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Anti-inflammatory effects of aloe vera on soy meal-induced intestinal inflammation in zebrafish

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ABSTRACT

Soybean meal is one of the most promising alternatives to replace fishmeal in the aquaculture industry. However, its ingestion triggers an intestinal inflammatory process that compromises fish health and nutrition. Therefore, finding strategies that reduce the deleterious effects of a soy protein-based diet are relevant. In this work we analyzed the effects of an aloe vera (*Aloe barbadensis* miller, AV) extract on intestinal inflammation and innate immunity of zebrafish by adding it to the water and by supplementing it in a soybean meal-based diet. To search for potential immunomodulatory effects of AV, we tested its effectiveness in two inflammation assays and compared fish fed with either fishmeal or soybean meal-based feed supplemented with AV. Our results show a strong anti-inflammatory effect of AV. Furthermore, while soy-based meal strongly induces the expression of inflammation markers, supplementation with AV reverted this effect. Finally, we show that fish fed with a soy meal diet are highly susceptible to bacterial infection, but that this condition is significantly reduced when the soy meal is supplemented with AV. Our results suggest that AV is a good candidate to be incorporated as an additive in farmed fish diets to facilitate the replacement of fishmeal by soybean meal, maintaining intestinal health.

1. Introduction

The exponential growth of the aquaculture industry has led to an increase in the requirement of new protein sources for the formulation of diets for farmed fish [14]. Soybean meal (SBM), is one of the most widely used alternatives to replace fishmeal (FM), mainly due to its high protein content [33]. However, its ingestion triggers an inflammatory process in the distal portion of the fish intestine that seriously compromises the animals' health. Intestinal inflammation in this context is characterized by morphological and functional alterations, with shortening of mucosal folds, a decrease in the number of absorption vacuoles, an increase in goblet cells and a decrease in epithelial enzymatic activity [3,7,27,42]. Despite these adverse effects, SBM remains one of the best options to replace FM due to it being a balanced, low-cost and widely available protein source. Therefore, it is crucial to explore the inclusion in SBM of additives that can reduce or prevent the harmful effects that this legume-based food produces in fish [25,37]. Under this scenario, aloe vera (*Aloe barbadensis* Miller, AV) may represent a viable and convenient supplement due to its natural origin and multiple beneficial properties originating from the more than 75 bioactive compounds present in the internal gel of its leaf [1,10,20,21,23,35]. The combined action of vitamins, amino acids, phenolic compounds, enzymes, minerals, organic acids, lipids and carbohydrates contained in this gel seems to be responsible for the multiple beneficial properties of AV. The polysaccharide acemanans are the best known because of their proven immunomodulatory effects [32,40]. Previous studies have shown that the inclusion of AV in the diet has a strong anti-inflammatory effect, decreasing the level of pro-inflammatory cytokines and histopathological damage in gastric mucosa in rats with gastric ulcers [12]. Likewise, the application of anthrones and chromones present in the AV leaf to a cell line of murine macrophages previously stimulated with lipopolysaccharides inhibits the cyclooxygenase pathway, decreasing the production of prostaglandin E2 and pro-inflammatory cytokines [31]. On the other hand, AV has been shown to have an immunostimulating effect. The administration of an ethanolic extract of AV to chickens with coccidiosis

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increases the lymphoproliferative responses and the levels of total IgG and IgM [2]. Similarly, studies in common carp and rainbow trout fed with different levels of AV showed increased phagocytic activity of macrophages and IgM levels [17]. This immunostimulating effect has also been confirmed by *in vitro* studies, revealing an antibacterial effect of AV gel by inhibition of the growth of resistant strains of *Helicobacter pylori* [5]. Despite these findings, there is still no knowledge of the potential protective effect of AV on intestinal homeostasis when it is added to the diet of fish. One of the possible causes of this lack of information is the high cost and long timeframes required for trials to test food additives in species used in aquaculture. A convenient solution to this problem is the use of the zebrafish (*Danio rerio*). By carrying out preliminary studies in this species, it is possible to achieve applicable results with significantly lower costs and shorter trial times. The zebrafish has other key advantages, such as transparency during the early larval stages, allowing *in vivo* monitoring of fluorescently labeled immune cells [38], a wide range of transgenic lines and a well-known biology. Additionally, zebrafish larvae fed with SBM diet, showed an inflammatory process with similar characteristics to those occurring in commercially cultured fish [18].

The current study evaluated the effects of two preparations of AV gel on the function of the zebrafish innate immune system [39]. Using a transgenic line that fluorescently labels neutrophils, Tg(BACmpo:GFP)^{i114}, we evaluated the effect of AV using two inflammation models: i) inflammation triggered by a water-borne chemical present in the water or ii) by the intake of a soybean meal based diet. To complement these experiments, we also measured the mRNA level of immune response gene markers by RT-qPCR as well as the influence of AV on the response triggered by infection with *Edwarsiella tarda*. Our results indicate that AV elicits a clear anti-inflammatory effect, protecting the intestine of fish fed with an SBM based diet.

2. Materials and methods

2.1. Zebrafish strains and maintenance

Zebrafish were maintained and raised in the Laboratory of Fish Immunology, Universidad Andrés Bello, Chile, according to standard protocols. The following strains of fish were used in this study: TAB5 (wild type), Tg(BACmpo:GFP)^{i114} [38] and Tg(Brn3c:mGFP) [44]. All embryos were collected through natural spawning according to Kimmel et al. [24]. Eggs were incubated in petri dishes at 28 °C in E3 medium (5mMNaCl, 0.17mMKCl, 0.33mMCaCl2, and 0.33 mM $MgSO_4$, with methylene blue, equilibrated to pH 7.0). Embryonic and larval stages are expressed in hours postfertilization (hpf) or days postfertilization (dpf).

2.2. Aloe vera growth conditions and gel preparation

The AV gel was obtained from the leaves of *Aloe barbadensis* Miller plants grown in the Experimental Station of the University of Chile, located in the IV Region of Coquimbo, Chile. It has been reported that the sugar structure (linear vs branched) found in the AV gel depends on the osmotic conditions of plant growth [39]. We hypothesized that the immunomodulatory effects of AV could vary between the two growth conditions used and, therefore, we decided to use AV gel from plants grown in normal conditions (AV1) or under hydric stress (AV2).Thus, plants were subjected to two irrigation treatments, which consisted of AV1: 2000 and AV2: 0 mL of water per plant supplied every day, for three months. 40 fresh leaves harvested from plants subjected to each treatment were disinfected with NaClO (50 mg/L), and then the gel was separated from the bark under sterile conditions. The extracted gel was liquefied and stored at −80 °C. When needed, a gel aliquot was lyophilized, homogenized and stored at room temperature.

Table 1
Dilutions and concentrations of aloe vera stock solution in E3 medium.

