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TRPV1 Receptor Identification in Rainbow Trout (*Oncorhynchus mykiss*) and Evaluation of the Effects Produced by *Ocimum basilicum* Super Critical Fluid Extract

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Abstract: Transient receptor potential vanilloid type 1 (TRPV1) has been investigated in humans and mammals; in recent years, some researchers have focused on this receptor in fishes. The present study aimed to identify TRPV1 receptors in cultures of RT-gill W1 cells and in the organs of rainbow trout (*Oncorhynchus mykiss*), in addition to evaluating the possible modulation induced by super critical fluid extract of basil (*Ocimum basilicum*), named F1-BEO. In vitro evaluation consisted of cell cultures and immunocytochemistry assays. During in vivo experimental sessions, eighty trout were divided into five groups that received a fish diet supplemented with 0, 0.5, 1, 2 and 3% *w*/*w* F1-BEO. Forty trout were euthanized after 15 and 30 days; organs were collected and processed according to the immunohistochemistry technique. Receptor expression was quantitatively measured using Image Pro Plus software. TRPV1 was identified in RT-gill W1 cells and in all organs, with a higher positivity in the muscle layers of the stomach, intestine and kidneys. F1-BEO induced an increased expression of TRPV1 in the stomach while a lower expression was appreciated in the bowel. No morphological alterations have been highlighted in the liver or kidneys. Further investigation will be necessary to evaluate the functionality of this receptor in rainbow trout.

Keywords: rainbow trout; *Oncorhynchus mykiss*; *Ocimum basilicum*; transient receptor potential vanilloid 1; identification; super critical extraction

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1. Introduction

The transient receptor potential vanilloid type 1 (TRPV1) has been characterized, in mammals, as a nonselective cation channel, structurally analogous to the transient receptor potential (TRP) family of proteins [1]. The interaction of TRP receptors with "transient" stimuli, such as light, temperature, low pH, and electrical charge has been demonstrated, as well as the possibility of evoking a response after exposure to xenobiotics [2]. The TRP family is divided into six subfamilies based on the amino acid homology: TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin) and TRPV (vanilloid) [3]. TRPV1 is one of the most widely-investigated receptors, and it has been cloned in organs and tissues in humans and mammalian animal species, both in physiological and pathological conditions [2,4–7]. Species of interest in veterinary medicine have been considered as experimental models for human pathologies or as the main target for the development of new TRPV1-based therapeutic strategies for the treatment of treat inflammatory and neoplastic diseases [6–11].

The tissue expression of the TRPV channels both in humans and other mammals has been widely investigated in the last few years and, accordingly, its distribution in several tissues and organs has been defined as ubiquitous [9]. These types of receptors can have multimodal effectors, sensitive to a large number of stimuli, including temperature [5,12–14],

ionic balance [15,16], pressure and stretching [17–19], pH, ligands and ions (such as calcium and magnesium) [20], H_2O_2, and lipids and lipid-derived metabolites (such as arachidonic acid, anandamide, N-arachidonoyldopamine and lipoxygenase) [13,14,21,22].

The presence of TRP receptors was investigated in fishes, with a particular interest in TRPV1 and TRPV4 receptors [23]. Both receptors have been identified in rainbow trout (*Oncorhynchus mykiss*) [24], Atlantic salmon (*Salmo salar*) [25] and zebrafish (*Danio rerio*) [26–28].

The information available in the literature mostly regards the comprehension of the role of TRPV1 receptors in maintaining homeostasis processes in fishes and how TRPV1 receptors can be involved in the regulation of food intake [29,30]. The identification of TRPV1 in the pineal gland and retinal tissues suggested that the receptor could be involved in thermal perception and photostimulation, and strictly related to variations in fish behavior. This is a characteristic of fishes in which the pineal gland directly conveys light information to the brain and produces melatonin; this mechanism is not present in mammals [24,25]. Moreover, some hypotheses have been proposed regarding the possible role of TRPV receptors in the behavioral fever response in fish. An experimentally-induced infection in *Salmo salar* demonstrated that fish spontaneously move in warmer water, and this contributed to an increase in body temperature and a significantly higher mRNA expression of TRPV1, leading to a significantly increased release of proinflammatory cytokines (IL-1β, Il-6, TNF-α and PgE2). This proved the possible involvement in immunomodulatory activities of TRPV1 in fish [31].

