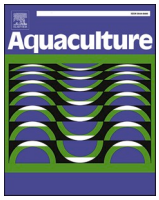
Aquaculture 599 (2025) 742109

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



# Thymol-carvacrol essential oil extract mitigates the mucosal stress-immune response induced by intraperitoneal immunization in sea bass (*Dicentrarchus labrax*)

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ARTICLE INFO

*Keywords:*
Vaccine
Mucosal stress-immune response
Gene expression
Cortisol
Plant-based sedative
Sea bass (*Dicentrarchus labrax*)

ABSTRACT

The present study investigates the mitigation of the stress response induced by a bivalent vaccine against vibriosis and pasteurellosis in sea bass through treatment with thymol-carvacrol oil extracts, known as FishEase-C (FEC) and FishEase-L (FEL). Plasma and skin mucus cortisol as well as mucosal transcript stress-immune response were measured at 1 and 24 h post-vaccination (hpv). Our results indicate that FEL but not FEC was capable of reducing plasmatic cortisol secretion by 24 hpv, in contrast to vaccinated and vaccinated plus treated with FEC groups. A similar pattern was noted in the skin mucus though secretion of cortisol was prevented by the FEL. The expression of stress-immune related gene transcripts (*gr1*, *gr2*, *crh*, *hsp70*, *cox2*, *il1β*, *il6*, *il8*, *il10*, *tgfβ1*, *lysozyme*, *igm*, *igt* and *igd/igt*) revealed that stress-immune gene regulation was not homogenous among mucosae. Gills were more responsive to the stress/inflammation induced by FEC, as shown by *crh*, *hsp70*, *cox2*, *il1β* and *il6* enhanced expression, suggesting higher absorption of FEC via this mucosae. The gills also appeared to be more resilient to the stressful effect of the vaccine, while higher regulation was observed in skin and intestine. In summary, administration of FEL was able to mitigate the stress response at physiological (e.g., cortisol) and the stress-immune genes activation in skin and intestine(*hsp70*, *il1β*, *il6*), mainly at 24 hpv. We recommend FEL as a customized formulation to farmers for use on sea bass prior to vaccination, in order to mitigate negative effect of vaccination procedure.

## 1. Introduction

Vaccination against bacterial and viral diseases is nowadays the most efficient method to induce protection against diseases in fish within the aquaculture environment. Disease resistance involves activation of the innate and aquired immune systems. However, the innate immune response preceeds the adaptive response and thus determines the nature of the response (Fearon and Locksley, 1996). Generally speaking, the vaccination proccess comprises different procedures and physiological reactions e.g., sedation, manipulation, as well as inflammation provoked both by injection and antigen exposure. Therefore, the vaccination process itself can induce stress in animals and potentially compromise vaccine success in addition to the stress response caused by antigens exposure (Ellis et al., 2007; Liu et al., 2019). Briefly, under stress situations, a hormonal cascade along the Hypothalamus-sympathtic-chromaffin (HSC) and Hypothalamic-Pituitary-Interrenal axes (HPI) is initiated, which elicits numerous physiological reactions (Weyts et al., 1998). Thus, in the short term, vaccination activated HPI axis involving different mechanisms at different functional levels e.g., molecular, cellular, and systemic responses (Evans et al., 2004; Khansari et al., 2018; Zheng et al., 2010). Similar to mammals, modulation of the immune system by stress in fish is explained by intimate bidirectional communication between the neuroendocrine and immune systems (Verburg-van Kemenade et al., 2011). The precise mechanism behind

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https://doi.org/10.1016/j.aquaculture.2024.742109
Received 17 May 2024; Received in revised form 23 December 2024; Accepted 30 December 2024
Available online 31 December 2024

this communication has yet to be fully elucidated which is mainly mediated by receptors and hormones as well as cytokines acting on both systems (Baigent, 2001; Tort, 2011). Effects of stress on the immune system in fish are mainly mediated by cortisol involving neutrophil elevation, reduction of lymphocyte number and antibody response (Weyts et al., 1998; Wojtaszek et al., 2002), decrease of complement activity (Sunyer et al., 1995), multi-omics analysis (X. Li et al., 2022a; Roh et al., 2020) and a reduction in cytokine production e.g., *tgfβ1* and *il6* (Khansari et al., 2017a). Furthermore, an inverse connection is also reported among regulatory systems i.e., neural, endocrine and immune systems. Hence, the production of cytokines namely *il12* and *il18* by cells of the central nervous system (CNS), hyphothalamus and pituitary can provoke the HPI axis (Tort, 2011). In this sense, our previous findings show this bidirectional relationship in fish inducing cytokines expression in the brain following vaccination (Liu et al., 2019).

Again, vaccination confers protection at both mucosal and systemic levels, with the effectiveness of the route of vaccination being determinant (Skov et al., 2018). In fish, the mucosal immune system (MIS), and particularly the mucosal-associated lymphoid tissue (MALT), has been an important target for vaccine development due to the importance of the mucosal barriers in protecting animal from external threats. Recent studies that have assessed the immune system, have put greater emphasis on the MIS, due to its importance in relation to its systemic counterpart (Koppang et al., 2015; Salinas et al., 2011; Xu et al., 2016). The mucosal barriers serve as the initial interface between the fish and its surroundings, being responsible for attaining information from the environment, processing it and sending the signal to the central nervous system to initiate an appropriate adaptive response for maintaining homeostasis. Hence, the fish MIS appears to be more complex than its mammalian counterpart due to the close interaction with the microbe-rich aquatic environment (Parra et al., 2015; Yu et al., 2020).

Fish MALTs include the skin-associated lymphoid tissue (SALT), the gill-associated lymphoid tissue (GIALT), the gut-associated lymphoid tissue (GALT), and the nose-associated lymphoid tissue (NALT). As mentioned, MALTs can independently initiate local responses, without requiring activation of the central nervous system and indeed this aspect is absent in the classical hierarchical view of stress reaction. Therefore, the rapid and primary local immune response exhibited by MALTs in comparison to systemic organs, can be described as one of their unique features (Caipang et al., 2011; Parra et al., 2015). It is therefore of paramount importance to reduce the stress response induced by vaccination at the mucosal level, in order to enhance the vaccine protection.

Recently there has been a growing interest in the use of plant-based anesthetics (essential oils; EOs) as potential substitutes for synthetic fish anesthetics (De Freitas Souza et al., 2019). This is due to their cost-effectiveness, low environemental impact, and, in particular, their immunestimulant capacity (Brown et al., 2008; Montero et al., 2024; Roldan-Juarez et al., 2023; Serradell et al., 2020). In contrast, synthetic drugs are only employed to facilitate vaccination, ensuring the well-being of the individuals and minimizing physical injuries. Indeed, there is a substantial body of research examining the impact of phytogenic sedatives/feed additives on stressed animals. However, in the case of vaccination, there is little consensus (Bahi et al., 2018; Dawood et al., 2020; Herrera et al., 2019; Serradell et al., 2022). Thus, the use of plant-based products for fish anesthesia as an alternative to chemical anesthetics may be a promising approach to reduce the stress associated with vaccination in aquaculture. Hence, it is important to point out that EOs are good candidates due to their lipophilic and liposolubility properties, which confer rapid dispersion through the biological membrane. An important caveat however, is the difficulty in precising factors such as the origin or the variety of the oil and the dose of administration for each fish species to determine the effectiveness of the product (Soltanian et al., 2018).

In the current study, juvenile european sea bass, an important species in Mediterranean aquaculture, was exposed to short-term sedation using two unpatented plant-based products (FishEase-C and FishEase-L) prior to intraperitoneal (IP) vaccination. The primary goal was to evaluate the sedative impact of these products in the mucosal barriers and to determine if stress mitigation by plant-based sedatives led to a decrease in stress hormones as well as inflammation induced by the vaccine. To achieve this aim, physiological indicators (plasmatic and skin mucus cortisol) and stress-immune transcript response of skin, gills and gut were evaluated at 1 and 24 h post immunization in sea bass.

## 2. Material and methods

### 2.1. Fish husbandry

Juvenile of sea bass, with a body weight of 10.42 ± 0.01 g and a standard length of 8.05 ± 0.05 cm, were obtained and housed within a Recirculating Aquaculture System (RAS) at the Technological Center for Aquaculture of Andalusia (CTAQUA, REGA NUMBER ES110270000411). The RAS had a daily water renewal rate of 5–10 % and was equipped with a biofilter, mechanical filtration, UV sterilization, a protein skimmer, and options for both automatic and manual feeding at a rate of 1 % body weight. The water chemistry parameters were automatically monitored and were as follows: $O_2$ 6.0 ± 1.0 mg/L, temperature 22.0 ± 1.0 °C, pH 7.5 ± 0.5, and salinity 38 ppt.

