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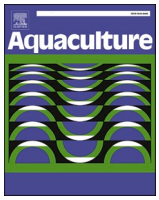

Phytogenics enhance welfare and vaccine efficacy against *Vibrio anguillarum* in European seabass (*Dicentrarchus labrax*) juveniles

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ABSTRACT

Disease prevention is pivotal in aquaculture, and while vaccines offer protective immunity, challenges such as cost and low efficacy persist. The present study investigated the potential of plant-derived compounds, known as phytogenics, to bolster the effectiveness of vaccines against vibriosis in European seabass. Two phytogenic blends, namely PHYTO1 (terpenes) and PHYTO2 (terpenes and flavonoids) were supplemented to a commercial diet to obtain three experimental diets: a non-supplemented control diet, PHYTO1 (a 200-ppm blend of garlic and Lamiaceae oils with 87.5 mg kg^{-1} terpenes), and PHYTO2 (a 1000 ppm blend containing citrus fruits, Asteraceae and Lamiaceae oils with 57 mg kg^{-1} terpenes and 55 mg kg^{-1} flavonoids). Following vaccination by bath immersion, juvenile European seabass were divided into groups and fed one of the three diets for 30 days. After this feeding period, fish were anesthetized and boosted with a single dose of vaccine through intraperitoneal injection. They continued to be fed their respective diets for another 30 days. At day 60, after the priming vaccination, fish were challenged with *Vibrio anguillarum* via intraperitoneal injection. Various parameters were measured at different time points post each vaccination, including total weight, circulating plasma cortisol and glucose levels, serum immunoglobulin M (IgM) titers, antioxidant power of leucocytes, and the expression of several antioxidant and immune-related genes. The results showed that fish fed with phytogenic supplements did not differ in weight compared to the control group. However, they exhibited lower plasma cortisol and glucose levels, increased IgM titers, and enhanced antioxidant protection and antioxidant power of head kidney leucocytes. In addition, phytogenics upregulated several immune-related genes in the gills and head kidney immediately after each vaccination. Notably, PHYTO2, enriched with flavonoids and terpenes, exhibited an even more pronounced positive effect on boosted fish by reducing vaccine-associated stress while improving antioxidant protection and modulating the vaccine-induced immune response. This synergistic effect of vaccination combined with phytogenics introduces new pathways for enhancing fish health in aquaculture.

1. Introduction

The aquaculture sector is currently facing the challenge of coping with increasing production while using new or alternative raw materials to replace fishmeal (FM) and fish oil (FO) in aquafeeds in a climate change context (Naylor et al., 2021). Marine fish fed low FM and FO diets can display a gut inflammation-like status (Torrecillas et al., 2017) or dysbiosis (Rimoldi et al., 2020), even when growth rate and feed efficiency are not altered (Torrecillas et al., 2017). Such issues can further affect fish health under intensive farming settings, such as high

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stock densities and repeated handling, which not only increase the vulnerability of farmed fish to infectious diseases but can also potentiate the spread of such diseases (Kotzmann and Stonebridge, 2022). Because pathogen proliferation and transmission rates are higher at higher temperatures, warm-water marine species, such as those from the Mediterranean, are more susceptible to disease than cold-water species, such as salmonids (Leung and Bates, 2013). The ability of the aquaculture industry to effectively handle aquatic diseases and improve fish robustness under the challenging situations of climate change and increased international transactions, which will alter the geographic distribution of pathogens and their interactions with hosts, will significantly affect its success (Moreira et al., 2021). Whilst historical data indicate that skillful disease management leads to profits for producers and advantages for consumers, large-scale disease outbreaks can threaten the role aquaculture is expected to play in global food production in the next decade (FAO, 2020). Indeed, the aquaculture industry faces an annual estimated economic loss of $6 billion USD worldwide due to diseases (World Bank, 2014).

To prevent disease outbreaks, extensive efforts have been directed toward the development of vaccines, which have been shown to be positively correlated with protective immunity (Mohd-Aris et al., 2019; Galindo-Villegas et al., 2019). To be commercialized, vaccines must be cost-effective, safe, suitable for large-scale production, and provide protection against multiple pathogens (Mondal and Thomas, 2022). Although vaccines can prevent animal mortality, reduce reliance on antibiotics, and improve animal welfare (Barnes et al., 2022), vaccination still faces challenges such as the occurrence of side effects, high production costs, and low efficacy (Galindo-Villegas, 2020; Semple and Dixon, 2020). Commercially available vaccines using inactivated pathogens as antigens, which have demonstrated efficacy in Mediterranean species, have limited applicability. These vaccines typically provide short-term protection lasting, only a few weeks. Administration is usually done through immersion during early life stages to prevent intraperitoneal injection (IP)-related lesions and stress-induced susceptibility to disease (Miccoli et al., 2019). However, it is important to note that vaccination by immersion is significantly less effective than IP vaccination (Du et al., 2022). In many cases, booster vaccination is recommended to enahnce or extend the immune protection conferred by a particular antigen (Ruyter et al., 2023).

Plant-derived feed additives (phytogenics), such as plant extracts and essential oils with beneficial health effects, are potential alternatives to chemotherapeutics in the management of diseases in aquaculture species (Galindo-Villegas et al., 2022). Phytogenics such as alkaloids, terpenoids, flavonoids, and polyphenols, have been shown to have growth-promoting, antimicrobial, immune stimulating, anti-inflammatory, sedative, and antioxidant properties in several animal species (Firmino et al., 2021b). In aquatic organisms, phytogenics prevented the harmful effects of heavy metal exposure and improved the health status of carp (*Cyprinus carpio*) (Mohiseni et al., 2017). Specifically, in tilapia (*Oreochromis niloticus*), phytogenics improved digestive enzyme and antioxidant production as well as immune response (Mohammady et al., 2022). In warm-water marine species, such as European seabass (*Dicentrarchus labrax*), dietary supplementation with phytogenics has been shown to improve overall health outcomes by providing protection against oxidative stress (Serradell et al., 2022; Torrecillas et al., 2021) and improving gut health (Rimoldi et al., 2020; Torrecillas et al., 2019). In addition, Serradell et al. (2020) observed reduced cortisol levels and improved immune function (increased serum lysozyme levels and downregulation of apoptosis-related gene expression) in response to infection. Interesting results have also been observed in various warm-water marine species, including greater amberjack (*Seriola dumerili*). When supplemented with phytogenics rich in Lamiaceae and garlic (*Allium sativum*) oils, greater amberjack presented a notable decrease in both plasma and skin mucus cortisol levels after a stress challenge, concomitant with an enhanced response in the skin-associated lymphoid tissue (Fernández-Montero et al., 2021). Despite their recognized potential to improve disease resistance and prevention in European seabass, the effects of phytogenics on vaccine efficacy have not yet been evaluated in this species, which is particularly susceptible to stress- and pathogen-related diseases (Toranzo et al., 2005).

The present study aimed to evaluate the effect of two phytogenic dietary supplements, one containing terpene only and the other a mixture of terpenes and flavonoids, on the efficacy of a commercial vaccine against vibriosis in juvenile European seabass. This disease is primarily caused by the bacterium *Vibrio anguillarum* and is the leading cause of mortality and associated economic losses in farmed *D. labrax* (Frans et al., 2011). Available vaccines against vibriosis for this fish species have shown moderate efficacy (Miccoli et al., 2019) and require of effective adjuvants or other strategies to potentate it (Galindo-Villegas et al., 2013). The effects of the two phytogenic supplements on stress response (plasma cortisol), immune function [serum immunoglobulin (Ig)M], expression of immune- and oxidative stress-related genes], and performance against pathogens (survival) after vaccination were tested.