Dilution	Concentration (g/L)
1:10	1
1:100	0.1
1:1000	0.01
1:5000	0.002

2.3. Incubation with aloe vera gel

For the incubation with AV, serial dilutions of both AV1 and AV2 gel were made. Briefly 1 g of the lyophilized AV gel was dissolved in 100 ml of Milli-Q water, obtaining a stock solution with a concentration of 10 g/L. From this stock solution the different dilutions were made in the E3 filtered medium (Table 1).

The different dilutions and stock solution were stored at −20 °C and thawed at room temperature overnight prior to conducting any experiment. 3 dpf Tg(BACmpo:GFP)^{i114} larvae were incubated for 24, 48, 72 and 96 h with two concentrations for both AV1 and AV2. As a control group, larvae were incubated in E3 medium. At least 15 larvae for each dilution were used, and each assay was performed in triplicate in three independent experiments.

2.4. Chemically induced inflammation assay (ChIn)

The protocol of damage to neuromasts with $CuSO_4$ was performed according to what was described by D'Alençon et al. [8]. Briefly, 3 dpf Tg(BACmpo:GFP)^{i114} larvae with less than 5 neutrophils in the dorsal region of the tail were selected to make groups of 15 larvae in 6-well plates (M8562; Sigma) in a volume of 6 ml of E3 solution. 10 mM $CuSO_4$ solution (copper II sulfate pentahydrate, catalog No. 102780, Merck, Darmstadt, Germany) were added to each well, and incubation was carried out for 40 min at 28 °C. Six treatment groups were performed: E3 (negative control), with $CuSO_4$ (positive control), and four groups previously incubated for 1 h with AV: AV1 0.1 g/L + $CuSO_4$; AV1 0.01 g/L + $CuSO_4$; AV2 0.1 g/L + $CuSO_4$ and AV2 0.01 g/L + $CuSO_4$. To ensure that hair cells were indeed damaged, Tg(Brn3c:mGFP) larvae (which have fluorescently labeled hair cells) were incubated in parallel with the same $CuSO_4$ solution. After incubation, larvae were washed and the number of neutrophils around the third neuromast (L3) was quantified *in vivo* in each larva.

2.5. Diets

Two basal diets were formulated and prepared to contain either fishmeal as the primary protein source or 50% soybean meal as partial fishmeal replacement (Table 2). One batch of each diet was supplemented with AV1 or AV2 in concentrations of 0.2 g/kg (SBM + AV1; SBM + AV2; FM + AV1, FM + AV2) and 0.4 g/kg (2SBM + AV1; 2SBM + AV2; 2FM + AV1, 2FM + AV2). All diets were supplemented with a standard vitamin and mineral premix and formulated to be isoenergetic and isonitrogenous. Diets were prepared by cooking-extrusion in a twin screw extruder (CLEXTRAL BC-21, Clextral, Firminy, France) with 2 mm diameter. The resulting moist pellets were oven-dried at 60 °C for approximately 8 h and then coated with fish oil, according to the formulation for each diet, using a laboratory vacuum coater (Dinnissen Model VC10, Sevenum, Netherlands). The pellets were subsequently crumbled, screened to the appropriate particle size (75 μm), and stored at −20 °C until use in the feeding trials.

2.6. Feeding strategy

The feeding strategy was performed as previously described by

Table 2
Ingredients and nutrient composition of basal diet.

Ingredients g/kg	FM	SBM
Fish meal	610	250
Soybean meal	0	500
Wheat grain meal	255	115
Starch	45	45
Fish oil	30	60
Vitamin mix	15	15
Mineral mix	15	15
Cellulose	30	0
Total	1000	1000
Analytical composition (dry base, %)		
Dry matter	96.21	95.23
Proteins	49.17	48.75
Etheric extract	3.47	1.69
Fiber	3.37	2.60
No nitrogenous extract	31.52	36.43
Ash	12.47	10.53
Gross energy (MJ/kg)	19.423	19.341

Hedrera et al. [18]. Briefly, 90 larvae were fed two times daily from 5 to 8 dpf. The last feed was given at least 16 h before larval fixing to promote intestinal emptying.

2.7. Immunohistochemistry

Immunohistochemistry was performed as previously described by Westerfield [46]. The following antibodies were used: rabbit anti-Green Fluorescent Protein (anti-GFP) (Cat. number A11122, Invitrogen); antirabbit peroxidase (Cat. number A8275, Sigma); rabbit anti-Cox2 (anti-Cox2; Cat. Number 12282, Cell Signaling, Technology). Stain was developed by using the ImmPactTM DAB peroxidase substrate kit (Vector, Cat: SK-4105). The neutrophils and Cox-2 positive cells present in the intestine were quantified within a defined area that included the mid and posterior intestine. At least 30 larvae were analyzed per diet in three independent experiments [13,18].

2.8. Quantitative polymerase chain reaction (qPCR)

Larvae fed with control and experimental diets were sampled at the end of each treatment for total RNA extraction. Total RNA was extracted from a pool of ~90 intestines per diet. The whole intestine was dissected from a larva anesthetized in tricaine methanesulfonate using sterile instruments. Samples were stored in RNAlater solution and then homogenized in TRIzol Reagent (Cat. Number 15596-026, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA samples were treated with DNAse (DNAse RQ1, Promega, Cat: M6101) and the corresponding cDNA libraries were synthesized from 1.5 μg of total RNA using SuperScript II Reverse Transcriptase (Cat. number 100004925, Invitrogen, Carlsbad, CA, USA) and Oligo-dt primers. Primer sequences are shown in Table 3. Each gene was analyzed in triplicate, with the average of the Ct values of each sample normalized against the average value of the Ct of the reference gene (Rpl13α). qPCR was performed with the ABI 7300 Real-Time PCR system using the Maxim SYBR Green/ROX qPCR Master Mix (2x) (Fermentas, Waltham, MA, USA) following the manufacturer's instructions. A 15 μL reaction volume was used, containing 1 μL of 10-fold diluted cDNA. The PCR was run with a ten minute activation and denaturation step at 95◦C, followed by 40 cycles of 30 s at 95◦C, 30 s at 57–60◦C, and 30 s at 72◦C. Reaction specificity was verified using melting curve analysis and by confirming the absence of primer dimers. Standard curves were obtained for each pair of primers by plotting Ct values against the $\log_{10}$ of seven different dilutions of a cDNA mix solution for all analyzed samples. Real-time PCR efficiency (E) was calculated from a standard curve according to the equation $E = 10(-1/\text{slope})$. Relative expression was calculated with the Pfaffl method [34].

Table 3
Primer sequences used for amplification of specific genes through RT-qPCR.