The experiment complied with the Guiding Principles for Biomedical Research Involving Animals (EU2010/63), the guidelines of the Spanish laws (law 32/2007 and RD 53/2013) and authorized by the Ethical Committee of the Universitat Autònoma de Barcelona (Spain) for the use of laboratory animals.

### 2.2. Plant-based products

Two prototypes of plant-based products (FishEase-C and FishEase-L) were received from TECNOVIT-FARMFAES S.L. Company, Spain. These products were formulated with a confidential blend of aromatic phytogenic compounds primarily derived from plants of the Lamiaceae family. Both products are based on thymol with a minor content of carvacrol, with the difference of a lower carvacrol content in the FishEase-L product. The concentrations of the two products used in the study, as well as the exposure time, were chosen based on previous experiences of the group according to already published protocols Jerez-Cepa et al., 2019. In this way, the concentration used was 30 ppm and the sedation time was 10 min (Cartán et al., 2023).

### 2.3. Vaccine

ICTHIOVAC® VR/PD is commonly employed in sea bass farming to provide protection against vibriosis and pasteurellosis (LABORATORIOS HIPRA, Spain). The composition of the ICTHIOVAC® VR/PD vaccine per 0.1 mL dose includes the following active substances: Inactivated *Photobacterium damselae* subsp. piscicida strain DI 21, and inactivated *Listonella anguillarum* serotype 01,02α, and serotype 02β.

### 2.4. Experimental design

For the experiment, 480 sea bass were evenly distributed into twelve 100 L tanks ($n = 40$, density 4 kg/m$^3$ aprox.). Fish were devided into six groups, each with two replicate tanks; ***(1) No FishEase(C/L)-Control (N·P. control*** were sampled in the morning and in the afternoon to take into consideration the influence of circadian rhythm. ***(2) vaccine group (N·P. vaccine):*** fish were intraperitoneally vaccinated with ICTHIOVAC® VR/PD vaccine according to the manufacturer's instructions. ***(3) FishEase-C + vaccine (FEC + vaccine)*** and ***(4) FishEase-L + vaccine (FEL + vaccine)*** groups were first treated with a 30 ppm waterborn concentration of the products and fish behavior was then monitored. After a 10 min sedation period, the fish were subsequently vaccinated in a manner similar to the vaccine group. Two additional groups were designated as control groups including ***(5) FishEase-C***

***(FEC)*** and ***(6) FishEase-L (FEL),*** non-vaccinated to identify the specific effects of the products only on non-vaccinated fish. The vaccine group (vaccine) were anesthetized prior to vaccination with 100 mg/L tricaine methane-sulfonate (MS-222; Sigma-Aldrich) instead for appriximately 5 min to follow the common practices used in aquaculture for the process of vaccination.

### 2.5. Skin mucus, blood and tissue sampling

Five fish from the two replicate tanks ($n = 10$) were randomly sampled at 1 and 24 h post-vaccination (hpv) and sacrificed by MS-222 overdose (300 mg/L). Skin mucus was collected according to Xu et al. (2013). Blood was drawn by cutting the caudal vein and collecting into capillary tubes. The blood was centrifuged at 5000*g* for 10 min for plasma collection. After skin mucus and blood collection, the skin tissue samples (the upper part of the lateral line, from the left side and roughly the same size) were meticulously taken to avoid muscle contamination. The second lamella on the left side of the gills was also sampled. For intestine sampling, the body cavity was opened laterally, and midgut and hindgut were removed using a scalpel and forcep. Feces and mucus were thoroughly removed with forceps. All samples were snap frozen in liquid nitrogen and then transferred to −80 °C to await further analysis.

### 2.6. Cortisol assay in plasma and skin mucus

The quantitative analysis of cortisol levels in plasma and skin mucus was measured by ELISA (Enzyme-Linked Immunosorbent Assay) using a kit (NEOGEN#402710). The plasma samples were diluted to 1:50 using an extraction buffer, while the skin mucus samples remained undiluted. Subsequently, all samples and standards were added to a cortisol antibody-coated 96-well plate following the manufacturer's instructions. After the addition of the diluted cortisol enzyme conjugate, the plate was gently shaken and incubated at room temperature (RT) for one hour. Following the incubation period, a three-step washing process was performed, and K-blue substrate was added to the wells and incubated for 30 min with gentle shaking. The reaction was halted using 100 % hydrochloric acid (HCL). Results were obtained by measuring and comparing the absorbance readings of the samples against the standards at 650 nm using the Tecan Infinite M200 Plate Reader.

### 2.7. IgM detection in skin mucus

ELISA was employed to assess total IgM levels in skin mucus at specific time points following vaccination, as descibed previously (Khansari et al., 2018). A 96-Well Microplate (Thermo Fisher Scientific) was employed for incubating 50 μL of samples per well, kept at 4 °C overnight. After two washes with PBS to minimize non-specific binding, 100 μL of 5 % non-fat milk in Phosphate-buffered saline (PBS) was added to each well and incubated for 1 h at (room temperature) RT. 50 μL of anti-European sea bass (*Dicentrarchus labrax*) IgM monoclonal antibody (diluted 1/500 in PBS; Aquatic Diagnostics Ltd., UK) was added to each well and incubated for 1 h at RT as the primary antibody. Next, the samples were washed three times with 200 μL of the washing buffer (PBS + 0.1 % Tween 20) and incubated for 1 h with 50 μL per well of the secondary antibody, goat-anti-mouse IgG conjugated with HRP (diluted 1/4000 in PBS; Jackson Immuno Research EUROPE LTD). The microplate underwent five washes, and then 50 μL/well of Ultra-TMB (3,3′,5,5′-tetramethylbenzidine; Thermo Fisher Scientific) was introduced as the substrate. Subsequent to an 8 min incubation at RT, 50 μL/well of $H_2SO_4$ (2 M) served as the stop solution, and absorbance readings were obtained at 450 nm using a microplate reader (Victor3; Perkin Elmer).

### 2.8. RNA isolation and cDNA synthesis

Total RNA from individual fish samples was isolated using TRI reagent (Sigma-Aldrich) following the manufacturer's instructions. The quality and quantity of total RNA were assessed by a NanoDropND-2000 spectrophotometer (Thermo Fisher Scientific). Finally, 2 μg of total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). All cDNA samples were immediately stored at −20 °C.

### 2.9. Real time quantitative PCR (qPCR)

Fish mucosal samples including skin, gills and intestine were analysed using qPCR analysis. The analysis included the evaluation of stress and immune related genes glucocorticoid receptor 1–2 (*gr1*–2), corticotropin-releasing hormone (*crh*), heat shock proten 70 (*hsp70*), cyclooxygenase 2 (*cox2*), interleukin 1 beta (*il1β*), interleukin 6 (*il6*), interleukin 8 (*il8*), interleukin 10 (*il10*), transforming growth factor beta 1 (*tgfβ1*), *lysozyme*, complement component 3 (*c3*), immunoglobulin M (*igm*), immunoglobulin T *igt* and immunoglobulin d/t (*igd/igt*). The specific primers used in this study are listed in Table 1. The secondary structure of the primers and their annealing specificity were assessed using OligoAnalyzer (version 3.1) and Primer-Blast software, respectively. Five housekeeping genes *ef1α*, *βactin*, *rpl13*, *18 s* and *RPS18* were evaluated to determine the most stable expression. Among them, *β-actin* and *18 s* were employed for quantification analysis using BIORAD method corrected for efficiency of each primer set. Real-time PCR reactions were carried out using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories) with a 1:15 cDNA dilution prepared for the genes of interest. The thermal conditions included a 3 min pre-incubation at