2. Materials and methods

2.1. Ethical considerations

Animal manipulation complied with ARRIVE guidelines, Directive 210/63/EU, and Spanish legislation (RD 53/2013), and was approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (REF: OEBA -ULPGC 25/2020).

2.2. Fish husbandry and feeding procedures

Dicentrarchus labrax juveniles ($n = 750$, mean weight $= 8.52 \pm 0.82$ g; sex is not differentiated at this stage) were housed in a recirculating aquaculture system at the Marine Biosecurity Station of Parque Científico-Tecnológico Marino (University of Las Palmas de Gran Canaria, Telde, Canary Island, Spain). Fish were distributed among nine 500-L cylindroconical tanks and acclimated at 6.6–6.1 ppm of dissolved oxygen, 20.2 °C, and a 12:12 light: dark photoperiod. They were fed thrice a day to satiation for 30 days. Three experimental diets were tested, all formulated to meet the nutritional requirements of *D. labrax* and following commercial standards (Table 1). The control diet remained without supplementation, whereas the experimental diets, denoted as PHYTO1 and PHYTO2, were enriched with a blend of phytogenics sourced from Delacon, Engerwitzdorf, Austria. Specifically, PHYTO1 comprised a 200-ppm liquid blend containing garlic and *Lamiaceae*-plants oil, with a terpene content of 87.5 mg kg^{-1} in pellet form. In contrast, PHYTO2 included a 1000 ppm solid blend consisting of citrus fruits, *Asteraceae*, and *Lamiaceae*-plants oil, with terpene content at 57 mg kg^{-1} and flavonoid content at 55 mg kg^{-1} in pellet form. Notably, Delacon's internal product codes for PHYTO1 and PHYTO2 were identified as SBPMH02 and SBPMH03, respectively.

All diets were industrially produced by extrusion at BioMar Tech-Centre (Brande, Denmark). To ensure product stability, the blends of phytogenics were homogenized with dietary oil and included by vacuum coating during the post-extrusion process. The stability of the functional products was checked before diet production and at the beginning of the feeding trial. The proximate composition of each diet (Table 1) was analyzed according to standard procedures (AOAC, 2023). Dry matter content was determined after drying in an oven (110 °C) to constant weight, and ash content was determined by combustion in a muffle furnace (600 °C, 12 h). Crude lipids were extracted as described in Folch et al. (1957) and crude protein content was determined (N Å ~ 6.25) using the Kjeldahl method (AOAC, 2023).

2.3. Vibrio anguillarum vaccination and challenge tests

The vaccination protocol entailed two sequential routes of

administration of the vaccine against *V. anguillarum* (de Ruyter et al., 2023; Miccoli et al., 2019) (Fig. 1). At the onset of the experiment ($t = 0$, t0), all fish ($n = 750$) were primed with the vaccine (Icthiovac VR©, HIPRA, Amer, Girona, Spain) for 1 h by bath immersion. The vaccine was added directly to the culture tank (1:500 dilution) and the water level was reduced to a minimum, following commercial recommendations, and mirroring industrial conditions. After vaccination, each group of fish was fed one of the three diets (control, PHYTO1, or PHYTO2) for 30 days. At this time point ($t = 30$), fish were anesthetized (1:1, 0.2 mL L^{-1} natural pure clove oil:EtOH 70%) and revaccinated with a single dose of Icthiovac VR/PD© (0.1 mL, HIPRA) via IP injection. Fish groups were fed the respective experimental diet for another 30 days. Blood samples were collected by caudal sinus puncture using 1 mL syringes at 7, 14, 21, and 30 days after vaccination by immersion (primed fish) and IP injection (boosted fish) and immediately prepared for determining plasma cortisol and glucose concentrations or serum IgM titers.

At 60 days after the first vaccination ($t = 60$), 20 fish per tank (60 per dietary treatment) were challenged with *V. anguillarum* via IP injection [10^5 colony forming units (CFU) mL^{-1}, strain 507, isolated from a clinical outbreak in the Canary Islands] and transferred to nine 500 L tanks (three tanks per diet) to evaluate responses to the challenge test. The *V. anguillarum* dose inoculated was previously determined using a bacterial concentration gradient in similar dietary and culture conditions; the 10^5 CFU mL^{-1} dose was selected by common linearization of sigmoidal curves. Fish survival was recorded daily and described through Kaplan-Meier curves for each dietary treatment. *V. anguillarum* was confirmed as the causative agent of death by standard biochemical procedures.

2.4. Plasma cortisol and glucose concentrations

An aliquot (approximately 0.5 mL) of each blood sample collected from four fish per tank (12 per diet) at 30 days after each vaccination ($t = 30$ and $t = 60$) was used to determine the concentrations of cortisol and glucose in circulating plasma. Immediately after collection, aliquots were placed in 1.5 mL Eppendorf tubes coated with heparin, to avoid clotting, and centrifuged for 5 min at 3000 ×*g* and 4 °C to obtain plasma samples. These were stored at −80 °C until plasma cortisol and glucose analyses. The concentration of circulating plasma cortisol was determined using the Access Cortisol assay kit (Beckman Coulter, Inc., Brea, CA, USA) at AnimaLab (Las Palmas de Gran Canaria, Canary Islands, Spain). The concentration of circulating plasma glucose was determined via the hexokinase method for in vitro diagnosis using glucose reactive OSR6521 (Beckman Coulter Inc.) and the chemistry analyzers AU2700® and AU5400® (both Beckman Coulter Inc.).

2.5. Serum IgM titers

An aliquot of each blood sample (approximately 1 mL) taken at 7, 14, 21, and 30 days after each vaccination step was allowed to clot overnight at 4 °C. After centrifugation for 5 min at 3000 ×*g* and 4 °C, a serum sample was obtained and stored at −80 °C until the enzyme-linked immunosorbent assay (ELISA) to quantify specific IgM titers against *V. anguillarum* was performed. Briefly, *V. anguillarum* were resuspended in phosphate-buffered saline (PBS) at 1×10^8 bacteria mL^{-1} and added to ELISA plates 100 µL per well. After overnight incubation at 4 °C, glutaraldehyde [50 µL, 0.05% (*v*/v) diluted in PBS] was added to each well and the plates were incubated for 20 min at room temperature (22 °C). Bovine serum albumin (BSA, 250 µL) was then added to each well to block non-specific binding. The plates were incubated at 22 °C for 2 h and then washed twice with low-salt wash buffer. Fish serum (100 µL, diluted 1:2 in PBS) or PBS (control) was added to each well and incubated for 3 h at 22 °C. The plates were washed five times with high-salt wash buffer and incubated for another 5 min. Reconstituted (0.006 µg $µL^{-1}$) anti-European Sea bass IgM monoclonal antibody (Aquatic Diagnostic Ltd., Stirling, Scotland) was added to each well (100 µL) and the plates were incubated for 60 min at 22 °C. After washing with high-salt wash buffer five times, plates were incubated for another 5 min and anti-mouse IgG-horseradish peroxidase conjugate (diluted 1:1000 in conjugate buffer, 100 µL, Sigma-Aldrich, St. Louis, MO, USA; secondary Ab) was added to each well. The plates were incubated for another 60 min at 22 °C, washed five times with high-salt wash buffer, and incubated for 5 min. A TMB chromogen solution (100 µL, Sigma-Aldrich) was added to each well and the plates were incubated for 10 min at 22 °C. The reaction was stopped by using a stop solution (50 µL, Sigma-Aldrich). The following controls were included in ELISA plates and factored into the computations: samples devoid of bacteria but with serum, primary Ab, and secondary Ab; samples devoid of serum but with bacteria, primary Ab, and secondary Ab; and samples containing bacteria and serum but without primary Ab. Plates were read in an ELISA reader (Multiskan GO, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 450 nm.