The information on the different distribution and the function of TRPV1 in rainbow trout is still limited, and further investigations are necessary.

Phytotherapy is a branch of traditional pharmacology that aims to use parts or extracts of plants to treat pathological conditions and has become more and more popular both in human and veterinary medicine, mostly for finding alternative strategies to antibiotic treatments [32,33]. Related to aquaculture, essential oils have been tested to evaluate their efficacy against bacteria and microorganisms, improving production and reducing the use of antimicrobial drugs [34]. Essential oils contain many bioactive components with powerful antibacterial, antioxidative and immunostimulant potential, suggesting their application for aquatic animals. Essential oils can be provided via diet and can benefit aquatic animals by improving their well-being and health status [35].

Basil (*Ocimum* spp.) belongs to the Lamiaceae family and is characterized by an intense aromatic smell. Its plant-derived compounds possess antimicrobial properties and can provide beneficial effects in aquaculture, such as growth promotion, anti-stress and immunostimulation [36,37]. Specifically, sweet basil (*Ocimum basilicum*) contains natural antioxidants such as flavonoids and phenolic acids, and the derived essential oil has proven efficacy as an antioxidant, antimicrobial, insecticidal, nematocidal and fungistatic agent [34].

Following the aforementioned factors, the aims of the present study were to: (i) confirm the identification and the expression of TRPV1 receptors in rainbow trout (*Oncorhynchus mykiss*) and (ii) to evaluate the modulation of TRPV1 in tissues and organs in which TRPV1 is present after the administration of a fish diet supplemented with a supercritical fluid extraction of basil (F1-BEO).

2. Materials and Methods

2.1. In Vitro Experiment

2.1.1. Cell Culture

RTgill-W1 cells (CRL-2523, ATCC, Manassas, VA, USA), a cell line derived from the gills of rainbow trout, were cultured according to the manufacturer's instruction in the presence of Leibovitz's L-15 Medium (30–200, ATCC, Manassas, VA, USA), supplemented with 10% fetal bovine serum (Sigma Aldrich, Milan, Italy). Cells were incubated at 18 ± 2 °C in a 100% air atmosphere in the laboratory of The Veterinary Medical Research Institute for Piemonte, Liguria and Valle d'Aosta (Turin, Italy). Cells were split upon reaching 70% of confluence to prepare the cells for the immunocytochemistry (ICC) technique.

Cells were seeded at a density of 5×10^3 cells/well on sterile round glass coverslips placed in 6-well plates and cultured in the presence of a complete medium, as described previously. F1-BEO was not added to the control wells, while the other wells were set up to incubate cells with F1-BEO that was added at 0.5, 1, 2 and 3% *v*/*v*. The extract F1-BEO is a dense liquid, and it was homogenized in a complete culture medium prior to being added to the different wells to allow an easier and complete mixing with the culture medium.

Cells were incubated for 3 and 7 days, then coverslips were rinsed twice in phosphate-buffered saline (PBS), fixed in 4% neutral buffered formalin (NBF) for 10 min at room temperature, and washed three times in PBS. Sodium azide (Sigma Aldrich, Milan, Italy) 0.03% solution in PBS was added to cover the coverslips that were stored at 4 °C prior to the ICC staining.