**Table 1**
Primer sequences for qPCR analyses.

| Gene | GenBank accession number | Sequence 5′–3′ | Product size |
|---|---|---|---|
| *βactin* | AJ537421 | Fw: ATGTGGATCAGCAAGCAGG<br>Rv: AGAAATGTGTGGTGTGGTCG | 147 |
| *18 s* | AM419038.1 | Fw: TCAAGAACGAAAGTCGGAGG<br>Rv: GGACATCTAAGGGCATCACA | 49 |
| *gr1* | AY549305 | Fw: GAGATTTGGCAAGACCTTGACC<br>Rv: ACCACACCAGGCGTACTGA | 401 |
| *gr2* | AY619996 | Fw: GACGCAGACCTCCACTACATTC<br>Rv: GCCGTTCATACTCTCAACCAC | 403 |
| *crh* | JF274994.1 | Fw: AACCCAAAACTCCCAGCAG<br>Rv: TGTTCCCAACTTTCCCTTGT | 200 |
| *hsp70* | AY423555.2 | Fw: GCTCCACTCGTATCCCCAAG<br>Rv: ACATCCAGAAGCAGCAGGTC | 172 |
| *cox2* | XM_051377074.1 | Fw: CATTCTTTGCCCAGCACTTCACC<br>Rv: AGCTTGCCATCCTTGAAGAGTC | 160 |
| *il1β* | AJ269472.1 | Fw: ATCTGGAGGTGGTGGACAAA<br>Rv: AGGGTGCTGATGTTCAAACC | 106 |
| *il6* | AM490062 | Fw: ACTTCCAAAACATGCCCTGA<br>Rv: CCGCTGGTCAGTCTAAGGAG | 170 |
| *il8* | AM490063.1 | Fw: GTCTGAGAAGCCTGGGAGTG<br>Rv: GCAATGGGAGTTAGCAGGAA | 110 |
| *il10* | AM268529.1 | Fw: CGACCAGCTCAAGAGTGATG<br>Rv: AGAGGCTGCATGGTTTCTGT | 199 |
| *tgfβ1* | XM_051399565.1 | Fw: GACCTGGGATGGAAGTGG<br>Rv: CAGCTGCTCCACCTTGTG | 225 |
| *lysozyme* | KJ433681.1 | Fw: TGATGCAGGTTGTTGATGTTAATC<br>Rv: TCCATCCCCCATATTGTAGGC | 194 |
| *c3* | HM563078.1 | Fw: ACCAAAGAACTGGCAACCAC<br>Rv: CTAGCAGTCGGTCAGGGAAC | 154 |
| *igm* | AJ400233 | Fw: GAGCTGCAGAAGGACAGTG<br>Rv: TCAGACTGGCCTCACAGCT | 223 |
| *igt* | KM410929 | Fw: CGGACTTCATTCAGTACTCTG<br>Rv: CAACTGTACACATCAGGGCC | 225 |
| *igd/igt* | KU132361 | Fw: CTCTACGTACTCCTGTGTTGTC<br>Rv: ACAATGGACTTGTCTCGTCCAG | 177 |

95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s and annealing/extension at 60 °C for 30 s. All reactions were carried out in duplicate using the CFX384 touch Real-Time System (Bio-Rad Laboratories). Values for each experimental condition were expressed as fold expression against those of the reference genes *β-actin* and *18 s*.

### 2.10. Statistical analysis

A two-way ANOVA followed by Tukey's multiple comparisons post-test was conducted to analyze differences at each time point between the control group (N·P. control) and the treatment groups (N·P. vaccine, FEC + vaccine, and FEL + vaccine), as well as differences between treatment groups for each assessed MALT tissue. GraphPad software (v10.4.1) was used to calculate the mean, standard error of the mean (SEM), and to perform statistical analyses. A *p*-value of $<0.05$ was considered statistically significant.

## 3. Results

Overall, the transcript expression of key stress-related genes (*gr1*, *gr2*, *crh*, *cox2*, *hsp70* and *il1β*) was primarlily upregulated in the skin by the sole effect of the FEC product, with a lesser effect observed in the gills (*crh* and *cox2*) and intestine (*il1β*) (Suplemmentary Fig.1).

### 3.1. Cortisol in plasma and mucus

The results showed elevation of plasmatic cortisol levels at 1 hpv in all vaccinated fish compared to control (N·P. control), whether they were non-sedated ($p_v \leq 0.0001$) or sedated with FEC or FEL ($p_v \leq 0.0001$, pv = 0.0001). However, this enhancement was reduced in fish treated with FEL + vaccine compared to fish treated with FEC + vaccine ($p_v = 0.01$) (Fig. 1). At 24 hpv, plasma cortisol levels remained significantly higher in N.P. vacine and FEC + vaccine groups respect to control ($p_v = 0.02$ & $p_v \leq 0.0001$). Fish treated with FEC + vaccine showed significantly elevated cortisol compared to fish from the N.P. vaccine and FEL + vaccine groups ($p_v = 0.01$ & $p_v \leq 0.0001$). However, fish from the FEL + vaccine group exhibited a significant reduction in cortisol levels showing similar levels to control fish ($p_v = 0.98$) (Fig. 1).

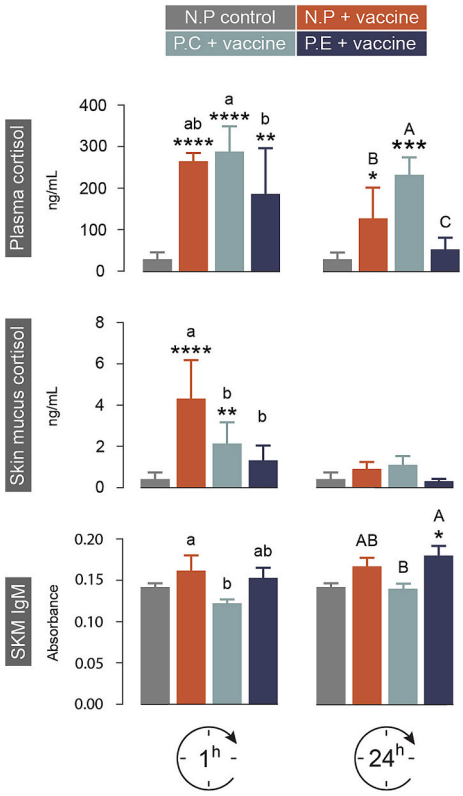


**Fig. 1.** Illustrates cortisol concentrations (ng/mL) in plasma and skin mucus, and total IgM levels following the administration of FishEase-C (FEC) and FishEase-L (FEL) and vaccination at 1 and 24 hpv. Different colors indicate different treatments. Data are represented as mean ± SE ($n = 10$ per sampling time point). (*) indicates significant differences between untreated (N.P. control) and treated groups (N.P + vaccine, FEC + vaccine, and FEL + vaccin) in each time point. The uppercase and lowercase letters indicate significant differences between the three treated groups within each time point ($p < 0.05$).

Cortisol levels in the skin mucus peaked at 1 hpv in the N.P. vaccine and FEC + vaccine groups ($p_v \leq 0.0001$, $p_v = 0.00$), but this enhancement was significantly prevented by sedative compound used (FEL; FEL + vaccine) ($p_v = 0.22$) (Fig. 1). At 24 hpv, the skin mucus cortisol levels exhibited a substantial and rapid decline, returning to baseline levels in all groups ($p_v = 0.72$, $p_v = 0.46$ & $p_v = 0.99$) (Fig. 1).

The total amount of IgM in the skin mucus was increased only in the FEL + vaccine ($p_v = 0.03$) group at 24 hpv. However, the IgM levels were lower in the PC + vaccine group compared to PE + vaccine ($p_v = 0.02$) treatment, with no significant difference between N.P. vaccine and FEC + vaccine, and control ($p_v = 0.25$ & $p_v = 0.99$) (Fig. 1).

### 3.2. SALT response

mRNA expression levels were used to evaluate the effects of plant-based sedatives on the expression of stress-immune related genes (*gr1*, *gr2, crh, hsp70*, *cox2*, *il1β*, *il6*, *il8*, *il10*, *tgfβ1*, *lysozyme*, *c3*, *igm*, *igt* and *igd/igt*) in the skin-associated lymphoid tissue (SALT). While, *gr1* did not show any significant cahnges following the vaccine and FEC/FEL + vaccine treatment, the mRNA levels of *gr2* was only affected by N.P. vaccine ($p_v = 0.01$) at 24 hpv with no significant alteration in the FEC + vaccine ($p_v = 0.99$), and FEL + vaccine groups ($p_v = 0.22$) (Table 2), thus, preventing an mRNA increase for *gr2* by the products. Fish treated with N.P. vaccine or FEC/FEL + vaccine did not exhibit significant cahnges in *crh* tarnscript expression at either 1 (pv > 0.05) or 24 (pv > 0.05) hpv (Table 2). *hsp70* mRNA levels were upregulated by the sole effect of FEC (FEC + vaccine) ($p_v = 0.00$) at 1 hpv. At 24 hpv, in addition to FEC + vaccine ($p_v = 0.00$), N.P. vaccine ($p_v = 0.04$) treatment upreulated the expression of *hsp70* transcript, whereas its expression remained at control levels in the FEL + vaccine ($p_v = 0.83$) (Table 2). *cox2* transcript expression was reduced in the N.P vaccine and FEL + vaccine groups at 1 hpv ($p_v = 0.00$ & $p_v = 0.00$) with significant difference compared to FEC + vaccine. The induced suppression remained in both groups up to 24 hpv ($p_v = 0.00$ & $p_v \leq 0.00$). Despite this, *cox2* was differentially regulated by FEC (FEC + vaccine) at 24 hpv being upregulated ($p_v = 0.02$) compared to control, N.P. vaccine and FEL + vaccine (Table 2).

