattern for SOD and CAT has been observed, since physiological, experimental or developmental state conditions may vary and induce such differences. It is also important to mention that the catalysis developed by CAT can also be fulfilled by glutathione peroxidase.[71] For this reason, future investigations should include this last enzyme for better understanding of the enzymatic antioxidant process.

Currently, three main types of hypothetical mechanisms of action for homeopathic medicines are under consideration: nanobubbles-related hormesis, vehicle-related electric resonance, and quantum non-locality.[72] Nonetheless, further studies are needed to corroborate whether and which of these hypotheses may be related to potential cellular effects of homeopathic preparations.[73] In our experience, rather than using physical-chemical methods to corroborate or prove any of these hypotheses, selective gene expression can be used instead to demonstrate the effect of homeopathic medication at transcriptomic and functional levels. In the effect of treatments with fish challenged with *V. parahaemolyticus,* H1 elicited an over-expression of IL-1β and MyD88 during the challenge, whereas H2 caused an over-expression of all the genes analyzed with respect to the unchallenged control. The H2 treatment contained *Phosphoricum acidum* and *Silicea terra*. Various investigations have indicated that nanoparticles (NPs) are formed during the dilution and succussion process, to which the biological effects of certain ultra-diluted substances have been largely attributed.[48,64,71,74]

Chikramane et al[74] mentioned that silver grinding with lactose and subsequent successive dilution and succussion up to 7c can be achieved to obtain 200 ng/mL of silver NPs. In our research, the biological effect of H2 during the challenge could have been due to the NPs that might be formed from *Phosphoricum acidum* and *Silicea terra* MTs during the dilution–succussion process up to 7c. Several investigations have mentioned the potential of certain NPs to stimulate the immune system: mainly silica, gold and silver.[75] In this regard, homeopathic medications containing *Phosphoricum acidum* and *Silicea terra* have biological and functional effects, such as improved growth (height and weight), as well as survival and enhancement of immune response against *V. alginolyticus* in juvenile Catarina scallop *A. ventricosus*.[14] Nevertheless, the reason that a greater relative expression of the evaluated genes was not provoked in unchallenged WS and EJ may be that the NPs that could be formed (dilution-succussion) by themselves did not cause a stimulating effect. The opposite effect observed before and after challenge was a marked feature of the homeopathy (H2: PhA 7c + SiT 7c) used in this work because H2-treated fish showed a gene over-expression after challenge. In our case, the challenge with *V. parahaemolyticus* could be classified as a stressor, which may be the reason that fish subjected to the H2 treatment have responded with a greater expression of the genes evaluated in response to such stimuli.

As discussed before, we are not sure that NPs were generated by the succussion process; nonetheless, vast information exists concerning the biological effect of silica NPs. Compared with other NPs, nanosilica has a well-documented ability to

mobilize the immune/inflammatory and stress response networks of the body.

The stimulatory potential of NPs has been reported to be closely linked to the size and shape of the particles.[76,77] There is a discussion concerning particle sizes and their effects on cytokine stimulation because highly variable and contradictory results – and even null cases – have been observed.[78–80] Some evidence has been reported that under certain conditions some NPs by themselves do not cause inflammation and require synergistic effects: for example, with other NPs to induce inflammatory responses in macrophages[81] or any type of immunosuppression.[82] This last reason allows us to suggest a pathway by which H2 could stimulate the expression of IL-1β challenged with *V. parahaemolyticus*, which has been described as having NPs as adjuvants (nano-vaccines) by Boraschi and Italiani[81] based on the principles of vaccines. In this potential scenario, the bacterial stimulus (PAMPs) would trigger activation via TLR-MyD88-NF-kB causing expression of IL-1β; furthermore, H2 would increase IL-1β expression through induced inflammasome activation by a slight stimulus of NPs of *Phosphoricum acidum* and *Silicea terra* recognized as damage associated with molecular patterns. On the other hand, over-expression of piscidin with H2 may have a direct relationship with over-expression of IL-1β rather than a possible stimulation per se of H2; as this cytokine promotes the recruitment of phagocytes and a greater number of cells stimulated by pathogens, its expression in the tissue may tend to be higher.[82]

Besides the activity pattern of antioxidant enzymes during challenge, we hypothesize that the increased SOD activity of phagocytes could have been induced because of the ability of IL-1β to enhance superoxide production to eliminate bacteria during the phagocytosis process. One of the main roles of this enzyme is to avoid oxidation of cell structures and thus oxidative stress.

Conclusion

This investigation observed that the homeopathic treatments used caused an effect that could be detected and evaluated at the molecular and enzymatic levels. Our results and recent literature have indicated that the cells have the capacity to receive these signals and translate them to maintain physiological balance.

In many cases, the non-expected responses in relation to homeopathic experimentation may have been due to differences in experimental methods between studies or forms and preparation of these medications (*i.e.*, succussion mechanism).

In our case, the use of tools such as *omics* (*i.e.*, transcriptomics, proteomics, metabolomics, metagenomics and interactomics) may continue to elucidate the possible routes of action of these compounds. Finally, we consider that the homeopathic medications used in this study in *S. rivoliana* can be used for preventive purposes which, together with an appropriate diet and good management practices, can improve welfare conditions of cultivated organisms at different levels of their development.

Highlights

- Effect of two homeopathic treatments on immune and antioxidant response in *Seriola rivoliana* was evaluated.
- Homeopathy generated from *Vibrio* pathogenic strains caused stimulation of immune system in *Seriola rivoliana*.
- The expression of MyD88 and IL-1β by nosode seems to initiate a cascade of intracellular signaling initiated by the recognition of a PAMP by the TLR-like receptor.

Supplementary Files

Supplementary File 1 Ethics Committee letter
Supplementary File 2 The ARRIVE Guidelines Checklist

Conflict of Interest
None declared.

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