Gene	Primer	Sequences (5' → 3')
rlp13a	F	TCTGGAGGACTGTAAGAGGTATGC
	R	AGACGCACAATCTTGAGAGCAG
il-1β	F	TGGACTTCGCAGCACAAAATG
	R	GTTCACTTCACGCTCTTGGATG
il-10	F	CACTGAACGAAAGTTTGCCTTAAC
	R	TGGAAATGCATCTGGCTTTG
muc2.2	F	ACACGCTCAAGTAATCGCACAGTC
	R	TCAGCGAGTGTTTGGCTCACTT
mmp9	F	CATTAAAGATGCCCTGATGTATCCC
	R	AGTGGTGGTCCGTGGTTGAG
b-def-1	F	CTCCTTGTCGTACTAGCATTGCAC
	R	ACACACTCCTTGTCTGCAAACACC
mbl	F	GTGAGGATGAGAATAAAGTGCT
	R	GTTAGTGAAAGTTAGAGGCTGG
gpx1a	F	AGGCACAACAGTCAGGGATT
	R	CAGGAACGCAAACAGAGGG
gstr1	F	CGATAAGAAGGAGCACCAGA
	R	GCCATTTCAGCAGGATTGT
cox2a	F	ACTACCCCTGAGCTTCTCACA
	R	GATGCTGTTGATGATATCCCAGATTG

2.9. Edwardsiella tarda challenge

The *Edwardsiella tarda* challenge was performed as previously described by Ulloa et al. [41]. Briefly, an *E. tarda* culture was grown overnight at 28 °C in a trypticase soy broth medium (TSB) with agitation. The overnight culture was diluted to 1:100 in fresh TSB and incubated at 28 °C to reach 10^8 CFU/mL. The culture was pelleted by centrifugation at 1500*g* for five minutes, washed with water from the aquarium system, and repelleted to recover bacteria. Clean *E. tarda* was resuspended again in water from the aquarium system to reach 10^8 CFU/mL. After four days of feeding, a group of 30 larvae were challenged for 5 h in 200 mL of the *E. tarda* water media and were subsequently transferred to a tank with new aquarium water. The larvae were fed during the entire challenge period, and larvae mortality was monitored every 12 h for four days. As a negative control for the challenge assay, larvae were maintained in aquarium water following the same feeding strategy as challenged larvae. Mortality was monitored in parallel to challenged larvae. This assay was performed at least in three independent times.

2.10. Imaging

Larvae images were taken using a QImaging MicroPublisher 5.0 RVT camera attached to an Olympus SZX16 stereoscope. All images were processed with Photoshop CS6 and ImageJ 1.44°, showing the representative effects of each treatment.

2.11. Statistical analysis

Neutrophil quantification was analyzed using one way ANOVA and RT-qPCR using *t*-tests with Welch's correction. Survival data from the *E. tarda* challenge were analyzed using Kaplan-Meier and group differences were analyzed by the log-rank test, using the Bonferroni correction for multiple comparisons. The RT-qPCR data was analyzed relative to the Ribosomal protein L13α transcript. All analyses were performed using Graph Pad Prism 6 Software (Graphad Software, Inc). Significance was established for all analyses at $P < 0.05$.

2.12. Ethical statement

This study was carried out in strict accordance with the recommendations included in the "Guidelines for the care and use of fish in research" [11]. The protocol was approved by the Bioethics

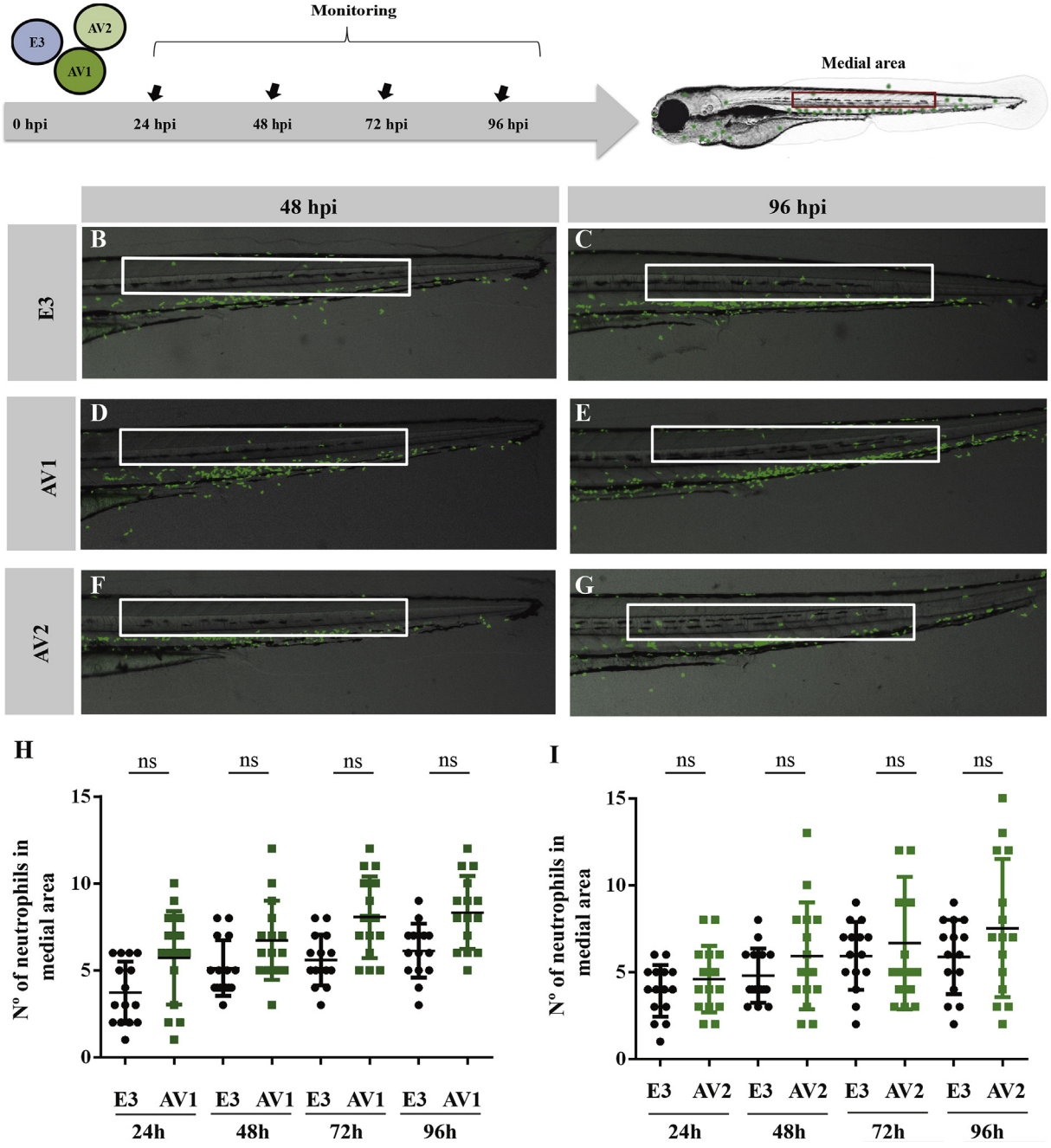

Fig. 1. Aloe vera on its own does not exert an immunostimulatory effect when added to the medium. (A) Assay Strategy. Tg(Bacmpx:GFP)i114 where incubated in aloe vera with normal water irrigation (AV1) or without water irrigation (AV2) and the effect on immune response was monitored every 24 h for 4 days. The presence of neutrophils at the medial area (red rectangle) was used as indicator that an immune response was triggered. (B–G) Lateral view of the caudal region of control and experimental larvae at 48 and 96 h post incubation (hpi). (H, I) Quantification of neutrophils present at the medial area in control and experimental larvae. At least 15 larvae per condition were analyzed in three independent experiments. Statistical analysis was performed using one way ANOVA. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns: no significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Committee of the Faculty of Biological Sciences University Andres Bello (008/2016).