All genes encoding proinflammatory cytokines and chemokine (*il1β*, *il6* and *il8*) were affected by all treatments and expression of these examined cytokines increased (Table 2). Generally, FEC enhanced pro-inflammatory cytokines at 1 hpv. The expression of *il1β* transcripts was not affected by N.P. vaccine ($p_v = 0.76$), while it was drastically stimulated, particularly by FEC (FEC + vaccine) ($p_v \leq 0.00$), and to a lesser extent by FEL (FEL + vaccine) at 1 hpv ($p_v = 0.03$); however, this expression returned to the control level at 24 hpv (Table 2). The expression of *il6* and *il8* showed similar pattern of change at 1 and 24 hpv except that the vaccine alone did not promote *il8* expression at 1 hpv ($p_v = 0.28$). FEC + vaccine increased *il6* ($p_v = 0.00$) and *il8* ($p_v = 0.00$) transcript expression whereas FEL did not induce any changes at 1 hpv ($p_v = 0.88$ & $p_v = 0.89$). At 24 hpv, a similar expression magnitude of *il6* ($p_v \leq 0.00$) and *il8* ($p_v \leq 0.00$) was induced by vaccine and it was the potent inducer of *il6* and *il8* in vaccinated fish compared to fish treated with the vaccine and products. Interestingly, neither *il6* ($p_v = 0.88$) nor *il8* ($p_v = 0.96$) expression were affected by FEL at either of the given time points (Table 2).

Regarding anti-inflammatory agents, *il10* was downregulated in the

**Table 2**
SALT mRNA level following administration of FishEase-C (FEC) and FishEase-L (FEL) and vaccination at 1 and 24 hpv. The mRNA levels of stress-immune gene transcripts were analysed by quantitative real-time PCR. Data are represented as mean ± SE (n = 10 per sampling time point). (*) indicates significant differences between untreated (N.P. control) and treated groups (N.P + vaccine, FEC + vaccine, and FEL + vaccin) in each time point. The uppercase and lowercase letters indicate significant differences between the three treated groups within each time point ($p < 0.05$).

| | | 1H | | | 24H | | |
|---|---|---|---|---|---|---|---|
| Gene | N.P.Control | N.P.vaccine | FEC + vaccine | FEL + vaccine | N.P.vaccine | FEC + vaccine | FEL + vaccine |
| *gr1* | 1 ± 0.09 | 1.20 ± 0.20 | 1.76 ± 0.24 | 1.13 ± 0.24 | 1.88 ± 0.33 | 2.84 ± 1.63 | 0.82 ± 0.25 |
| *gr2* | 1 ± 0.12 | $1.28^{ab}$ ± 0.07 | $1.39^{a}$ ± 0.16 | $0.88^{b}$ ± 0.17 | $1.60^{*A}$ ± 0.20 | $0.95^{B}$ ± 0.12 | $0.64^{B}$ ± 0.19 |
| *crh* | 1 ± 0.11 | 1.52 ± 0.34 | 1.33 ± 0.27 | 0.72 ± 0.14 | 1.38 ± 0.42 | 3.41 ± 1.93 | 90 ± 0.98 |
| *hsp70* | 1 ± 0.11 | $1.43^{b}$ ± 0.14 | $2.37^{***a}$ ± 0.24 | $0.88^{b}$ ± 0.12 | $1.81^{*A}$ ± 0.36 | $2.02^{**A}$ ± 0.30 | $0.75^{B}$ ± 0.20 |
| *cox2* | 1 ± 0.12 | $0.33^{***b}$ ± 0.08 | $0.85^{a}$ ± 0.08 | $0.36^{***b}$ ± 0.07 | $0.41^{***B}$ ± 0.09 | $1.41^{*A}$ ± 0.22 | $0.11^{****B}$ ± 0.05 |
| *Il1β* | 1 ± 0.09 | $6.01^{b}$ ± 1.71 | $29.63^{****a}$ ± 7.28 | $14.66^{*b}$ ± 5.32 | 2.72 ± 0.79 | 2.06 ± 0.43 | 0.43 ± 0.27 |
| *Il6* | 1 ± 0.12 | $2.61^{*}$ ± 0.23 | $2.93^{**}$ ± 0.45 | 1.41 ± 0.34 | $5.13^{****A}$ ± 0.90 | $1.96^{B}$ ± 0.23 | $0.87^{B}$ ± 0.28 |
| *Il8* | 1 ± 0.11 | $2.33^{ab}$ ± 0.75 | $4.00^{**a}$ ± 0.43 | $1.53^{b}$ ± 0.34 | $5.28^{****A}$ ± 1.20 | $1.50^{B}$ ± 0.15 | $0.66^{B}$ ± 0.22 |
| *Il10* | 1 ± 0.09 | $0.49^{*}$ ± 0.09 | 0.67 ± 0.07 | 0.82 ± 0.20 | 0.61 ± 0.18 | 0.60 ± 0.19 | $0.24^{***}$ ± 0.07 |
| *tgfβ1* | 1 ± 0.10 | 2.01 ± 0.41 | $2.53^{**}$ ± 0.31 | 1.53 ± 0.49 | $1.93^{A}$ ± 0.42 | $1.54^{AB}$ ± 0.19 | $0.74^{B}$ ± 0.19 |
| *lys* | 1 ± 0.11 | $0.37^{**b}$ ± 0.09 | $1.19^{a}$ ± 0.20 | $0.30^{***b}$ ± 0.08 | $0.41^{***AB}$ ± 0.09 | $0.60^{*A}$ ± 0.07 | $0.11^{****B}$ ± 0.05 |
| *c3* | 1 ± 0.10 | 1.81 ± 0.64 | 2.04 ± 0.55 | 1.42 ± 0.41 | $6.72^{****A}$ ± 1.73 | $1.46^{B}$ ± 0.07 | $0.59^{B}$ ± 0.27 |
| *igm* | 1 ± 0.11 | $0.56^{*}$ ± 0.13 | 0.84 ± 0.14 | 0.66 ± 0.14 | $1.41^{A}$ ± 0.17 | $0.71^{B}$ ± 0.10 | $0.42^{**B}$ ± 0.09 |
| *igt* | 1 ± 0.09 | 0.90 ± 0.50 | 1.47 ± 0.41 | 0.72 ± 0.16 | 0.62 ± 0.31 | 1.11 ± 0.25 | 0.60 ± 0.30 |
| *Igd/igt* | 1 ± 0.12 | 0.63 ± 0.12 | 1.13 ± 0.20 | 0.79 ± 0.28 | $1.86^{AB}$ ± 0.34 | $2.50^{A}$ ± 1.32 | $0.42^{B}$ ± 0.20 |

N.P. vaccine ($p_v = 0.03$) group at 1 hpv. While at 24 hpv, this suppression recovered the basal levels in the vaccinated fish, *il10* mRNA level was reduced in the FEL + vaccine group ($p_v = 0.00$). *lysozyme* and *c3* showed a different regulation pattern; *lysozyme* mRNA levels decresed in the N.P. vaccine and FEL + vaccine groups ($p_v = 0.00$, $p_v \leq 0.00$) at 1 hpv compared to control with the exception of FEC + vaccine, which did not change *lysozyme* expression ($p_v = 0.50$) at 1 hpv. However, at 24 hpv *lysozyme* transcript expression was downregulated in all groups ($p_v = 0.00$, $p_v = 0.04$, $p_v \leq 0.00$ respectively) with lower suppression noted for FEL + vaccine compared to FEC + vaccine ($p_v = 0.00$). *c3* transcript expression was significantly increased only by N.P. vaccine at 24 hpv compared to control ($p_v \leq 0.00$) and FEC/FEL treatment ($p_v \leq 0.00$). Therefore, reduction of *lysozyme* at 24 hpv was notable by all treatments. The expression of *igm* transcripts was reduced in the N.P. vaccine group ($p_v = 0.04$) at 1 hpv and in the FEL + vaccine group ($p_v \leq 0.01$) at 24 hpv with the dominant effect observed for FEL ($p_v \leq 0.01$). The vaccine group could recover to the basal level by 24 hpv (Table 2). No significant changes were noted for *igt* and *igd/igt* at any time point.