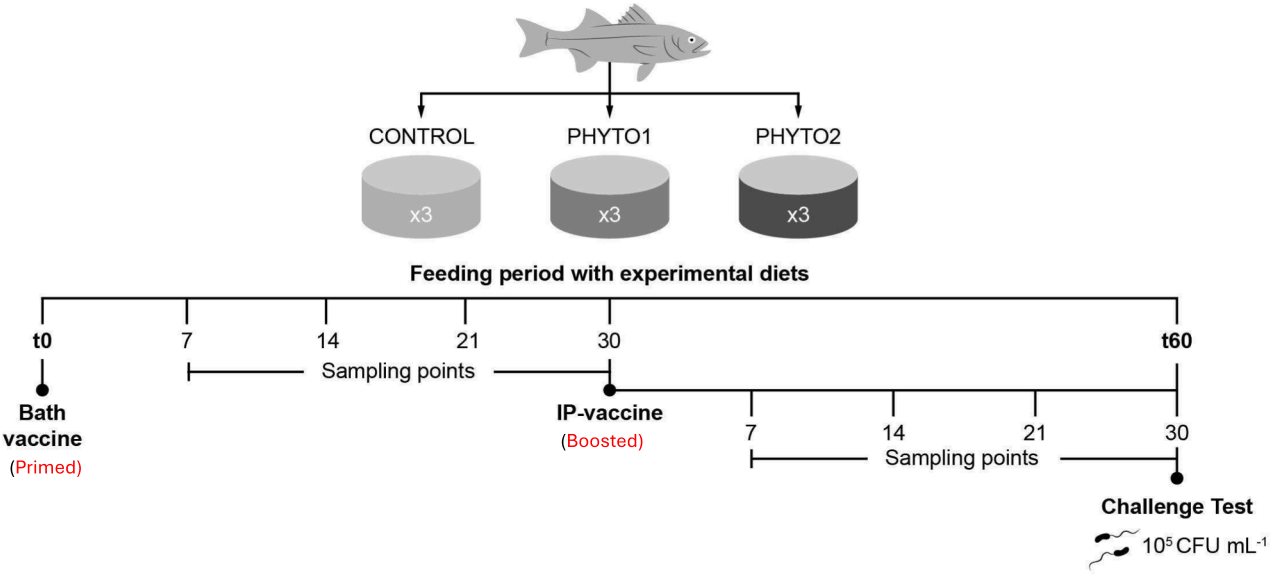

Fig. 1. Experimental timeline in *D. labrax* juveniles, including the dietary treatments, vaccination protocol, and challenge tests against *V. anguillarum*.

2.6. Leucocyte total antioxidant capacity

Just before IP vaccination (0 h, primed fish) and at 24 h, 14 days, and 30 days post-IP vaccination, three fish per dietary treatment were sampled for analyzing the total antioxidant capacity of head-kidney leucocytes as their ferric reducing antioxidant power (FRAP). Cells were isolated as previously described (Román et al., 2015). Viable cells were then stained with trypan blue, counted in a Neubauer chamber, and transferred to Cell Freezing Medium-dimethyl sulfoxide (DMSO) 1× (Sigma-Aldrich) at 10^6 cells mL^{-1} and froze at −80 °C until analysis. Prior to antioxidant capacity measurement, the Cell Freezing Medium-DMSO 1× solution was washed from leucocytes by dilution in 500 μL PBS followed by centrifugation for 15 min at 1100 ×*g* and 4 °C. This procedure was repeated four times to completely remove DMSO. A final step of sonication for 1 min at 30 Hz was performed to lyse the leucocytes. The FRAP of leucocytes was determined at 560 nm using a Ferric Antioxidant Status detection kit (Invitrogen, Thermo Fisher Scientific, Inc.). Final optical density (OD) measurements were performed in an Infinite® PRO200 plate reader (Tecan Trading AG, Männedorf, Switzerland).

2.7. Gene expression

At $t = 7$ and $t = 30$ before priming and after booster IP vaccination, six fish per tank (18 per dietary treatment) were euthanized by an overdose of anaesthetic (clove oil). Head-kidney and gill samples were then collected to assess the relative expression of antioxidant- and immune-related genes through real-time quantitative PCR (RT-qPCR) analysis as previously described (Torrecillas et al., 2021). Briefly, total RNA was extracted from tissue samples using TRI Reagent (Sigma-Aldrich) and a RNeasy® mini-kit (QIAGEN, Hilden, Germany), according to the manufacturers' instructions. The RNA was quantified by spectrophotometry (NanoDrop 1000, Thermo Fisher Scientific Inc.) and its integrity assessed on a 1.4% agarose gel using Gel Red™ (Biotium Inc., Hayward, CA, USA). Total RNA was reverse transcribed in a 20 μL reaction volume containing 2 mg of total RNA using a ThermoScript™ Reverse Transcriptase Kit (Invitrogen, Thermo Fisher Scientific Inc.) and a Mastercycle® Nexus GSX1 thermocycler (Eppendorf AG, Hamburg, Germany), following the manufacturers' instructions. Samples were then diluted (1:10) in Milli-Q® water (Sigma-Aldrich) and stored at −20 °C. An aliquot of the cDNA obtained by reverse transcription was then amplified by RT-qPCR using a primer set specifically designed for each target gene based on the cDNA sequences available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) for *D. labrax*. The target and reference (β-actin) genes, their GenBank accession numbers, and the sequences of the primers used are listed in Supplementary Table 1.

The RT-qPCR was carried out in a final volume of 20 μL, containing 3 μL of cDNA (100 ng), 10 μL of iTaq Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), and 500 nM of each primer, and using the CFX96 thermocycler (Bio-Rad Laboratories), according to the manufacturer's instructions. The profile was 1 min at 95 °C followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. A blank sample containing nuclease-free water instead of the cDNA template was included in each assay as a negative control. Relative expression levels were calculated using the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001) and toward *β-actin* levels. Considering that the amplification efficiencies of the target and *β-actin* genes must be approximately equal, the CFX Maestro™ Software (Bio-Rad Laboratories) was used to calculate the efficiency of each primer set. The same software allowed selecting the appropriate reference gene based on the average M value and analyzing gene stability by means of the reference gene selection tool. To calculate expression levels, we related the PCR signal of the target transcripts in the treatment groups (PHYTO1 and PHYTO2) to that of control samples.

2.8. Statistics

Observations >1.5 times the interquartile range (1.5 IQR) below the first quartile or >1.5 IQR above the third quartile were considered outliers (Hoaglin and Iglewicz, 1987) and excluded from the analyses. To assess survival rates over time, Kaplan-Meier curves were applied to the different dietary treatments. Differences between survival curves were evaluated by means of a log-rank test (Dudley et al., 2016; Ranstam and Cook, 2017). Quartile normality of residuals was tested using a Kolmogorov-Smirnov test, and residual homogeneity of variance was assessed through Levene and Bartlett tests. When significant heterogeneity of residuals was found, the data were square root- or log-transformed. If this approach did not eliminate heterogeneity, the analysis was performed on non-transformed data using the F-test. This test was used for analyzing plasma cortisol levels with "diet" and "vaccination method" as predictor variables. Three-way analysis of variance (ANOVA) with "diet", "vaccination method", and "time" as predictor variables was applied to IgM titers and gene expression. Two-way ANOVA with "diet" and "vaccination method" as predictor variables was applied to gene expression for a given time and to leucocyte FRAP with "diet" and "time" as predictor variables. Post-hoc multiple comparisons were performed on diet upon statistical significance returning Tukey-corrected *p*-values. The admissible error was set at $\alpha = 0.05$ (Underwood, 1997).