3. Results

3.1. Aloe vera on its own does not exert an immunostimulatory effect when added to the medium

To determine if AV1 or AV2 added to the fish medium on their own triggers an immune response in zebrafish we examined the behavior of neutrophils present in the larval body after exposure to each extract (Fig. 1 A). Since neutrophils are the first immune cells to respond after tissue damage, these granulocytes can be used as markers for an inflammatory process. Normally, they reside in the caudal hematopoietic tissue (ventral to the somites) and their presence outside of this area is indicative of inflammation. We incubated 3 days post fertilization (dpf) Tg(BACmpo:GFP)^{i114} transgenic larvae, which have neutrophils fluorescently labeled, in 0.1 or 0.01 g/L AV1, and quantified their recruitment to the medial area (in the vicinity of the horizontal myoseptum) at 24, 48, 72 and 96 h post incubation (hpi) (Fig. 1B–G). The results

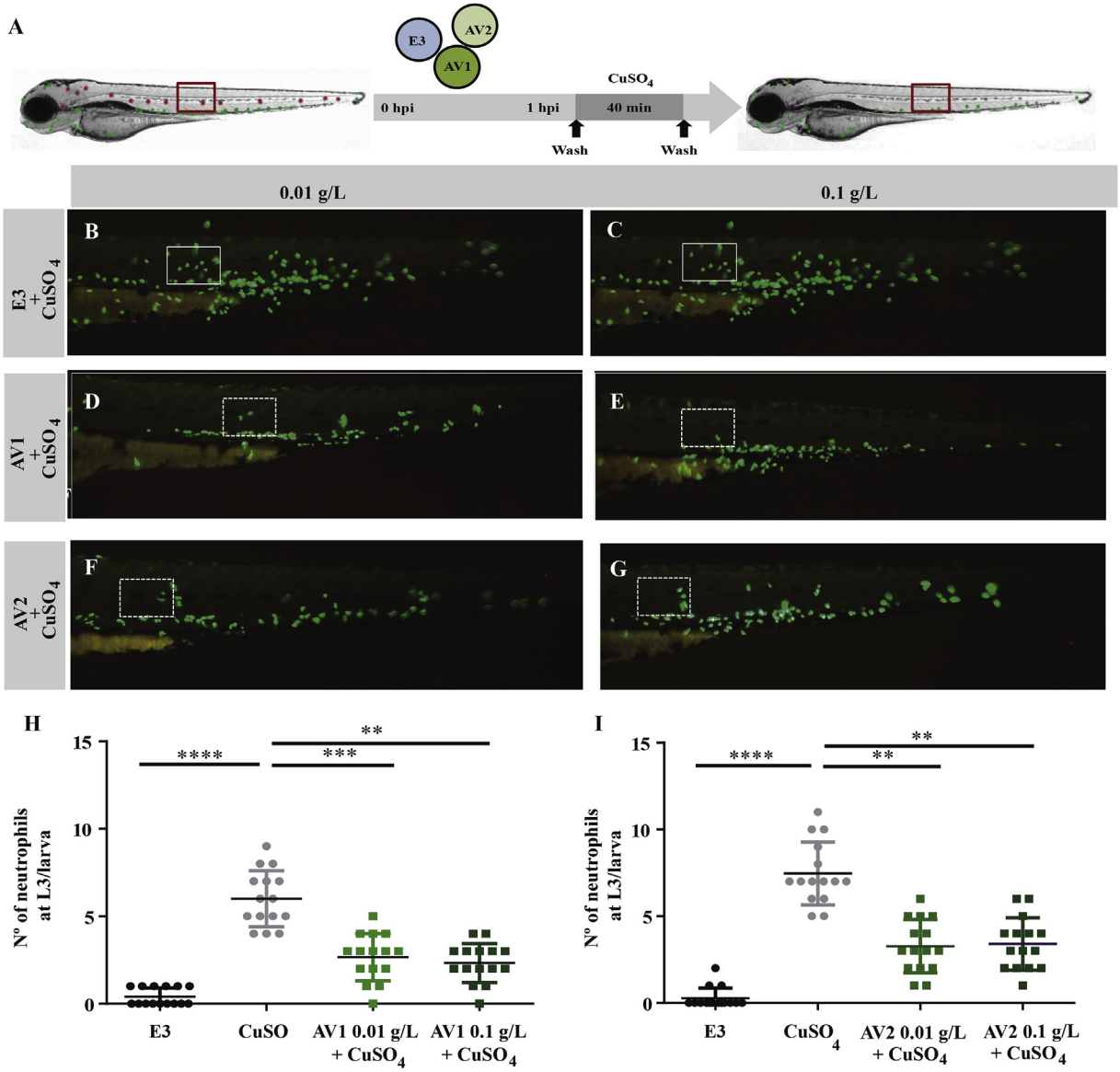

Fig. 2. Aloe **vera exerts anti-inflammatory effect when added to the medium.** (A) Scheme of CHIn assay. Larvae were incubated with AV1 or AV2 during 1 h, treated with CuSO4 for 40 min, and then the number of neutrophils (green dots) present around L3 neuromast (red dot delimited with a red rectangle) was quantified. (B–G) Lateral view of a segment from the tail of control and experimental Tg(Bacmpx:GFP)i114 larvae with L3 neuromast delimited in white. (H, I) Quantification of neutrophils present at the L3 region in control and experimental larvae. At least 15 larvae per condition were analyzed in three independent experiments. Statistical analysis was performed using one way ANOVA. **p < 0.01; ***p < 0.001; ****p < 0.0001; ns: no significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

obtained showed no significant increase in the recruitment of neutrophils to the medial area at any time point analyzed using either AV1 (Fig. 1 H) or AV2 (Fig. 1 I). Therefore, AV extracts dissolved in the water do not, on their own, induce an inflammatory response in zebrafish larvae.