### 3.3. GIALT response

Stress-related genes (*gr1*, *gr2*, *crh*, *hsp70* and *cox2* showed the same regulation pattern in the gills. FEC was the exclusive driver of expression of these stress genes at 1 hpv *gr2*; ($p_v = 0.00$), *hsp70*; ($p_v = 0.00$), and *cox2*; ($p_v \leq 0.00$) except *gr1* ($p_v = 0.14$) and *crh* ($p_v = 0.82$) exhibited no significant changes in mRNA levels (Table 3). However, at 24 hpv the transcript expression of these genes: *gr1* ($p_v \leq 0.00$), *gr2* ($p_v \leq 0.00$), *crh* ($p_v = 0.00$), *hsp70* ($p_v \leq 0.00$) and *cox2* ($p_v \leq 0.00$) were significantly upregulated. Particularly *crh* and *cox2* were found to be remarkably upregulated by FEC at 24 hpv, and no changes were induced by N.P. vaccine or FEL + vaccine (Table 3). Interestingly, similar expression patterns were observed for the pro-inflammatory cytokines *il1β* ($p_v = 0.00$), *il6* ($p_v \leq 0.00$), and anti-inflammatory cytokines *il10* ($p_v = 0.00$) and *tgfβ1* ($p_v \leq 0.00$) at 24 hpv (Table 3). FEC appeared to be a potent stimulator of these genes at 24 hpv compared to both control (N.P. control and N.P. vaccine) and FEL + vaccine. However, at 1 hpv, FEC did not alter the mRNA of these genes. Similarly, for immune genes *c3* ($p_v = 0.00$), *lysozyme* ($p_v = 0.00$), *igm* ($p_v \leq 0.00$), *igt* ($p_v \leq 0.00$) and *igd/igt* ($p_v \leq 0.00$), FEC + vaccine remarkably increased their transcript expression at 24 hpv compared to control, N.P. vaccine and FEL + vaccine No significant changes were observed at 1 hpv for these genes, nor were they induced by N.P. vaccine or FEL + vaccine treatments (Table 3).

### 3.4. GALT response

All genes encoding stress genes *gr1*, *gr2*, *hsp70* and *crh* were mainly

**Table 3**
GIALT mRNA level following administration of FishEase-C (FEC) and FishEase-L (FEL) and vaccination at 1 and 24 hpv. The mRNA levels of stress-immune gene transcripts were analysed by quantitative real-time PCR. Data are represented as mean ± SE (n = 10 per sampling time point). (*) indicates significant differences between untreated (N.P. control) and treated groups (N.P + vaccine, FEC + vaccine, and FEL + vaccin) in each time point. The uppercase and lowercase letters indicate significant differences between the three treated groups within each time point ($p < 0.05$).

| | | 1H | | | 24H | | |
|---|---|---|---|---|---|---|---|
| Gene | N.P.Control | N.P.vaccine | FEC + vaccine | FEL + vaccine | N.P.vaccine | FEC + vaccine | FEL + vaccine |
| *gr1* | 1 ± 0.06 | 0.93 ± 0.16 | 3.89 ± 0.79 | 0.52 ± 0.06 | $0.77^{B}$ ± 0.12 | $9.79^{****A}$ ± 2.36 | $0.56^{B}$ ± 0.19 |
| *gr2* | 1 ± 0.05 | $0.69^{b}$ ± 0.08 | $3.73^{**a}$ ± 0.77 | $0.72^{b}$ ± 0.26 | $0.68^{B}$ ± 0.13 | $5.33^{****A}$ ± 1.39 | $0.47^{B}$ ± 0.16 |
| *crh* | 1 ± 0.11 | 1.25 ± 0.33 | 21.32 ± 6.45 | 0.65 ± 0.31 | $1.14^{B}$ ± 0.27 | $73.04^{***A}$ ± 31.18 | $0.83^{B}$ ± 0.30 |
| *hsp70* | 1 ± 0.07 | $0.87^{b}$ ± 0.03 | $8.91^{**a}$ ± 1.50 | $0.48^{b}$ ± 0.07 | $0.52^{B}$ ± 0.08 | $18.38^{****A}$ ± 4.20 | $0.38^{B}$ ± 0.04 |
| *cox2* | 1 ± 0.06 | $0.18^{b}$ ± 0.05 | $9.23^{****a}$ ± 1.29 | $0.46^{b}$ ± 0.20 | $0.11^{B}$ ± 0.01 | $18.53^{****A}$ ± 3.55 | $0.14^{B}$ ± 0.13 |
| *Il1β* | 1 ± 0.08 | 0.76 ± 0.18 | 1.78 ± 0.36 | 1.19 ± 0.40 | $0.59^{B}$ ± 0.10 | $5.47^{***A}$ ± 1.72 | $0.58^{B}$ ± 0.08 |
| *Il6* | 1 ± 0.10 | 0.66 ± 0.19 | 10.60 ± 3.79 | 1.73 ± 0.59 | $0.64^{B}$ ± 0.18 | $30.54^{****A}$ ± 8.34 | $0.70^{B}$ ± 0.41 |
| *Il8* | 1 ± 0.08 | $0.74^{b}$ ± 0.19 | $19.98^{**a}$ ± 3.02 | $1.52^{b}$ ± 0.65 | $1.65^{B}$ ± 0.42 | $27.87^{****A}$ ± 8.55 | $0.98^{B}$ ± 0.81 |
| *Il10* | 1 ± 0.08 | 0.65 ± 0.18 | 2.27 ± 0.39 | 1.15 ± 0.38 | $0.57^{B}$ ± 0.09 | $5.16^{**A}$ ± 2.04 | $0.56^{B}$ ± 0.17 |
| *tgfβ1* | 1 ± 0.08 | 0.92 ± 0.15 | 5.01 ± 0.83 | 0.54 ± 0.08 | $0.77^{B}$ ± 0.09 | $12.73^{****A}$ ± 4.33 | $0.48^{B}$ ± 0.14 |
| *lys* | 1 ± 0.09 | $0.32^{b}$ ± 0.04 | $10.44^{a}$ ± 3.93 | $0.56^{b}$ ± 0.19 | $0.19^{B}$ ± 0.03 | $19.61^{***A}$ ± 6.87 | $0.14^{B}$ ± 0.05 |
| *c3* | 1 ± 0.10 | 1.50 ± 0.52 | 5.25 ± 0.33 | 0.64 ± 0.20 | $1.20^{B}$ ± 0.24 | $16.97^{***A}$ ± 6.61 | $0.54^{B}$ ± 0.27 |
| *igm* | 1 ± 0.09 | 0.59 ± 0.13 | 1.74 ± 0.19 | 0.80 ± 0.21 | $0.87^{B}$ ± 0.07 | $9.44^{****A}$ ± 2.84 | $0.50^{B}$ ± 0.14 |
| *igt* | 1 ± 0.08 | 0.53 ± 0.15 | 5.22 ± 0.78 | 0.56 ± 0.21 | $0.51^{B}$ ± 0.09 | $19.98^{****A}$ ± 4.66 | $0.55^{B}$ ± 0.23 |
| *Igd/igt* | 1 ± 0.08 | 0.74 ± 0.15 | 3.57 ± 0.79 | 0.67 ± 0.18 | $0.79^{B}$ ± 0.15 | $13.71^{****A}$ ± 4.77 | $0.45^{B}$ ± 0.12 |

downregulated in the intestine by all treatments (N.P. vaccine, FEC + vaccine and FEL + vaccine (Table 4).

At 1 hpv, *gr1* transcript expression was decreased by FEL (FEL + vaccine) only ($p_v \leq 0.00$), but at 24 hpv, a similar reduction was observed in all groups ($p_v \leq 0.00$, $p_v = 0.00$ & $p_v \leq 0.00$) (Table 4). However, a stronger downregulation was induced by the combination of vaccine and FEL compared to FEC + vaccine ($p_v = 0.02$). In contrast, for *gr2* expression a general reduction was observed at 1 and 24 hpv as a result of vaccine and vaccine+products exposure. At 1 hpv, the reduction trend begun with N.P. vaccine ($p_v \leq 0.00$), followed by a reduction induced by FEC + vaccine ($p_v \leq 0.00$) and *gr2* expression appeared to be highly affacted by FEL ($p_v \leq 0.00$) (Table 4). Contrary to this trend, *gr2* expression at 24 hpv in the FEC + vaccine group was less downregulated than N.P. vaccine ($p_v = 0.00$) and FEL + vaccine groups ($p_v = 0.00$) with no significant difference between N.P. vaccine and FEL + vaccine ($p_v = 0.59$). A similar reduction pattern to *gr2* at 24 hpv was also observed for *hsp70*. The observed regulation at 1 hpv was similar to that of *gr2* but with no suppressing effect of FEC ($p_v = 0.99$), thus differed from the N.P. vaccine ($p_v = 0.01$) and FEL + vaccine ($p_v \leq 0.00$). In contrast, the expression of *cox2* transcripts was differentially regulated at 1 hpv. *cox2* was upregulated by the effect of FEC ($p_v = 0.00$) and returned to the basal level at 24 hpv ($p_v = 0.99$), and vaccine ($p_v = 0.12$) and FEL + vaccine ($p_v = 0.10$) remained unaltered at 24 hpv (Table 4).