2.1.2. Immunocytochemistry Protocol

A standard ICC protocol was performed using the Dako REAL™ EnVision™ Detection System, Peroxidase/DAB + Rabbit/Mouse (cat. K 5007, Dako Agilent, Santa Clara, CA, USA), according to the manufacturer's instructions. Endogenous peroxidase activity was briefly blocked using 2.5% H_2O_2 in methanol (Sigma Aldrich, Milan, Italy) at room temperature. The cells on coverslips were rinsed in PBS, permeabilized using 0.03% Tween-20 (Sigma Aldrich, Milan, Italy) in PBS and finally treated with 1.5% bovine serum albumin (BSA, Sigma Aldrich, Milan, Italy) in PBS for 30 min to block non-specific binding. Next, the cells were incubated for 48 h in a humidity chamber at 4 °C with the primary antibody against TRPV1 (sc-398417, Santa Cruz Biotechnology Inc. Dallas, TX, USA) diluted in 1.5% BSA/PBS (dilution 1:100). The cells were then washed three times in PBS containing 0.03% Tween-20 and then processed using DAKO Real EnVision kit.

Negative controls followed the same protocol, with the omission of the primary antibody, and consistently yielded negative results.

MCF-7 and CF-41 cell lines have been enrolled as positive controls.

2.2. In Vivo Trial

2.2.1. Ethical Statement

The study protocol was designed according to the guidelines of the European Union council 2010/63/EU for the use and care of experimental animals, ARRIVE guidelines, respecting the principle of the 3Rs. The study protocol was approved by the Institutional Review Board of the Italian Ministry of Health (authorization no. 196/2020-PR). The project was divided into three distinct branches: the first two parts have already been described in published papers [34,38].

2.2.2. Chemical Profile of Supercritical Fluid Basil Extract (F1-BEO)

The basil supercritical fluid extract (F1-BEO) procedure was previously described by Pastorino et al. [34,38]. F1-BEO was extracted in a two-step procedure using a supercritical fluid extractor (SCF-100; Separeco s.r.l, Pinerolo, Italy) processing clean and dried sweet basil leaves (ranging from 0.3 to 0.5 cm, with residual humidity < 10%) in the laboratory of the Exenia Group s.r.l. (Pinerolo, Turin, Italy). The first fraction (F1-BEO; yield 3.7% *w*/*w*) was chosen for the purposes of this study, while the second fraction (F2- BEO; yield 4.8% *w*/*w*) was almost totally composed of volatile organic compounds (VOCs) and has been deemed inadequate for experimental use. Total polyphenol content and total flavan-3-ol content were determined by spectrophotometric analysis [34,38]. The polyphenolic compounds were identified by HPLC-ESI-MS/MS, while total fatty acid content was determined by GC–MS and GC-FID analyses [34,38].

2.2.3. Diet Formulation and Rainbow Trout Nutrition

The diet was prepared at the experimental facility of the Department of Agricultural, Forest and Food Sciences of the University of Turin (Carmagnola, Turin, Italy), previously reported by Pastorino et al. [34]. Commercial feed was supplemented with the basil extract

(Alterna Eel, Skretting Italia, Verona, Italy; ingredients: fish meal, fish oil, wheat gluten, poultry blood meal, soybean protein concentrate, swine hemoglobin, whey powder; and proximate composition: protein 48%, fat 11%, ash 8%, fiber 1%, vitamin A, vitamin D3, zinc sulfate, potassium, manganese, copper, iron sulfate, anhydrous calcium iodate). The fraction derived from the supercritical fluid extraction of basil was added to the commercial feed in proportions of 0.5%, 1%, 2% and 3% *w*/*w* F1-BEO. The control diet was composed only of commercial feed without the F1-BEO. The mixture was added to obtain material for pellets, which were obtained using a 4.0 mm die meat grinder and dried at 30 °C for 48 h. The five diets have been prepared A: control; B: 0.5%; C: 1%; D: 2% and E: 3% *w*/*w* F1-BEO. All diets were stored in dark bags at 4 °C until use.

2.2.4. Rainbow Trout

To pursue the goals of this study, 430 sex-reversed female rainbow trout exhibiting a sterile filiform gonad were purchased from a private fish farm in northwest Italy. The fish were conditioned for 20 days before the study began, in the same tanks and environmental conditions as those used during the experimental sessions.