3.2. *Aloe vera exerts an anti-inflammatory effect when added to the medium*

To analyze whether AV1 or AV2 have an anti-inflammatory effect on zebrafish larvae we used a previously developed test, the ChIn assay, where an inflammatory process is triggered by exposure to $CuSO_4$ [8]. 3 dpf Tg(BACmpo:GFP)^{i114} larvae were pre-incubated for 1 h with 0.1 and 0.01 g/L AV1 or AV2, an then treated with 10 μM copper sulfate for 40 min. The number of neutrophils recruited to the third neuromast was quantified and compared to control animals (Fig. 2 A). To verify that $CuSO_4$ exposure effectively elicits neuromast damage in our hands, we treated Tg(Brn3c:GFP) larvae in parallel. In these fish, exposure to $CuSO_4$ causes the disappearance of neuromast hair cells (Fig. 2 B, C). The fluorescently labeled cells in this transgenic line also allowed the visualization of the position of the third neuromast (L3, white square), where neutrophil quantification was performed. The quantification revealed that both AV1 and AV2 exerted an anti-inflammatory effect (Fig. 2D–I). Whereas in copper treated controls an average of 7 ± 0.5 neutrophils were detected at the third neuromast, in AV1 treated fish the number of neutrophils diminished to 3 ± 0.3 with the two different concentrations used (Fig. 2 H) ($p < 0.001$). In the case of both concentrations of AV2, the reduction of neutrophils present in the third neuromasts was 3 ± 0.4 (Fig. 2 I) ($p < 0.01$).

3.3. *Aloe vera exerts an anti-inflammatory effect when added to the diet*

To analyze the effect of AV supplemented feed on soybean meal triggered intestinal inflammation, 6 diets were prepared: a fishmeal

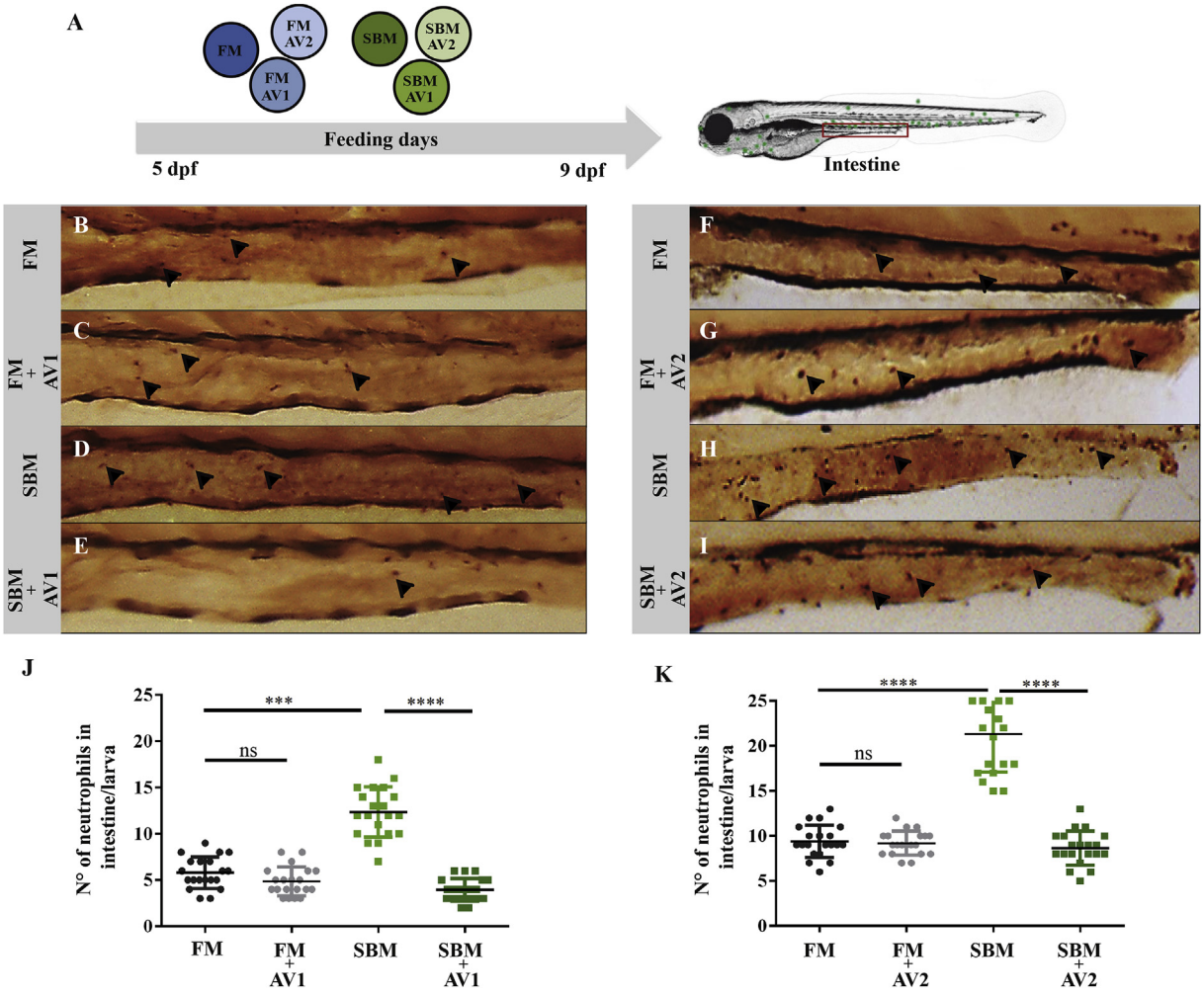

Fig. 3. Aloe vera exerts an anti-inflammatory effect when added to the diet. (A) Assay strategy. Scheme highlighting the intestinal territory selected for quantification (red rectangle). (B, I) Lateral view of the intestine of tg(Bacmpx:GFP)i114 larvae fed with control and experimental diets after anti GFP immunohistochemistry, highlighting the presence of GFP + cells (black arrowhead). (J–K) Quantification of the number of neutrophils present at intestine in control and experimental larvae. At least 15 larvae per condition were analyzed in three independent experiments. Statistical analysis was performed using one way ANOVA. ***p < 0.001; ****p < 0.0001; ns: no significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

based diet (FM) as a negative control, a soybean meal based diet (SBM) as intestinal inflammation positive control, and a batch of each diet supplemented with AV1 or AV2 at a concentration of 0.2 g/kg (FM + AV1, FM + AV2, SBM + AV1 and SBM + AV2). 5 dpf Tg (Bacmpo:GFP)^{i114} larvae were fed during 4 days with each diet, according to the protocol established by Hedrera et al. [18]. After this period, neutrophils present in the intestine were quantified (Fig. 3 A). As expected and described previously [18], larvae fed FM have an average of 6 ± 0.4 and 9 ± 0.4 neutrophils present in the intestine (Fig. 3 B, F). This value doubled in the case of larvae fed with SBM (Fig. 3 D, H) ($p < 0.001$), corroborating the development of an inflammatory process induced by the soybean meal. The addition of AV1 or AV2 to the FM diet did not significantly alter the number of neutrophils present in the intestine (Fig. 3 C, G), showing an average of 5 ± 0.3 and 9 ± 0.3, respectively. On the contrary, the supplementation of the SBM diet with AV1 or AV2 led to a strong decrease in the number of neutrophils present in the intestine when compared with larvae fed with SBM alone (Fig. 3 E, I). The decrease was from an average of 13 ± 0.6 to 4 ± 0.3 ($p < 0.0001$) in the case of AV1 (Fig. 3 J), and from 20 ± 0.3 to 8 ± 0.4 ($p < 0.0001$) in the case of AV2 (Fig. 3 K). These results are indicative that neither AV1 nor AV2 exert an immunostimulatory effect in a normal dietary condition (FM diet), but both extracts induce a potent anti-inflammatory effect when incorporated into the SBM diet.