3. Results

3.1. Growth

After 8 weeks of starting the trial, the final weight and number of fish in each cage were recorded to measure growth performance following 24 h of starvation. Neither growth nor natural mortality were promoted or affected by any phytogenic extract. The values obtained for each treatment were as follows: Initial weight T0 (8.58 ± 0.12 g; 8.68 ± 0.35 g; 8.75 ± 0.18 g), and Final weight T60 (36.52 ± 6.12 g; 36.93 ± 8.94 g; 35.67 ± 7.05 g), values corresponding to the Control, PHYTO1, and PHYTO2 treatments, respectively.

3.2. Cortisol and glucose concentrations in circulating plasma

Plasma cortisol and glucose levels were found to be higher ($p < 0.05$) in boosted fish (Fig. 2 B, D) compared to primed fish (Fig. 2 A, C). Surprisingly, both primed and boosted European seabass fed the PHYTO diets exhibited significantly lower plasma cortisol ($F = 38.28$, $p < 0.001$) and glucose ($F = 19.59$, $p < 0.0001$) concentrations than fish fed the control diet (Fig. 2). The effect of PHYTO diets in reducing plasma cortisol concentration relative to that of control fish was more evident in primed fish (PHYTO1 by 66% and PHYTO2 by 63%) than in boosted fish (PHYTO1 by 34% and PHYTO2 by 50%). However, the effect of PHYTO1 was only statistically significant in primed fish ($p = 0.008$, Fig. 2 A), whereas PHYTO2 was significantly effective ($F = 32.13$, $p < 0.0004$, Fig. 2 A, B) throughout the experimental period. As for plasma glucose concentrations (Fig. 2 C, D), PHYTO1 was more effective in boosted fish than in primed fish (31% vs. 18%) whereas PHYTO2 presented the opposite trend (by 32% in primed fish and by 30% in boosted fish) ($F = 23.41$, $p < 0.0001$).

3.3. Serum IgM titers

Serum IgM titers (Fig. 3) were apparently higher in boosted than in primed fish, although these differences were not significant ($F = 3.993$, $p = 0.051$), and generally increased with time post vaccination ($F = 133.746$, $p < 0.001$). Moreover, fish fed the PHYTO diets always produced significantly higher levels of serum IgM than those fed the control diet, irrespective of the vaccination protocol ($F = 12.246$, $p < 0.001$). Although IgM-specific titers of fish fed PHYTO2 were higher than those

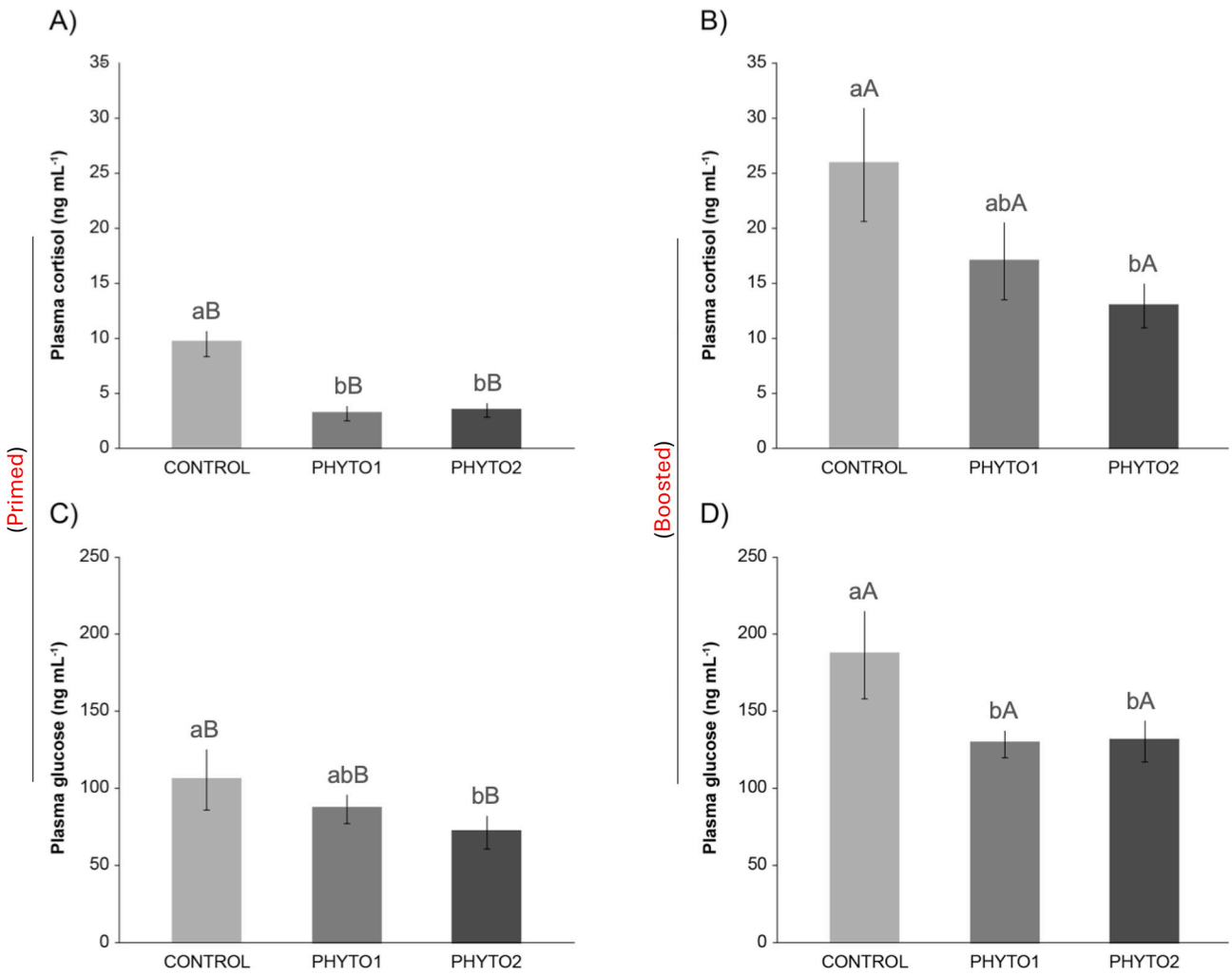

Fig. 2. Plasma cortisol (A, B) and glucose (C, D) concentrations (ng mL^{-1}) in primed (A, C) and boosted (B, D) *D. labrax* juveniles. Fish were fed a control diet or phytogenic-supplemented diets: PHYTO1, containing terpenes only, or PHYTO2, containing terpenes and flavonoids. Data are mean ± standard deviation. Different letters above the bars indicate statistically significant differences between the diets.

of fish fed PHYTO1, these differences were not significant. No significant effect was found for the interaction between diet and time post vaccination ($F = 3.640$, $p = 0.018$).

3.4. Head-kidney leucocyte FRAP

In primed fish, those fed the PHYTO2 diet showed significantly higher ($F = 11.02$, $p < 0.05$) FRAP values than those fed the other two diets (Fig. 4, 0 h). Although time post-IP vaccination showed no significant effect on the FRAP of leucocytes ($F = 5.8$, $p = 0.011$), a significant interaction between diet and time was observed in boosted fish ($F = 8.4$, $p < 0.001$). This interaction resulted in different FRAP patterns according to diet until the end of the trial. While fish fed the control diet displayed the highest FRAP at 14 days after IP vaccination (1144.1 ± 236), those fed the PHYTO diets showed the highest FRAP values at 24 h post IP vaccination (927.5 ± 57 for PHYTO1 and 893.3 ± 536 for PHYTO2). Notably, the FRAP of control-fed fish markedly decreased from 14 to 30 days after IP vaccination to a level below that observed in primed fish (Fig. 4), indicating a marked decrease in the antioxidant capacity of head-kidney leucocytes. Changes in the FRAP of boosted fish fed the PHYTO diets were not significant but those on PHYTO 2 diet showed a noticeable decrease in FRAP until the conclusion of the trial.