Genes encoding *il1β*, *il6* and *il8* were significantly regulated by the vaccine effect compared to the skin and gills, and considerable variation was observed with regard to their expression profiles (Table 4). The genes encoding *il1β* and *il6*, the key proinflammatory cytokines, showed a similar expression pattern particularly at 24 hpv with higher magnitude for *il1β*. But at 1 hpv, increased gene transcript expression was more pronounced for *il1β*, all treatment N.P. vaccine ($p_v = 0.02$), FEC + vaccine ($p_v \leq 0.00$) and FEL + vaccine ($p_v \leq 0.00$) significantly increased *il1β* with no significant differences observed between the treatments (Table 4). However, the expression of *il6* transcripts increased solely due to the effect of FEC + vaccine ($p_v = 0.02$). At 24 hpv, *il1β* expression remained upregulated in both N.P. vaccine ($p_v = 0.04$) and FEC + vaccine ($p_v \leq 0.00$) groups with FEL + vaccine returning its expression to the control levels ($p_v = 0.82$). Additionally, vaccine promoted *il16* expression ($p_v = 0.04$) at 24 hpv, while *il6* expression continued to increaese in the FEC + vaccine group ($p_v \leq 0.00$) (Table 4). *Il6* remained unaltered in the FEL + vaccine at both time points. *Il8* transcript expression exhibited a slightly different expression pattern. At 1 hpv, no significant regulation was detected, whereas at 24 hpv, an increased transcript expression was noted fro *il8* induced by both N.P. vaccine ($p_v = 0.00$), and FEC + vaccine ($p_v \leq 0.00$) treatments (Table 4).

Anti-inflammatory agents *il10* and *tgfβ1* expression were decresed at 1 hpv in the FEC + vaccine ($p_v = 0.01$) and the FEL + vaccine ($p_v = 0.00$) group, respectively. At 24 hpv, a general reduction was recorded in all groups for *il10* (N.P. vaccine; $p_v = 0.03$, FEC + vaccine; $p_v = 0.01$, FEL + vacine; $p_v = 0.01$) and *tgfβ1* (N.P. vaccine; $p_v = 0.00$, FEL + vacine; $p_v \leq 0.00$), except that FEC + vaccine did not alter *tgfβ1* ($p_v = 0.95$) mRNA levels. Unlike *c3* which showed no significant ulteration, *lysozyme* exhibited suppression in the N.P. vaccine ($p_v \leq 0.00$) and FEL + vaccine ($p_v \leq 0.00$) groups at 1 hpv, and further suppression was also induced at 24 hpv in the FEC + vaccine group ($p_v = 0.00$). However, less suppression was triggered by FEC compared to N.P. vaccine ($p_v \leq 0.00$) and FEL + vaccine ($p_v \leq 0.00$) (Table 4). Fish exposed to the treatments-N.P. vaccine, FEC + vaccine and FEL + vaccine were unable to alter mRNA levels for *igm*, *igt* and *igd/igt* (Table 4).

## 4. Discussion

Our findings indicate that the application of waterborne sedatives (FEC and FEL) prior to IP vaccination exerts distinct mitigatory/stimulatory effects on different mucosa (skin, gill, and intestine). Overall, FEL alleviated the stress response to vaccination, whereas FEC elicited a pronounced stress response in the gills.

### 4.1. SALT response

Plasma cortisol elevation in all groups as a result of the vaccination process is consistent with that previously reported in fish submitted to intraperitoneal vaccination, and can be explained by the fact that this process involves sedation, handling and inflammation induced by antigen exposure/and injection (Tort, 2011). In this way, *V. anguillarum*, *E. ictaluri* and *A. hydropila* have also been shown to activate BSC and HPI axes, thus releasing adrenaline and cortisol, which may compromise vaccine success (Bilodeau et al., 2003; Bowers et al., 2000; Khansari et al., 2018; Tort, 2011). Previous reports have shown that *Aloysia triphylla* and *Lippia alba* EOs, when used as sedatives, reduced plasma cortisol in fish (da Cunha et al., 2010; Zeppenfeld et al., 2014). Moreover, previously phytogenic blends (terpenes and flavonoids) and garlic supplementation was shown to decrease plasma cortisol in European seabass and carp (Montero et al., 2024; Yousefi et al., 2020). Under this context, FEL reduced cortisol secretion in plasma by 24 hpv, supports a protective role respect to cortisol enhancement associated to vaccination procedure. In fact, EOs due to possessing lipophilic properties and ability to rapidly diffuse through brain cell membrane, or by regulating the gamma-aminobiotyric acid receptor complex (GABA), can modulate

**Table 4**
GALT mRNA level following administration of FishEase-C (FEC) and FishEase-L (FEL) and vaccination at 1 and 24 hpv. The mRNA levels of stress-immune gene transcripts were analysed by quantitative real-time PCR. Data are represented as mean ± SE (n = 10 per sampling time point). (*) indicates significant differences between untreated (N.P. control) and treated groups (N.P + vaccine, FEC + vaccine, and FEL + vaccin) in each time point. The uppercase and lowercase letters indicate significant differences between the three treated groups within each time point ($p < 0.05$).

| | | 1H | | | 24H | | |
|---|---|---|---|---|---|---|---|
| Gene | N.P.Control | N.P.vaccine | FEC + vaccine | FEL + vaccine | N.P.vaccine | FEC + vaccine | FEL + vaccine |
| *gr1* | 1 ± 0.07 | $1.07^{a}$ ± 0.10 | $1.05^{a}$ ± 0.12 | $0.48^{****b}$ ± 0.04 | $0.34^{****AB}$ ± 0.03 | $0.56^{***A}$ ± 0.07 | $0.26^{****B}$ ± 0.02 |
| *gr2* | 1 ± 0.09 | $0.69^{****a}$ ± 0.06 | $0.47^{****b}$ ± 0.03 | $0.25^{****c}$ ± 0.03 | $0.20^{****B}$ ± 0.02 | $0.42^{****A}$ ± 0.06 | $0.14^{****B}$ ± 0.02 |
| *crh* | 1 ± 0.11 | $0.26^{***ab}$ ± 0.09 | $0.58^{*a}$ ± 0.15 | $0.05^{****b}$ ± 0.00 | $0.14^{****B}$ ± 0.05 | $0.60^{A}$ ± 0.22 | $0.09^{****B}$ ± 0.06 |
| *hsp70* | 1 ± 0.07 | $0.74^{*b}$ ± 0.06 | $1.02^{a}$ ± 0.12 | $0.32^{***c}$ ± 0.04 | $0.35^{****B}$ ± 0.06 | $0.64^{**A}$ ± 0.07 | $0.21^{****B}$ ± 0.02 |
| *cox2* | 1 ± 0.08 | $0.45^{b}$ ± 0.05 | $2.73^{***a}$ ± 0.78 | $0.44^{b}$ ± 0.13 | 0.16 ± 0.01 | 0.90 ± 0.06 | 0.13 ± 0.02 |
| *Il1β* | 1 ± 0.08 | $18.22^{**}$ ± 1.81 | $25.19^{****}$ ± 6.60 | $25.81^{****}$ ± 4.5 | $13.57^{*B}$ ± 0.75 | $30.23^{****A}$ ± 2.6 | $4.87^{B}$ ± 1.4 |
| *Il6* | 1 ± 0.11 | 2.85 ± 0.15 | $3.75^{*}$ ± 1.05 | 2.27 ± 0.58 | $3.68^{*B}$ ± 0.41 | $11.31^{****A}$ ± 0.46 | $1.71^{B}$ ± 0.13 |
| *Il8* | 1 ± 0.10 | 3.53 ± 1.09 | 4.24 ± 0.60 | 4.54 ± 1.27 | $9.04^{***A}$ ± 1.30 | $11.25^{****A}$ ± 2.55 | $3.52^{B}$ ± 0.58 |
| *Il10* | 1 ± 0.09 | $1.05^{a}$ ± 0.23 | $0.52^{*a}$ ± 0.04 | $0.84^{ab}$ ± 0.07 | $0.58^{*}$ ± 0.11 | $0.53^{*}$ ± 0.10 | $0.52^{*}$ ± 0.05 |
| *tgfβ1* | 1 ± 0.07 | $0.99^{a}$ ± 0.12 | $1.07^{a}$ ± 0.21 | $0.33^{***b}$ ± 0.03 | $0.48^{**B}$ ± 0.05 | $0.92^{A}$ ± 0.07 | $0.31^{****B}$ ± 0.02 |
| *lys* | 1 ± 0.08 | $0.44^{****b}$ ± 0.07 | $0.98^{a}$ ± 0.14 | $0.19^{****b}$ ± 0.02 | $0.19^{****B}$ ± 0.03 | $0.63^{**A}$ ± 0.12 | $0.09^{****B}$ ± 0.00 |
| *c3* | 1 ± 0.12 | $0.46^{ab}$ ± 0.17 | $1.51^{a}$ ± 0.61 | $0.07^{b}$ ± 0.00 | $1.01^{AB}$ ± 0.23 | $1.27^{A}$ ± 0.47 | $0.35^{B}$ ± 0.14 |
| *igm* | 1 ± 0.11 | 0.99 ± 0.36 | 1.08 ± 0.47 | 0.32 ± 0.14 | 0.37 ± 0.12 | 0.38 ± 0.22 | 0.29 ± 0.16 |
| *igt* | 1 ± 0.10 | $1.27^{ab}$ ± 0.31 | $1.69^{a}$ ± 0.45 | $0.69^{b}$ ± 0.19 | 0.77 ± 0.16 | 1.30 ± 0.13 | 0.54 ± 0.04 |
| *Igd/igt* | 1 ± 0.09 | $1.14^{ab}$ ± 0.20 | $1.34^{a}$ ± 0.16 | $0.64^{b}$ ± 0.08 | $0.66^{B}$ ± 0.13 | $1.29^{A}$ ± 0.28 | $0.43^{B}$ ± 0.06 |