3.4. Effect of aloe vera on the immune response, mucosal function and antioxidant status markers

To corroborate the anti-inflammatory effect exerted by AV1 and AV2, described above, we quantified the number of Cyclooxygenase 2 (Cox-2) expressing cells in the intestine of larvae fed with control and supplemented diets by immunohistochemistry. As expected, the number of intestinal Cox-2 positive cells in larvae fed SBM is significantly higher than that observed in larvae fed FM diet, 30 ± 1.7 and 12 ± 1.7 respectively (Fig. 4 A, B) ($p < 0.0001$). The addition of AV1 or AV2 to the FM diet had no effect on the number of Cox-2 positive cells, while the addition of AV1 or AV2 to the SBM diet drastically reduced their number from 30 to 16 ± 0.9 in the case of AV1 and from 30 to 18 ± 1.5 by the addition of AV2 ($p < 0.01$ and $p < 0.05$ respectively).

To complement the neutrophil migration assay and the Cox-2 immunohistochemistry results, we analyzed the effect of AV1 and AV2 on the transcription of genes related to inflammatory processes (*il-1β, il-10, mmp9, cox-2a and mbl*), mucosal barrier function (*muc2.2 and β.def-*

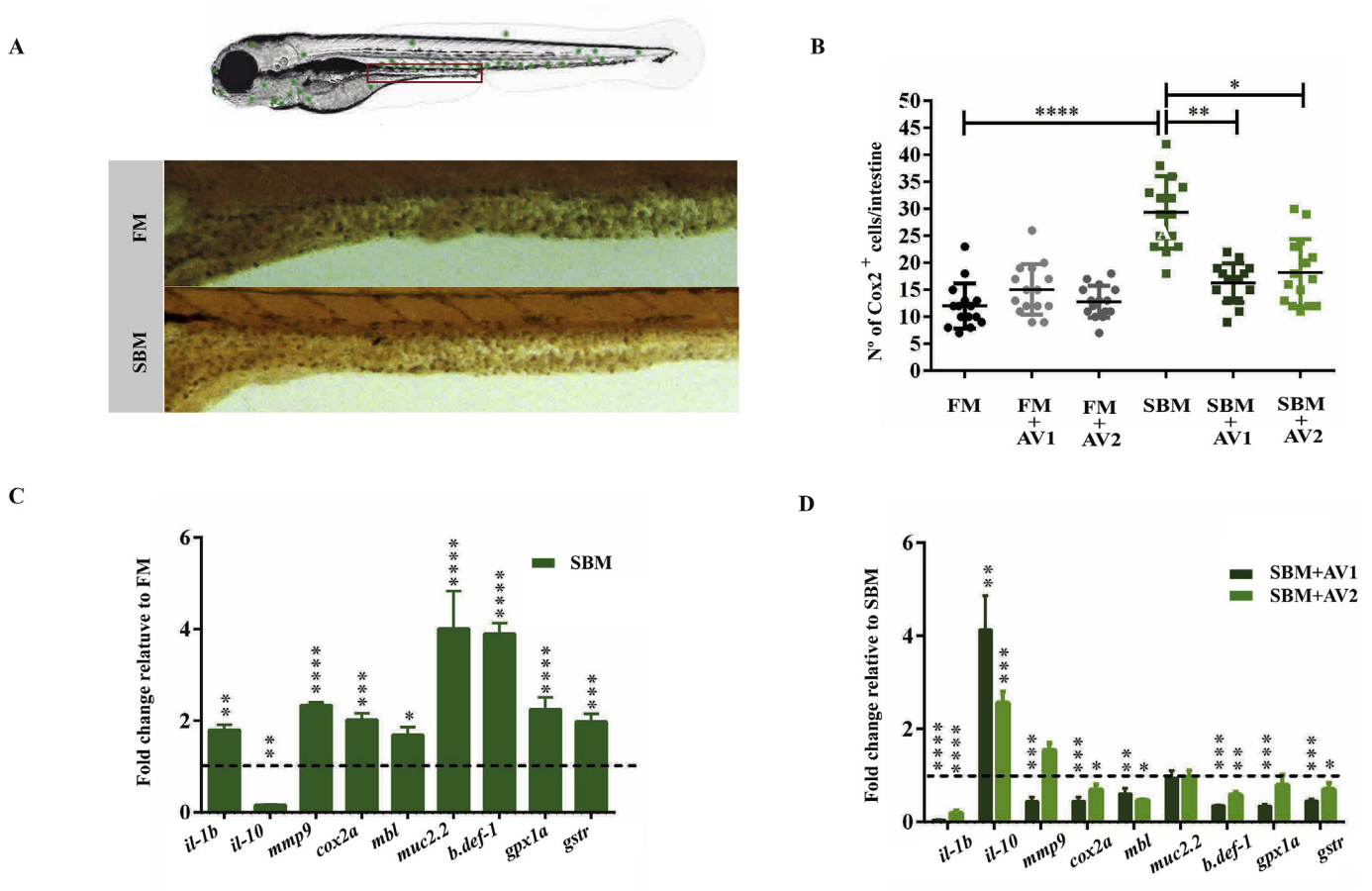

Fig. 4. Effect of aloe vera on the immune response, mucosal function and antioxidant status markers. (A) Lateral view of mid intestine of a larva fed FM or SBM diet after anti Cox-2 immunohistochemistry. (B) Quantification of the number of Cox-2 + cells in the intestine of control and experimental larvae. (C–D) Quantification of the relative expression of the mRNA level of immune, mucosal barrier and antioxidants gene markers. The data was normalized against the housekeeping gene *rpl13* and compared to those obtained in larvae fed with FM (C) or SBM (D). At least 15 larvae per condition were analyzed in three independent experiments. Statistical analysis was performed using one way ANOVA. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.000$.

1) and antioxidant activity (*gpx1a and gstr*) by qPCR. The results obtained (Fig. 4 C), show a significant increase in the transcription of genes involved in the inflammatory processes, mucosal barrier and antioxidant enzymes, in the SBM condition compared to FM fed fish. In contrast, when AV1 or AV2 was incorporated to the SBM diet, an anti-inflammatory effect was detected. This was reflected in a reduction in the mRNA level of genes related to inflammation (*il-1β, mmp9, cox-2a* and *mbl)* and an increase in the anti-inflammatory cytokine *il-10* (Fig. 4 D). In the case of the mucosal barrier markers, only *b.def*-1 mRNA showed a significant decrease in larvae fed with SBM + AV1 or SBM + AV2 when compared to those fed with SBM. In addition, neither AV1 nor AV2 affected *muc2* transcription. Finally, the incorporation of AV1 to the SBM diet decreased the mRNA level of both antioxidant enzymes genes analyzed, *gstr* and *gpx1a*. On the contrary, the addition of AV2 only decreased transcription of *gstr* (Fig. 4 D). All together, these results support the conclusion that AV elicits anti-inflammatory effects as predicted by the neutrophil migration assay result.