3.5. Survival after V. anguillarum infection

Following *V. anguillarum* infection, PHYTO1 and PHYTO2 diets significantly improved ($p < 0.0001$) the survival of boosted fish, by approximately 50% and 70%, respectively, compared to fish fed the control diet (Fig. 5).

3.6. Gene expression

The three-way ANOVA performed revealed that the interaction "diet × vaccination × time" had no significant effect on the expressions of antioxidant- and immune-related genes in the head kidney, although it was significant for all genes in the gills ($p < 0.001$). However, the effect of "time" alone was not always significant. Hence, we present results for the two-way ANOVA conducted using "diet" and "vaccination" and their interaction at 7 and 30 days after each vaccination.

3.6.1. Antioxidant-related genes

Seven days after each vaccination, the expression of genes encoding the antioxidant enzymes catalase (*cat*), superoxide dismutase (*sod*), and glutathione peroxidase (*gpx*) was higher in the gills and head kidney of primed and boosted fish fed the PHYTO diets than in those of fish fed the control diet (Supplementary tables 2 and 3). In the head kidney of primed fish, the highest expressions of *cat* and *sod* were shown by fish

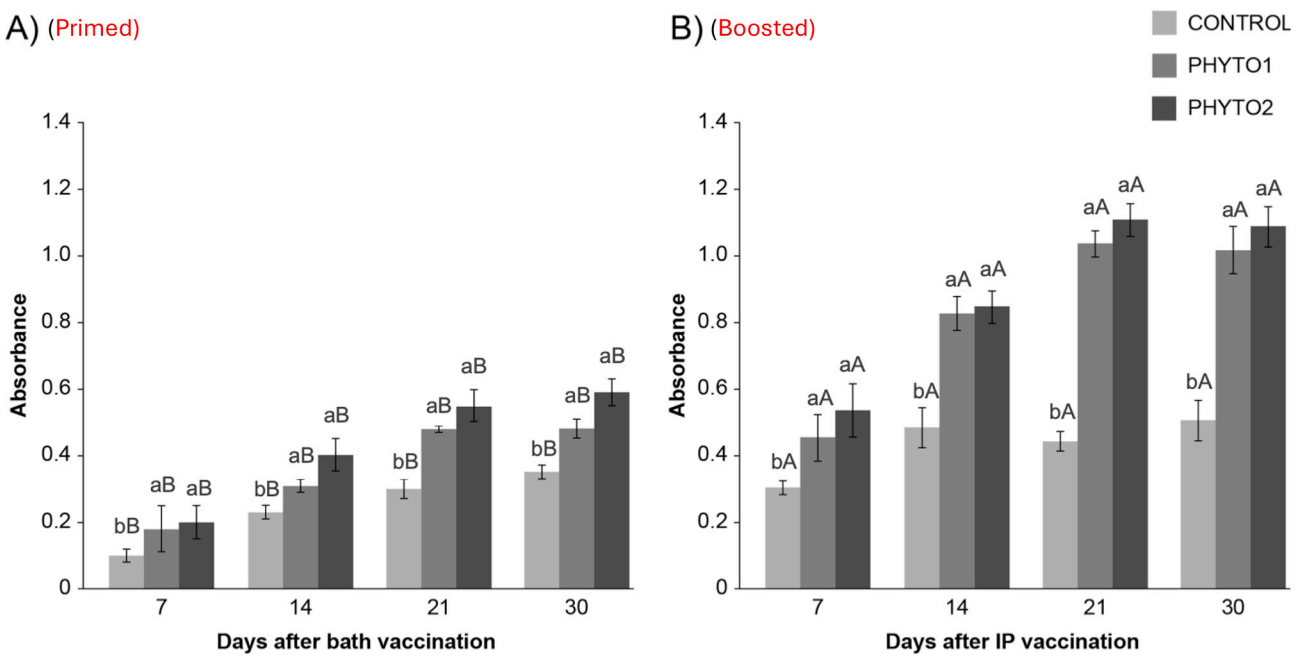

Fig. 3. Serum IgM titers in primed (A) and boosted (B) *D. labrax* juveniles at 7, 14, 21, and 30 days after each vaccination. Fish were fed a control diet or phytogenic-supplemented diets: PHYTO1, containing terpenes only, or PHYTO2, containing terpenes and flavonoids. Data are mean ± standard deviation. Different letters above the bars indicate statistically significant differences between the diets.

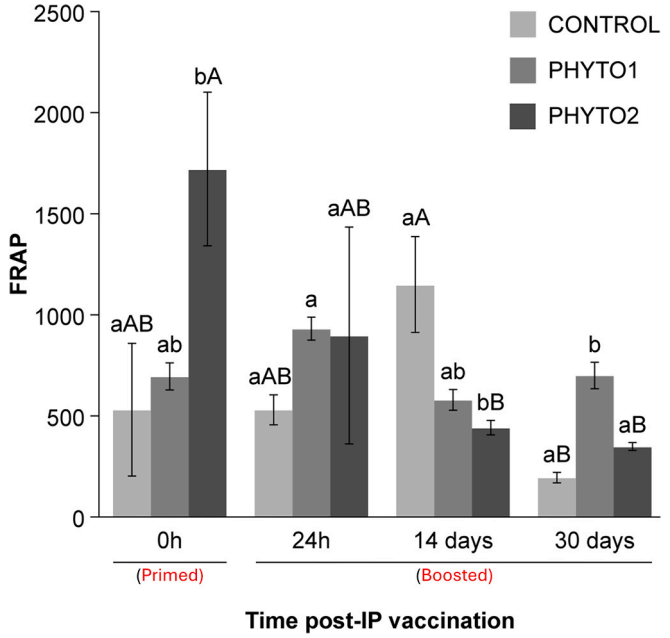

Fig. 4. Mean ferric reducing ability power (FRAP) of head kidney leucocytes in primed fish (0 h, just before IP vaccination) and boosted fish measured at several time points until the end of the trial (24 h, 14 days, and 30 days). Different low-case letters denote significant differences ($p < 0.05$) between dietary treatments at each sampling point and different capital letters denote significant differences ($p < 0.05$) between sampling points for each dietary treatment (Two-way ANOVAs: Diet × Time, Tukey post-hoc test). Data are mean ± standard deviation.

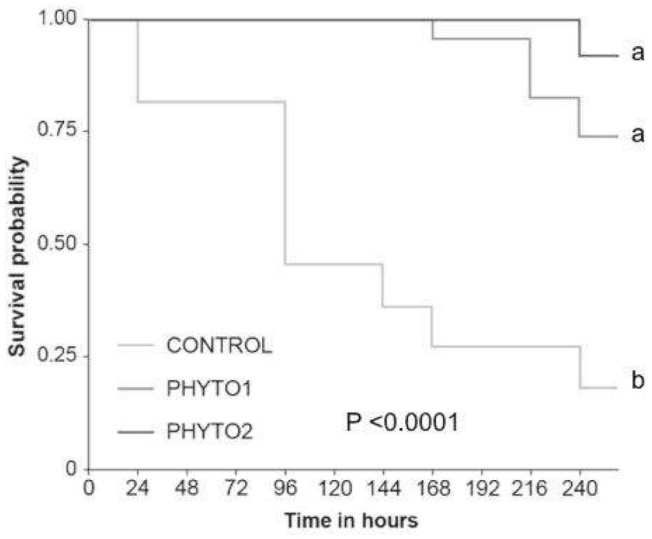

Fig. 5. Survival rate of boosted *D. labrax* juveniles over time (Kaplan-Meier curves) after the in vivo challenge test with *V. anguillarum*. Fish were fed a control diet or phytogenic-supplemented diets: PHYTO1, containing terpenes only, or PHYTO2, containing terpenes and flavonoids. Significant differences were detected ($p < 0.001$).