the brain activation and thereby mitigate the stress response (Bakkali et al., 2008; Chioca et al., 2013). Unlike FEL, FEC increased plasma cortisol level at 24 hpv, indicating that, similar to anesthetics such as benzocaine and tricaine (MS222), this product may be somehow stressful for sea bass and other teleost depending on the carvacrol content (Jerez-Cepa et al., 2019). However, the noted inconsistency with previous reports may imply i) the thymol-based product has a sedative effect, which could be influenced by the concentration used or its interaction with carvacrol; and ii) substantianal variations among fish species in response to sedative/anesthetics (Zahl et al., 2012).

In addittion to plasma, mucus is other matrix where cortisol can be detected and measured. The secretion of cortisol in the skin mucus post vaccination agree with our previous results, and its reduction by both products, particularly FEL (no significant increase), could indicate mitigation of stress response at skin level (Firmino et al., 2021; Khansari et al., 2018). It is interesting to remark the differential effect of FEC in the skin, compared to its systemic effect, and this deserves future research.

In the present study, IgM enhancement as an important immunoglobulins (Igs), as affected only by FEL + vaccine treatment in the skin mucus aligns partially with our previous results, where *V. anguillarum* vaccination by immerssion similarly did not enhance IgM level in sea bream skin mucus. This points out the significant role of the FEL treatment in combination with vaccination although species-specific differences can't be ruled out (Khansari et al., 2018; Muñoz-Atienza et al., 2021). This finding is consistent with the results of Guardiola et al. (2016), where dietary thymol raised Igs levels. Nonetheless, no change in IgM levels in vaccinated fish can compromise the general health status of the animal and thus make it more suceptible to upcoming infections (Ghafarifarsani et al., 2022).

Cortisol exerts its genomic effect through glucocorticoid receptors (GRs) in the target tissues, which have varying sensitivity to cortisol concentration (Pavlidis et al., 2011; Vijayan et al., 2003). Align with previous findings that proposed a major role for *gr2* in GR-cortisol ligand formation in sea bass, in the current study, the changes driven by vaccination were only pronounced in *gr2* with no role for *gr1* (Di Bella et al., 2008). Furthermore, the lack of *gr2* regulation by FEC/FEL + vaccine in the skin reinforce the hypothesis of a protective mechanism to prevent more allostatic load associated with vaccination.

Heat shock proteins (HSPs) are essential during stress situation, functioning as chaperones in collaboration with GRs while also protecting cells (Sathiyaa and Vijayan, 2003). Under this context, *hsp70* increased expression in the FEC + vaccine and vaccinated group indicated a stress-associated effect of vaccine consistent with our previous results in the skin of rainbow trout and sea bream (Khansari et al., 2024, 2018). However, FEL apparently offers cellular protective properties in accordance with the recent results of our research group, where genes of the HSP family (*dnajc8*, *dnajb9* and *hspa14*) were down-regulated in the skin mucus following thymol and garlic supplementation (Firmino et al., 2021). *cox2* is a potent mediator of inflammation and its regulation is different in response to a wide variety of challenging conditions (Khansari et al., 2018; Otto and Smith, 1996; Ricciotti and Fitzgerald, 2011). Therefore, different regulation of *cox2* by vaccine and FEC in our study suggested an additional stress effect imposed by FEC in the skin.

*Il1β* plays several systemic biological roles and is crucial for initiating the inflammatory response after infection/vaccination (Benjamin et al., 1977). Thus, FEC, and to a lesser extent FEL, in combination with the vaccine, induced a remarkable transient inflammation in the skin. However, *il1β* dropped to the basal level by 24 hpv. This aligns with previous results, where *il1β* expression was stimulated with different stressors/pathogens *I. multifiliis* and *V. anguillarum* (Alipio et al., 2023; Moreira et al., 2017). Interestingly, *Il6*, which is also an inflammatory agent, showed a lower threshold of activation than *il1β* and was enhanced in vaccinated and FEC + vaccine groups. However, its reduction by the effect of FEC by 24 hpv and the lack of *il6* expression in the FEL + vaccine group might be attributed to the anti-inflammatory influence of both products particularly FEL. This has previously been reported in Nile tilapia, with thymol decreasing *il1β* and *tnfα* expression following *A. hyrophila* infection (Khalil et al., 2023). As a balance between proinflammatory and anti-inflammatory response, the induction of anti-inflammatory agents within the inflammatory response is of paramount importance to control the inflammation (Wang and Secombes, 2013). Therfore, as with previous results, it is not surprising to observe *tgfβ1* expression induced by FEC + vaccine, which results in minimizing tissue damage. The different regulation pattern observed for *il10* suggests a higher involvement of *tgfβ1* in controlling inflammation in the skin of sea bass treated with FEC (Moreira et al., 2017; Pinto et al., 2007). Unlike the transient *il8* expression early post vaccination in the FEC + vaccine, the vaccine alone later induced *il8* expression. This is probably due to its primary function in recruiting and attracting neutrophils to the site of inflammation in the vaccine group (Carriero et al., 2020; Omaima Harun et al., 2008). Other agents present in the skin involved in erradicating bacteria and microbes include *c3* and *lysozyme*. In contrary to our previous data (Khansari et al., 2018), a general decrease was detected for *lysozyme* expression, whereas only vaccine was able to enhance extrahepatic *c3* expression in skin suggesting species-specific response to vaccine/antigen in the skin. However, as previously observed (Bahi et al., 2018), a decrease or no change in *c3* and *lysozyme* activity, induced by EOs, may increase suceptibily to pathogens.

The early suppression of *igm*, a major marker of B cells also present in SALT, following vaccination is in line with the different effect of vaccine/stress on *igm* transcripts previously reported in fish that might be related to: a) antigen-specific response and; b) EOs' immune-modulatory properties because of a later repression observed for *igm* (FEL + vaccine) (Galindo-Villegas et al., 2013; Khansari et al., 2018). In contrast to rainbow trout *igt*, no regulation on sea bass *igt* and *igd/igt* i.e., a newly decribed Ig in European sea bass by vaccine may indicate lesser contribution of this Ig in the skin of sea bass (Buonocore et al., 2020, 2017).

### 4.2. GIALT response

The increased expression of stress related genes (*gr1, gr2, crh*, *hsp70* and *cox2*) in the FEC + vaccine group was likely due to the synergic stress effect of the vaccine combined with thymol and carvacrol, present in the FEC employed in this study. As mentioned before, the slow effect (genomic) of glucocorticoids (GCs) (mainly cortisol) are mediated by intracellular GR, although in certain situations a fast effect of cortisol can be induced by membrane GRs (Aedo et al., 2019; Das et al., 2018). Although high cortisol levels were also observed in vaccine and FEL + vaccine groups, cortisol modulated only gill *gr1* and *gr2* expression in FEC + vaccine group, indicating higher responsiveness of this tissue to stress effect of FEC. Moreover, unlike skin, the regulation of *gr1* and *gr2* in sea bass gill indicates that i) both receptors were involved in mediating cortisol's genomic effects and; ii) confirming a greater implication of *gr2* also in the gill. It is also important to note that the drastic *crh* expression in the gills shows the degree of stress induced by FEC. As previously mentioned, transcription of *hsp70* and GRs is interconnected due to its chaperone role and thus expression of this HSP by FEC is not surprising. Consistent with our previous data showing different regulation of *hsp70* in rainbow trout and sea bream gills in response to *V. anguillarum*, we confirmed the specificty of the response in each fish species (Khansari et al., 2018). Furtheremore, high regulation of gill *hsp70* expression supports a stress-inducing effect of FEC at the tested concentration (Realini and Marcos, 2014). In addition to *hsp70*, the remarkable gill *cox2* expression in FEC treated fish following vaccination reinforces the hypothesis of higher absorption of this compound through gills compared to skin and gut. This may be the reason for the more pronounced stress response in gill respect to other tissues assessed. Of note, no induction of *cox2* by the vaccine in the sea bass gill is consistent with results obtained in rainbow trout, but not sea bream,