3.5. Aloe vera increases the survival of larvae challenged with Edwardsiella tarda

To determine whether, in addition to the anti-inflammatory effect of AV, we could enhance immune performance against pathogens in zebrafish larvae, we challenged larvae fed with control and supplemented diets with *Edwarsiella tarda* and determined the cumulative mortality up to 72 h post challenge. First, we determined that larvae fed the SBM diet have significantly higher mortality compared to those fed with the FM diet when challenged with bacterial infection (Fig. 5 A) ($p < 0.01$). Further, when larvae were fed the FM diet supplemented with AV1 or AV1 and then challenged, no change in cumulative mortality was observed when compared to challenged larvae fed with FM alone, indicating that no protective effect against bacteria was exerted by AV1 or AV2 under normal (non-inflammatory) conditions. In contrast, when larvae were fed with SBM + AV1 or SBM + AV2 and then challenged, a significant decrease in mortality was observed when compared to challenged larvae fed with SBM alone ($p < 0.001$ and $p < 0.01$ respectively). Importantly, the cumulative mortality of larvae fed either with SBM + AV1; SBM + AV2; FM; FM + AV1 or FM + AV2 were indistinguishable from each other.

We wished to determine if the protective effect observed with AV1/AV2 added to SBM could be enhanced or whether it could be extended to animals fed with FM. We doubled the amount of AV, from 0.2 g/kg to 0.4 g/kg (2FM; 2FM + AV1; 2FM + AV2; 2SBM; 2SBM + AV1; 2SBM + AV2), both for AV1 and AV2. Despite the increase in AV extract added to the feeds, the cumulative mortality induced by *E. tarda* infection remained similar to that observed with the lower AV concentration (Fig. 5 B). This result indicates that, while AV does not *per se* protect against *E. tarda* infection, the decrease in mortality observed in larvae fed with AV supplemented SBM is likely due to the improvement in intestinal health and reduced inflammation induced by AV that we have revealed in these experiments.

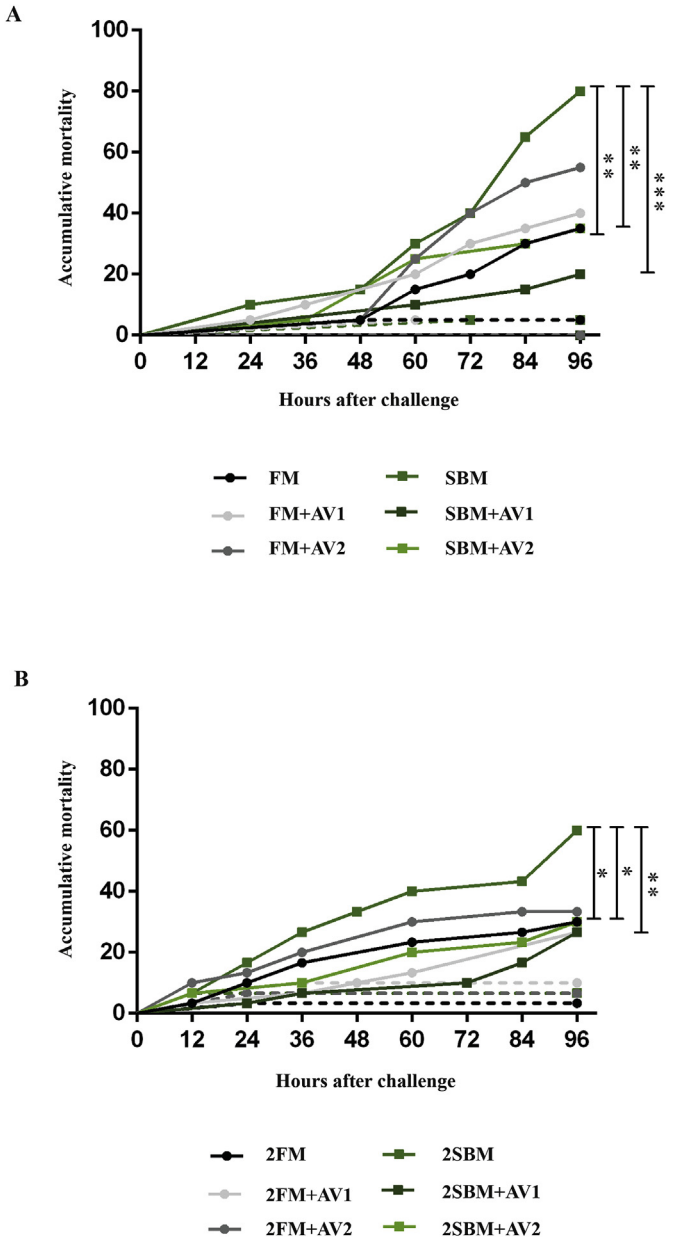

Fig. 5. Aloe vera increases the survival of larvae challenged with *Edwardsiella tarda*. 9 dpf larvae were challenged with *Edwardsiella tarda* after four days of feeding with different diets (FM, FM + AV1, FM + AV2, SBM, SBM + AV1 and SBM + AV2), where AV was at 0.2 g/kg (A) or 0.4 g/kg (B). Mortality was monitored immediately after the challenge and every 12 h over four days until 13 dpf. Statistical analysis was performed using survival curve analysis with the log-rank test against FM and SBM diets. *p < 0.05; **p < 0.01; ***p < 0.001. Continuous lines represent challenged larvae, while dotted lines represent control larvae.

4. Discussion

It is known that AV has multiple medicinal properties, among others, its immunostimulating and anti-inflammatory effects, through different forms of administration, as topical or in feed [35]. However, there is very little information about its effect on fish as an aquatic or dietary additive [47,48]. In this work, we demonstrated that the addition of AV to the medium of zebrafish larvae subjected to chemical damage of neuromasts by $CuSO_4$, has a protective effect at the surface level, decreasing the number of neutrophils in the affected area. Additionally, we observed that the AV added to a soybean meal-based diet prevented the induction of intestinal inflammation normally caused by this food source. The anti-inflammatory effect was evidenced by a reduction in the number of neutrophils present in mid-intestine and by changes in gene expression.

The effect of AV on neutrophil recruitment to the affected area could occur due to a decrease in the inflammatory response and/or to the total or partial inhibition of the damage generated by the soy-derived components. Since we observed diminished neutrophil infiltration in a superficial sensory organ damage assay (ChIn) where sensory cells were killed with $CuSO_4$, we conclude that the AV did not prevent the damage caused by $CuSO_4$, but rather, affected neutrophil migration or the generation of pro-inflammatory signals by the damaged tissue.