fed PHYTO2 while that of *gpx* was shown by fish fed PHYTO1. In the head kidney of boosted fish, the expressions of *cat* and *gpx* were highest in fish fed the PHYTO2 diet whereas *sod* expression was highest in fish fed PHYTO1. However, "diet" and "diet × vaccination" had no significant effect ($0.155 < p < 0.852$ and $0.655 < p < 0.947$, respectively) and "vaccination" only significantly affected the expression of *sod* ($F = 7.524$, $p = 0.018$). As for the gills, primed fish fed PHYTO1 presented the highest expression of all three genes, while boosted fish fed PHYTO1 showed the highest values of *cat* and *sod* and fish fed PHYTO2 the highest expression of *gpx*. The two-way ANOVA results confirmed the significant effect "diet" ($33.340 < F < 166.340$, $p < 0.001$ in all tests), "vaccination" ($36.410 < F < 176.560$, $p < 0.001$ in all tests), and "diet × vaccination" ($16.820 < F < 271.730$, $p < 0.003$ in all tests). However, expression of *gpx* was found to be significant only between primed fish fed the control diet and either of the PHYTO diets. On the other hand, the expression of *sod* only showed significant differences between

primed fish fed the control diet and boosted fish fed PHYTO1, as well as all fish in the other groups. Additionally, *cat* expression differed significantly among all groups of fish, except for primed fish fed PHYTO1 and boosted fish fed the control diet.

At 30 days after each vaccination (Supplementary tables 4 and 5), in the head kidney, boosted fish displayed higher values of *cat, sod,* and *gpx* than primed fish, and fish fed PHYTO1 consistently displayed the highest expressions of these antioxidant-related genes, regardless of vaccination. The two-way ANOVA indicated the significant effect of both "vaccination" ($19.997 < F < 176.101$, $p < 0.001$ in all tests) and "diet" ($4.072 < F < 8.348$, $p < 0.05$ in all tests) in the expression of all three genes, but the effect of "diet × vaccination" was significant for *sod* ($F = 3.967$, $p = 0.048$) and *gpx* ($F = 7.337$, $p = 0.005$) but not for *cat* ($F = 2.010$, $p = 0.177$). As so, *cat* expression differed significantly between primed fish fed the control or PHYTO2 diets and boosted fish fed the PHYTO diets, *sod* expression differed significantly between primed fish fed the control or PHYTO2 diets and all other groups, and *gpx* expression differed significantly between primed fish fed PHYTO1 and the other two groups of primed fish, and between all groups of primed fish and boosted fish, irrespective of diet; no significant differences were found between groups of boosted fish. In the gills, *cat* expression was higher in primed than in boosted fish while the other two genes presented the opposite trend. Nevertheless, in both groups, fish fed PHYTO2 generally presented the highest expression of all three genes; the exception was primed fish fed PHYTO1 which displayed higher expression of *sod* than primed fish fed PHYTO2, although this difference was not significant. In fact, no significant differences were found in the expression of *sod* for primed or boosted fish fed the different diets; differences were only significant between primed fish fed PHYTO1 and boosted fish in all groups, and between boosted fish fed the control diet and primed fish in all groups. The expression of *cat* only differed significantly between primed fish fed PHYTO2, primed fish fed the other two diets, and boosted fish fed the control diet. As for *gpx*, boosted fish fed the PHYTO diets displayed significantly different expression of this gene toward all the other groups but not between each other. These patterns are supported by the significant effects of "vaccination" ($15.658 < F < 133.910$, $p < 0.05$ in all tests) and "diet" ($5.594 < F < 17.980$, $p < 0.05$ in all tests) in the expression of all three genes, and significant effect of "diet × vaccination" for *gpx* only ($F = 11.720$, $p = 0.008$).

3.6.2. Immune-related genes

The expression of immune-related genes, including cluster of differentiation 4 (*cd4*), cyclooxygenase-2 (*cox-2*), γδ-T-cell receptor (*γδ-tcr*), IgM (*igm*), interleukin-10 (*il-10*), interleukin-1β (*il-1β*), and nuclear factor kappa *β* subunit 2 (*nfkβ2*) in the head kidney of primed and boosted fish at 7 days after each vaccination was generally higher in fish fed the PHYTO diets, particularly PHYTO2, than in fish fed the control diet (Supplementary tables 2 and 4). However, no significant effect of "vaccination", "diet", or "diet × vaccination" was found for these genes. At 30 days after each vaccination, the pattern of a generally higher expression of these genes in fish fed the PHYTO diets was maintained and boosted fish showed significantly higher expression of *cox-2, γδ-tcr, igm, il-10,* and *nfkβ2* in boosted fish than in primed fish (Supplementary tables 3 and 5). However, within primed and boosted fish groups, significant differences according to diet were only found between primed fish fed PHYTO1 and the other two groups. These patterns are supported by the two-way ANOVA results, according to which "vaccination" had a significant effect in the expression of *cox-2, γδ-tcr, igm, il-10,* and *nfkβ2* ($30.223 < F < 151.88$, $p < 0.001$ in all tests) whereas "diet" only significantly affected *γδ-tcr* expression ($F = 8.131$, $p < 0.05$) and "diet × vaccination" significantly affected *γδ-tcr* ($F = 15.312$, $p < 0.001$) and *nfkβ2* ($F = 5.844$, $p < 0.05$).

In the gills, at 7 days after each vaccination, the expression of tumor necrosis factor-α (*tnf-α*), *cox-2,* Mx protein (*mx*), caspase (*casp*), and glucocorticoid receptor 2 (*gr2*) were higher in boosted fish than in primed fish and generally higher in fish fed the PHYTO diets within each vaccination group; the exception was *mx* in boosted fish, which was higher in fish fed the control diet. Moreover, a significant effect of "vaccination" ($77.520 < F < 400.350$, $p < 0.001$ in all tests), "diet" ($45.220 < F < 167.780$, $p < 0.001$ in all tests), and "diet × vaccination" ($15.490 < F < 321.830$, $p < 0.05$ in all tests) was found. However, significant differences between primed fish fed PHYTO1 and PHYTO2 diets were not found, and only *tnf-α* and *mx* differed significantly between boosted fish fed PHYTO1 and PHYTO2. At 30 days after each vaccination, the expressions of *tnf-α, casp,* and *gr2* were higher in boosted fish than in primed fish and the opposite pattern was found for *cox-2* and *mx.* Moreover, expression of these genes was generally higher in fish fed the PHYTO diets than in those fed the control diet, except for *mx* and *casp* in boosted and primed fish fed the control diet, respectively. The two-way ANOVA revealed the significant effects of "vaccination" ($15.890 < F < 703.310$, $p < 0.05$ in all tests) and "diet × vaccination" ($5.781 < F < 66.970$, $p < 0.05$ in all tests) in all genes, and of "diet" ($6.602 < F < 30.077$, $p < 0.05$ in all tests) in all genes except *mx* ($F = 1.268$, $p = 0.347$). However, differences between fish fed PHYTO1 and PHYTO2were only found for *tnf-α* in primed fish and *gr2* in primed fish. Primed fish showed significantly different expression of *tnf-α* and *casp* between fish fed the control and PHYTO1 diets and *casp* and *gr2* between fish fed the control and PHYTO2 diets. Boosted fish showed significantly different expression of *tnf-α*, *cox-2*, and *gr2* between fish fed the control and PHYTO1 diets and *cox-2* between fish fed the control and PHYTO2 diets.