indicating again a species-specific response of the gills (Khansari et al., 2018). It is also worth noting that, analogous to the situation in mammals, *cox2* and *hsp70* expression in fish may play an important role in biphasic pro and anti-infllamatory responses as a result of gill exposure to FEC (Stolte et al., 2009). Furthermore, in humans, *il1β* is an inducer of *cox2* in stress situations, and thus modulation of *cox2* in the present study may be related to the *il1β* expression due to stimulating macrophages (Ansari and Haqqi, 2016). On the other hand, overexpression of pro-inflammatory cytokines and chemokines (namely *il1β*, *il6* and *il8*) after exposure to environmental stimuli was suggested to be associeted with an immunotoxic effect. This immunotoxic inflammatory response in our study culminated in a marked up-regulation of *il1β*, *il6* and *il8* (Abou-Zeid et al., 2021). Therefore, concomitant induction of anti-inflammatory cytokines *il10* and *tgfβ1* was required to maintain the general status of gills due to involvement in other physiological functions e.g., oxygen exchange and ion homeostasis (Hughes and Morgan, 1973). EOs, namely thymol, have previously shown antioxidant properties that can alleviate inflammation caused by environmental stressors (De Freitas Souza et al., 2019; Khalil et al., 2023). Since, oxidative stress triggers proinflammatory cytokines, it is plausible that unlike FEL, FEC at this concentration stimulated reactive oxygen species (ROS) and thereby eliciting an inflammatory response (Liang et al., 2017).

Furthermore, the later scenario is activation of NF-κB transcription factor suggested by Wang et al. (2014). This could be associated with cytokine expression in the gills treated with FEC. This high regulation of pro/anti-inflammatory cytokines in the gill might be related to an immune cell accumulation in the interbrachial lymphoid tissue (ILT), resembling the mammalian lymph node (Parra et al., 2015). *lysozyme* and *c3* also showed significant upregulation triggered by FEC in gills, but no changes were noted by the vaccine. Consideration should be given to the increase of these two antibacterial components of the mucosal immune system and thus we thought that this may be as a result of the integrated gill stress response in response to FEC. However, our previous findings showed different thresholds of responsivenes of extrahepatic *c3* and *lysozyme* to *V. anguillarum* vaccine in the gills of sea bream and rainbow trout (Khansari et al., 2018).

On the other hand, clove oil previously showed enhanced lysozyme and bactericidal activities at a concentration of 25 mg/L in skin mucus of rainbow trout, whereas a concentration of 55 mg/L reduced hemolytic complement activity in sea bream. This indicates the importance of fish species and concentration of EOs employed (Bahi et al., 2018; Soltanian et al., 2018). In addition to the concentration of thymol examined in this study, its combination with carvacrol or trace component of FEC was found to be highly stressful for sea bass specimen. Thus, the stress response may impair the actual immune function of the gills. As such, the consequent *igm*, *igt* and *igd/igt* enhancement expression could also be a result of stress induction in gills exposed to FEC, reflecting the innate features of B cells.

### 4.3. GALT response

Unlike gills and skin, *gr1* and *gr2* expression was mainly reduced in intestine across all experimental groups and different regulation of GRs in MALTs may indicate adoption of a protective mechanisms to reduce GCs effect on intestine as previously reported in stress situations (Teles et al., 2013). However, considering the reduction of both GRs in intestine as a protective mechanism, milder stress-immune genes transcription would be expected. Therefore, reduction of *crh* and *hsp70* transcript levels in the intestine may confirm lower stress induction by the vaccine despite previous insight that animals usually show a rapid *crh* regulation (local CRH system) as a consequence of rapid stress hormone secretion (Gorissen and Flik, 2016; Tort, 2011).

Thanks to the evaluation of HSPs in response to stress and pathological conditions, recent results have shown increased levels of these proteins in fish gut, which is not in line with our recent results. The observed downregulation of *hsp70*, primarily induced by the vaccine is probably due to the tissue/pathogen specific response (Song et al., 2016).

An increase in intestinal permeability is considered as one of the deleterios influences of cortisol secretion (Olsen et al., 2012). Thus, a reduction of permeability is essential for homeostasis through cyclooxygenase proteins (e.g., *cox2*). Therefore, increased *cox2* expression in the FEC + vaccine group despite no changes or a tendency for suppression in the N.P. vaccine and FEL + vaccine indicates a transient contribution of the FEC to increasing the intestine permeabilty. *il1β* transcription was enhanced in all groups in agreement with previous findings in sea bass showing increased expression of this pro-inflammatory cytokine in the gut following *V. anguillarum* infection. However, at 24 hpv, only FEL could alleviate the inflammation and hence reduced *ilβ* expression (Torrecillas et al., 2017). A similar effect of FEL was observed which interestingly inhibited *il6* expression during the antigen exposure by the vaccine. This confirms the alleviatory influence of FEL in gut during inflammation. Thymol and other EOs, such as oregano, have previously been shown to reduce inflammation by decreasing the transcript expression of *il1β* and *tnfα* in fish gut, liver and spleen (Khalil et al., 2023; M. Li et al., 2022b). This mitigatory effect might be associated with inhibiting the transcription factor NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways to avoid overexpression of the pro-inflammatory cytokines, as suggested by Liang et al. (2014). Analogous to this, the expression of chemokine *il8* in intestine was inhibited by FEL in contrast to its increased expression in the N.P. vaccine and FEC + vaccine. Despite the immunological need for activating anti-inflammatory agents during episodes of inflammation, the observed general dowregulation of anti-inflammatory agents indicates a specific response of gut to the injected vaccine, or control of the inflammation by another anti-inflammatory agent. *Lysozyme* expression was also suppressed by the vaccinaton procedure, but less repression was regulated by the effect of FEC. Furthermore, a general decrease at 24 hpv indicates i) a specific response of sea bass gut to vaccine, as it was previously observed in sea bream and rainbow trout gut (Khansari et al., 2018); ii) the adverse effects of the EO (FEC/FEL) on *lysozyme* expression is inconsistent with previous findings that have shown enhanced lysozyme activity in rainbow trout and silver catfish following EO exposure (clove oil, *H. ringens* and *O. americanum*). This may be related to the combined effect of vaccine and FEC/FEL in our study (Soltanian et al., 2018; Sutili et al., 2015). Additionaly, a contributing factor could be a decrease in neutrophilic number, resulting in *lysozyme* reduction as previously shown in silver catfish fed with *Aloysia triphylla* despite promoting bacterial resistence (dos Santos et al., 2017). Like rainbow trout GALT, sea bass gut $IgT^+$ B cells are the dominant population (30 %) and $IgM^+$ B cells comprise 2–10 %, therefore the lack of significant regulation of *igm* or *igt*/and *igd/igt* may represent a specific response of the sea bass intestine to the injected bivalent vaccine (Picchietti et al., 2021).

## 5. Conclusion

Our findings revealed that IP vaccination triggers the onset of stress responses event in the fish mucosal barriers. Observational and behavioral examinations following administration of the products confirm that thymol derived products with lower carvacrol content showed a sedative effect and a quick recovery within 10 min. Furthermore, physiological stress indices e.g., plasma and skin mucus cortisol as well as mucosal transcript response of stress-immune related genes revealed sedative properties of oil FEL, thus mitigating the adverse stress effects of vaccination. In contrast, FEC appear to be an stressing agent rather than a sedative at 30 ppm concentration in the gill, suggesting that the product was primarily absorbed through the gills. Differences on the effects of the products (FEC and FEL) on stress-immune related genes may be related to carvacrol content which affects secondary consituents and trace components, as both tested products are primarily thymol-based.

## CRediT authorship contribution statement

**Sheida Azizi:** Writing – original draft, Methodology, Investigation, Formal analysis. **Mahsa Shahbazi:** Methodology. **Sara Cartan:** Methodology, Investigation. **Ismael Jerez-Cepa:** Methodology, Investigation. **Juan M. Mancera:** Writing – original draft, Supervision, Methodology. **Darragh Doyle:** Writing – original draft, Methodology. **Lluis Tort:** Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition. **Ali Reza Khansari:** Writing – original draft, Methodology, Investigation, Formal analysis.

## Declaration of competing interest

The authors have declared no conflicts of interest.

## Data availability

Data will be made available on request.

## Acknowledgements

Thanks are given to Pilar Tudela (UAB), the University of Cadiz team, and Iria Iglesias for their technical help and all the facilities given to the researchers during the experiments. This work is part of the Project PISCIBIEN supported by the Next Generation funds from the Ministry of Agriculture of Spain supported by the European Union under the Plan de Recuperación, Transformación y Resiliencia (RD 685/2001) and also the grant (PID2020-117557RB-C21-C22) funded by MCIN/AEI/10.13039/ awarded to LT and JMM (Spain). Thanks are also given to TECNOVIT-FARMFAES and Javier Feito for providing the experimental Thymol oil products.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.742109.

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