In the case of the effect of AV as an additive in a diet with high SBM content, the reduction in the infiltration of neutrophils in the intestine could be the product of the following effects:

1 Total or partial inhibition of the inflammatory process through a physical effect, generated by the sequestration or quenching of the toxic components present in the SBM, without allowing them to contact the intestinal mucosa. Through this means, AV would avoid the increase in intestinal permeability reported by different studies, which leads to the subsequent recognition of dietary antigens or the intestinal flora that are naturally present [26]. This physical effect could also be given by the protection of the intestinal epithelium with the AV components themselves, for instance by coating the mucosal layer that would prevent direct contact with the SBM derivatives.
2 Through a prebiotic effect of AV, as reported by Guo et al. [15,16]. In this case, AV would strengthen elements of the intestinal microflora (which can be modified by SBM consumption), decreasing the proliferation of pathogenic bacteria in the environment, which influences or triggers enteritis [3].
3 AV could act by decreasing the inflammatory process by promoting rapid recovery to the SBM toxicity, due to the activation of macrophages [50]. This would lead to a more efficient arrival of neutrophils to the inflamed area and a rapid inflammatory resolution. This possibility is supported by our observation that after 4 days of feeding SBM + AV, neutrophil numbers and gene expression levels returned to levels similar to the FM control condition.
4 A decrease in the SBM-induced inflammatory process due to AVs modulation of different cellular mechanisms, such as acting as an antioxidant decreasing the level of reactive oxygen species (ROS) in the cellular environment, and thus indirectly diminishing the inflammatory process [22].

In this work, we did not examine the effect of AV on the intestinal permeability, the intestinal microflora, activation of macrophages or the level of the inflammation prior to the 4 days of feeding. For this reason, the 3 first explanatory theories on the effect of AV on inflammation cannot be dismissed or proven.

Through this study, it is possible to verify the effect of AV as an anti-inflammatory agent through various cellular mechanisms. In the case of the FM diet supplemented by AV (FM + AV), no reduction was observed on neutrophil migration, the differences of what occurred when added to the SBM diet (SBM + AV), indicating its effect only in the inflammatory conditions, and not in the homeostasis conditions. In line with the reduction in the number of neutrophils present in the intestine, a decrease in Cycloxygenase 2 protein levels and the mRNA level of pro-inflammatory cytokines and other immune-related genes, such as *il-1β, cox-2, mmp9* and *mbl*, was observed. Even though an anti-inflammatory effect exerted by AV has been reported in several animal models, using different forms of administration [4,21,51], the mechanism through which it is generated is not yet fully known. There is evidence correlating the AV anti-inflammatory effect with the inhibition of critical signaling pathways such as NF-κB, P38, JNK and ERK that result in a lower activation of the inflammosome Nrlp3 protein complex,

necessary to activate the cleavage of pro IL-1, thus suppressing the expression of pro-inflammatory cytokines downstream of IL-1 [4]. Considering that IL-1β is a key cytokine during the early inflammatory response, the reduction of neutrophils observed in the intestine could be a direct consequence of this mechanism. Also, and in agreement with our results measuring Cox-2 protein, it has been reported that AV induces a reduction in the levels of prostaglandin F2α, a substance produced by COX-2 from arachidonic acid [6,12,19].

In addition to the anti-inflammatory results, we observed an antioxidant effect exerted by the AV gel extract, as evidenced by a reduction in the mRNA level of two antioxidant enzymes, glutathione peroxidase (GPx) and glutathione S-transferases (GST). It is reported that, in the presence of oxidative stress, the transcription of GPx and GST increase in order to control the high level of ROS produced [45]. In agreement with that, we observed that in larvae fed the SBM diet, an increase in the transcription level of GPx and GST in the intestine was induced, suggesting that oxidative stress was increased. On the contrary, the inclusion of AV to the SBM diet decreased these enzymes' transcription level to those observed in larvae fed the control diet (FM). There is evidence to indicate that the internal gel of the AV leaf has multiple components that act in a synergistic manner to activate an antioxidant system; inhibiting enzymes involved in the production of ROS [6], and increasing the bioavailability of antioxidants [29], among others. For example, present in this gel are anthraquinones a group of molecules that comprise aloe-emodin, barbaloin, aloin, among others. These compounds have been shown through mouse model and *in vitro* studies, to have a high capacity to capture ROS, reducing their concentration in the environment, and thus decreasing cellular damage [46]. Likewise, in zebrafish it has been shown that an enzymatic extract prepared from AV showed a protective effect against an oxidative stress-inducing agent and cell death [22]. In addition, in obese rats, has been demonstrated that the level of GSH and catalase activity increases significantly when treated with AV gel, at the same time that oxidative markers decrease their levels [36].

We did not detect an immunostimulatory effect of the AV gel extract, either when added to medium or when incorporated into the diet. In the latter situation, fish fed FM + AV1 or FM + AV2 did not increase the amount of neutrophils outside the caudal hematopoietic tissue compared to fish fed FM, as would have been expected. In addition, AV did not decrease mortality after the bacterial infection challenge. Several reports indicate that AV has an immunostimulant effect, increasing resistance to pathogens [2,5,17]. It is proposed that the polysaccharide acemannan, present in the AV gel, exerts this effect, because it has a structure similar to the ligand for the mannose receptors present in macrophages, thus activating them and enhancing the immune response [6,30]. The lack of an immunostimulant effect in this study may be due to multiple reasons, but the most likely is that we measured an end point variable (mortality). Other studies have considered direct effects on the immune response, such as antibody titer [9], phagocytic activity, respiratory burst [47], as well as lysozyme and complement activity [49]. As in our study, Zanuzzo et al. [48], incorporated AV in a diet for steelhead trout, and after 6 weeks of feeding did not observe significant effects on resistance of the fish to *Aeromona salmonicida*. In the case of the bacterial challenge, larvae fed with the diets SBM + AV1 or SBM + AV2, showed a significant reduction in accumulated mortality, in relation to the SBM group. The mortality observed in SBM + AV1 or SBM + AV2, was similar to the FM group. This improvement in mortality observed when adding AV to the SBM group is possibly a product of better general health status, due to the reduction in intestinal inflammation observed in previous studies [28].

We subjected AV plants to two irrigation treatments, AV1 to 2000 ml/d and AV2 at 0 ml/d, this to seek for changes arising from the hydric stress that the plants are subjected to. In our experiments, no significant differences were observed between AV1 and AV2. It is known that hydric stress treatment of the AV plant, although it modifies the structure of polysaccharides such as acemanans and fructans present in the internal gel [40]. However, our results indicate that these changes do not necessarily generate a greater or lesser immunomodulatory effect. Importantly, the effects of AV are not altered by irrigation regimes, and the beneficial properties of the extract are maintained despite the growth conditions of the aloe plants.

The present study highlights the positive effect of AV gel extract when added to fish feed or in the growth medium of larvae, demonstrating a potent anti-inflammatory effect during acute or chronic inflammation processes. Critically for farmed fish, we show that the addition of this extract in soybean meal-based feed can be a solution to the toxicity imposed by the plant proteins in the diet and can contribute to the replacement of fish meal-based diets. We also suggest that benefits may include reduction of oxidative stress, improved resistance to pathogens and enhanced regenerative abilities in tissues.

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