4. Discussion

Vaccines represent a bottleneck for increasing aquaculture production due to their production costs as well as specificity and efficacy of vaccination strategies (Mondal and Thomas, 2022). The current study aimed to determine if supplementing with phytogenics improves vaccine efficacy. Although no significant change in fish weights or natural mortality were noted, there was an observed increase in survival rates to *V. anguillarum* infection among boosted fish fed either of the phytogenic-supplemented diets. This suggests that these supplements may indeed enhance the effectiveness of the full vaccination protocol, aligning with prior research findings (Serradell et al., 2020). This effect was first observed at the very early stages of vaccination (24 h) and might be related to the innate immunomodulating/immunostimulatory properties of phytogenics (Firmino et al., 2021b). Garlic and Lamiaceae plant extracts have been shown to stimulate immune function in many aquaculture species, including European seabass (Muzaffar et al., 2017; Serradell et al., 2020). The results obtained in the present study at 30 days of feeding trial align with previous results, as this period was sufficient to observe a positive effect of PHYTO diets on the stress, antioxidant, and immune responses of European seabass juveniles.

Vaccination procedures have been shown to induce a stress response, with a subsequent increase in circulating plasma cortisol (Skinner et al., 2010) and glucose (Vargas et al., 2018) levels. Both cortisol and glucose are known to be particularly enhanced in fish administered IP injection vaccines compared to fish subject to bath vaccination (Liu et al., 2019; Schulz et al., 2020). Accordingly, in the present study, primed (bath vaccinated) fish displayed lower plasma cortisol and glucose levels than boosted (bath + IP injection vaccinated) fish. This vaccination-induced stress can strongly influence growth and disease resistance (Schulz et al., 2020). It has been suggested that the combination of cortisol elevation plus vaccination induces an allostatic overload due to hypersensitivity to adrenocorticotropic hormone coupled to a reduced efficiency of the negative feedback system with subsequent elevated baseline levels of plasma cortisol (Iversen and Eliassen, 2014). As a consequence of stress, insulin suppression leads to increased levels of plasma glucose in fish (Aluru et al., 2010; Malini et al., 2018). Thus, strategies focused on the decrease of plasma cortisol and glucose levels (both pre- and post-vaccination) could prevent the negative effect of cortisol and glucose elevation induced by vaccination. The use of phytogenics has been

described to reduce plasma cortisol in different animals (Cho et al., 2023; Choubey et al., 2016; Rodrigues et al., 2019), including fish (Firmino et al., 2021b; Firmino et al., 2021a), and specifically European seabass (Serradell et al., 2020). In the present study, both primed and boosted fish fed on the PHYTO diets showed decreased cortisol levels, particularly boosted fish fed the PHYTO2 diet. This effect suggests that flavonoids (exclusively present in the PHYTO2 diet) or the combination of flavonoids with terpenes can reduce stress levels over time, particularly when fish are vaccinated by IP injection. Flavonoids have been described to reduce the increase in cortisol levels associated to exhaustion (Ruiz-Iglesias et al., 2022). Hence, the beneficial effects of flavonoids and terpenes observed in the present study support those found in previous studies. For example, common carp (*Cyprinus carpio*) fed for 21 days on a diet supplemented with 200 mg kg^{-1} diet of quercetin, a flavonoid present in garlic, also showed reduced cortisol levels (Nasirin et al., 2023). European seabass fed a similar diet including terpenes also showed significantly reduced cortisol levels following a *V. anguillarum* challenge test (Serradell et al., 2020). Significantly lower glucose levels were also observed in gilthead seabream (*Sparus aurata*) fed a diet supplemented with a phytogenics' blend (garlic essential oil, carvacrol, and thymol) than in gilthead seabream fed a control (non-supplemented) diet (Firmino et al., 2021a).

The effect of PHYTO supplementation on the immune response to vaccination was assessed by serum IgM specific titers, as this is the major systemic Ig responsible for the development of an affinity response in the recently discovered splenic germinal-like structures (Matz et al., 2023). High specific IgM titers have been detected in teleost after ectoparasite infections (Yu et al., 2020), suggesting that even though IgT is the major mucosal specialized Ig, IgM also plays an important role in mucosal tissues and/or in the antigen presenting cells that reach the germinal-like structures where the germinal-like-centers reaction are set. As expected, and in agreement with previous results obtained for European seabass (Galeotti et al., 2013), increasing serum specific IgM titers due to B cell response were observed from day 7 to day 30 after each vaccination procedure. Dietary phytogenics have also been shown to increase serum specific IgM titers in several species (recently reviewed by Soltani et al., 2019). Nile tilapia (*Oreochromis niloticus*) fed on a diet supplemented with a lemon (*Citrus limon*), onion (*Allium cepa*), and garlic blend (20 mL kg^{-1} diet) for 70 days also displayed increased serum specific IgM titers (Mohammady et al., 2022). In turbot, (*Psetta maxima*), administered a pentavalent vaccine (inactivated *V. anguillarum*, *V. scophtalmi, V. harveyi*, *V. alginolyticus,* and *Edwardsiella tarda*) by IP injection and using *Astragalus* sp. polysaccharides as the adjuvant (Zheng et al., 2012) also presented an increase in serum Ab titers. These results therefore indicate that dietary phytogenics favor the development of immunocompetence after vaccination. Furthermore, the longer the time after vaccination the higher the serum specific IgM titers, suggesting that flavonoids and terpenes not only enhance but also prolong the duration of vaccine protection. Similar results were obtained for fish fed on diets supplemented with phytogenics derived from Lamiaceae (Chari et al., 2020), citrus fruits (Harikrishnan et al., 2020), and garlic (Li et al., 2019), all included in PHYTO supplements evaluated in the present study.

Dietary supplements can increase antioxidant defenses, as evidenced by the elevated FRAP values of head kidney leucocytes of primed fish and in the first hours after vaccination by IP injection in boosted fish; however, this effect was decreased with time. The iron-chelating activity of flavonoids (Mladěnka et al., 2011; Wang et al., 2021) decrease the availability of iron ions to be oxidized by hydrogen peroxide into hydroxyl radicals, which cannot be detoxified by antioxidant enzymes (Marciano and Vajro, 2017; Spiers et al., 2014). The decreased in FRAP observed throughout the trial might therefore be due to a plateau in the iron-chelating ability of the flavonoids present in PHYTO1 and PHYTO2.

Vaccination is known to induce changes in the responses of oxidative stress and antioxidant enzymes in a tissue-dependent manner, with gills being one of the most susceptible tissues to these phenomena (Tkachenko et al., 2014; Castejón et al., 2021). In the present study, boosted fish generally showed an upregulation of *cat*, *sod*, and *gpx* expression compared to primed fish at 7 days after each vaccination procedure, with higher expressions in the head kidney than in the gills, except for *cat*, which generally displayed higher values in the gills than in the head kidney. At 30 days after each vaccination, primed fish showed an upregulation of *cat* and *sod* but a downregulation of *gpx* in the gills when compared to boosted fish, but the latter showed higher expression of all genes in the head kidney. Moreover, fish fed the PHYTO diets always showed upregulation of *cat, sod,* and *gpx* expression when compared to fish fed the control diet. These results indicate that vaccine reinforcement via IP injection is needed to improve the immune response provided by bath vaccination, and that diet supplementation with phytogenics increases the activity of antioxidant enzymes. Increased *sod* transcripts have also been described in the gills of gilthead seabream after vaccination (Vargas et al., 2018), suggesting an increase in gill metabolism driven by the unfolding immune response and regulatory response to the secretion of reactive oxidative species (ROS) by gill phagocytes. However, in rainbow trout (*Oncorhynchus mykiss*), *cat* and *gpx* were the main defense factors against oxidative stress induced by vaccination against *Yersinia ruckeri* (Tkachenko et al., 2016). The magnitude of *cat, sod,* and *gpx* response to oxidative stress therefore seems to differ among fish species but also according to tissue, in agreement with previously reported data for yellow perch (*Perca flavescens*) (Dautremepuits et al., 2009).

Dietary supplementation with plant-derived compounds has been associated with increased antioxidant enzyme activity in teleost (Pérez-Jiménez et al., 2012; Sahin et al., 2014; Yonar et al., 2019). Antioxidant production not only prevents oxidative damage of cells, but also participates in immune defense through cell signaling. At 7 days post each vaccination, both the gills and head kidney of fish fed the PHYTO1 diet showed increased *cat*, *sod*, and *gpx* gene expression; fish fed the PHYTO2 diet, showed increased *gpx* expression relative to control fish in both tissues, increased *sod* and *cat* in the head kidney, decreased *sod* in the gills, and opposite patterns for *cat* in the gills of primed and boosted fish. At 30 days post each vaccination, primed, and boosted fish fed the PHYTO diets showed upregulated *cat* and *sod* expressions relative to control-fed fish in both the gills and head kidney but *gpx* expression was downregulated in the gills of primed fish fed PHYTO1 and head kidney of primed fish fed PHYTO2; in boosted fish, *gpx* expression was upregulated in both tissues of fish fed the PHYTO diets. These results indicate that the expressions of *cat, sod,* and *gpx* in the gills and head kidney are affected differently by diet supplementation and that the effect of supplementation is most obvious in boosted fish and at 30 days after vaccination. This suggests that immersion bath vaccination may not be sufficient to induce host defenses against pathogens through the antioxidant system, and this is considered a major bottleneck in mucosal vaccines (Salinas et al., 2022, 2021). Moreover, fish gills might be more reliable organs than the head kidney for assessing the antioxidant status in relation to diet (Tkachenko et al., 2014). Indeed, differences in *cat, sod,* and *gpx* expression were evidenced at only 7 days after each vaccination in the gills but not in the head kidney. As the primary function of gills is gas exchange, this organ is particularly susceptible to ROS effects, which may explain the faster response of antioxidant enzymes secreted by gill cells when compared to that of head kidney cells (Di Giulio and Hinton, 2008). The production of CAT, SOD, and GPx in gills may be sufficient to remove excessive ROS from the organism in the first days after vaccination, without requiring extra production of these enzymes by head kidney cells. However, at 30 days post vaccination the patterns in the head kidney were more consistent, particularly in boosted fish, likely because gill cells have exceeded their capacity to remove circulating ROS.

Antioxidant defenses participate in the functioning of immune response against pathogens, but the imbalance favoring free radicals versus antioxidants can compromise the balance of immune system function. The induction of an immune response may cause oxidative

stress, which might be a key factor mediating both the short- and long-term costs of immune system activation (Marri and Richner, 2015). The stress induced by vaccination (via bath or IP injection or both) generates a cascade of immune responses where different genes are up- or down-regulated and interact with each other in different feedback mechanisms to increase the fish physiological response to the stress. Although the mode of action and immune-related pathways activated by essential oils and/or herbal extracts in fish are not well known, studies in mammals have shown that these compounds can stimulate immune cells to produce interleukins mostly via the *nfkβ2*, *il-1β*, and *tnf-α* pathways (Arreola et al., 2015; Soltani et al., 2019). The upregulation of *nfkβ2* and *tnf-α* can further stimulate the upregulation of pro-inflammatory *cox-2* by immune cells (Galindo-Villegas et al., 2016). Increased expression of *casp* also promotes the expression of *tnf-α* (McIlwain et al., 2013), and both *casp* and *tnf-α* further induce the expression of *cox-2* to synthesize prostanoids (lipid mediators of fish immunity) via the COX-2 pathway, as this enzyme is upregulated in response to infection (Grasso et al., 2015; Severin and El-Matbouli, 2007). The pro-inflammatory cytokine *il-1β* also enhances *tnf-α* and *cox-2* production, and stimulation of *tnf-α* increases *nfkβ* signaling and *il-1β* release; on the contrary, upregulation of *il-10* decreases the production of *tnf-α* and *il-1β* but increases *igm* production (Zou and Secombes, 2016). The *cd4* gene encodes a transmembrane glycoprotein expressed on the surface of T-helper cells that is essential for triggering vaccine-induced immunity and lead immune cells to produce different types of pro-inflammatory cytokines (Ashfaq et al., 2019). In teleost, only a small fraction of T cells express *γδ-tcr*, although the protein it encodes can directly recognize antigens (Kordon et al., 2021), similar to immunoglobulins. Mx proteins belong to the type I interferon (IFN) system of antiviral defense and *mx* expression has been used as an indicator of *ifn* activity, as its short production and half-life hinder its detection (Wu and Chi, 2007). Elevations of cortisol, even at low concentration, are recognized by *gr2,* which is upregulated to produce further physiological and immune responses, including the decrease in *tnf-α* (Vallejos-Vidal et al., 2022).

In the present study, vaccination by IP injection led to a significant upregulation of the expression of immune-related genes at 7 days post vaccination in the gills and at 30 days post vaccination in the head kidney. Significantly upregulated expression of *tnf-α* and *gr2* were also detected in the gills at 30 days post vaccination by IP injection. Moreover, at 7 days post vaccination, European seabass fed the diets supplemented with phytogenics showed upregulation of *tnf-α, cox-2*, *mx* (in primed fish only)*, casp,* and *gr2* in the gills and *cd4* (only in boosted fish)*, cox-2, γδ-tcr, igm, il-1β, il-10*, and *nfkβ2*) in the head kidney to increase the production of pro-inflammatory cytokines. At 30 days post vaccination, *tnf-α, cox-2,* and *gr2* were still upregulated in the gills of fish fed both PHYTO diets but *mx* was downregulated in the gills of boosted fish and *casp* was downregulated in the gills of primed fish. Simultaneously, in the head kidney, all immune-related genes except *il-10* and *il-1β* in primed fish and *igm* in boosted fish were upregulated in fish fed the PHYTO2 diet. Therefore, the blends of phytogenic tested in the present study, particularly PHYTO2, can modulate the immune response of fish to the vaccination-induced inflammation by promoting the production of pro-inflammatory cytokines and the antigen recognition mechanisms that then activate the adaptive immune system (*igm, gr2,* and *cd4* production). The increased efficacy of the complete vaccination protocol after the *V. anguillarum* challenge observed in fish fed the PHYTO diets compared to fish fed the control diet, further supports these immunomodulatory effects. The combination of the full vaccination protocol (bath + IP injection) with phytogenic supplements, which have demonstrated an adjuvant-like function, has been reported by other authors (Soltani et al., 2019) and opens new opportunities for the fish health market. Indeed, continued research that enhances our comprehension of the diverse beneficial effects of phytogenic compounds has the potential to facilitate the industrial-scale incorporation of each of these substances or their blends. Such integration could play a substantial role in fostering economically effective and sustainable fish production practices.

5. Conclusions

The present study assessed the effects of supplementing the diet of farmed European seabass with a phytogenic blend to improve vaccination efficacy against *V. anguillarum.* The results obtained suggest that PHYTO2, rich in flavonoids and terpenes, and to a lesser extent PHYTO1, promoted survival in boosted fish, by mitigating vaccine-associated stress levels while conferring increased antioxidant protection and modulating immune response during the vaccine-associated inflammatory cascade. We therefore propose using flavonoid and terpene blends as a proactive strategy to enhance the protection bestowed by full vaccination and boost immunocompetence development in farmed European seabass.

CRediT authorship contribution statement

Daniel Montero: Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **Silvia Torrecillas:** Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **Antonio Serradell:** Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **Artem Nedoluzhko:** Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **Álvaro Fernández-Montero:** Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **Alex Makol:** Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **Luis Monzón-Atienza:** Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **Victoria Valdenegro:** Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **Ignasi Sanahuja:** Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **Jorge Galindo-Villegas:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Felix Acosta:** Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

D. Montero reports financial support was provided by Spanish Ministry of Economy and Competitiveness. Aquaculture Editor in Chief. JG-V. Co-author employed by Delacon. AM If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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