Prior to the beginning of the different experimental procedures and to evaluate clinical conditions, 30 rainbow trout were randomly selected for anatomopathological, parasitological, bacteriological and virologic analysis following standardized methods. The specimens were necropsied and evaluated for possible pathological alterations. For the parasitological exam, tissue scrapings from skin and gill filaments were prepared in a drop of water, covered with a clean cover slip (wet mount preparation) and examined under an optical microscope (Olympus BX40, Olympus) at 10× to 40× magnification. For bacteriological analysis, Columbia blood Agar (Liofilchem, Italy) was inoculated with kidney and brain samples and incubated at 22 °C for 72 h. Finally, for virologic examination, pools of the main fish organs (spleen, kidney, brain and heart) were homogenized (using a stomacher or a homogenizer) and then centrifuged in a refrigerated centrifuge at 3750 rpm for 15 min; the supernatant was then incubated at 4 ± 1 °C overnight. The virological examination was conducted on the cell monolayers using the EPC and BF2 cell lines. For each pool, two 7–10-day serial passages were performed. All fish were considered healthy since bacteriological, virologic and parasitological analyses tested negative.

Fish were fed by hand to apparent visual satiation seven days a week. After an acclimatization period, 400 fish were lightly anesthetized with tricaine methane sulfonate (MS-222; 70 mg L^{-1}; Sigma-Aldrich, Milan, Italy), individually weighed (mean body weight: 250 ± 50 g) and randomly equally distributed in the tanks. Fish weight was also monitored during the experiment.

The fish were kept for 30 days in 20 square fiberglass tanks (capacity, 400 L) supplied with artesian well water (constant temperature 13 ± 1 °C) in an open system (flow-through); each tank had a water inflow of 8 L min^{-1}. Dissolved oxygen was measured every day (range, 8.4–9.5 mg L^{-1}; water pH 7.5 ± 0.6). Fish were exposed to a controlled photoperiod (12 h light/12 h dark). The five experimental treatments (A; B; C; D; E) were randomly assigned to the 20 tanks (20 fish per tank; four replicate tanks per diet). Fish were fed by hand seven days a week. The daily feed quantity was set at 1% of tank biomass. The tank biomass was kept constant at 20 kg m^{-3}, this was achieved by lowering the water level in each tank when fish was removed for analysis at the different experimental time points. Mortality was checked every day.

For the specific purposes of this study, 32 fish from each experimental group (eight fish per tank; four replicates per diet) were sampled at the middle (15 days; T1) and at the end (30 days; T2) of the experiment. Fish were captured using a landing net and euthanized using an overdose (170 mg kg^{-1}) of MS-222 (Sigma-Aldrich, Milan, Italy). A necroscopic dissection was performed and order to carefully and entirely collect the brain, eye, gills, heart, stomach, liver, intestine, spleen, and kidneys immediately after death. Organs were checked to make sure they were whole and intact, then they were washed two times with cold PBS and stored in 4% NBF and transported to the laboratory of the Department of Veterinary Sciences of

Turin (Italy) within a few hours. Specimens were then rinsed with PBS, and residues of blood and intestine content were removed. Organs were checked to ensure they were whole and intact, then fixed again in 4% NBF and stored prior to further processing.

2.2.5. Histology Protocol

After 24 h of fixation, samples of the liver and kidneys were embedded with paraffin. Sections of 5 µm thickness were prepared in order to proceed with Mayer's hematoxylin-eosin (H-E) staining for histological evaluation.

Briefly, after dewaxing and rehydration, slides were immersed in Mayer's hematoxylin for 15 min, washed in lukewarm running tap water for 15 min, rinsed in distilled water and placed in 80% ethyl alcohol for 2 min.

Later, slides were counterstained in eosin–phloxine solution for 2 min. Slides were then dehydrated and cleared through two changes of 95% ethyl alcohol, absolute ethyl alcohol and xylene for 2 min each and mounted with DPX resin.2.2.7. according to immunohistochemistry protocols.

After 24 h of fixation, samples were embedded with paraffin and sections 5 µm thick were prepared for the immunohistochemistry (IHC) procedure to identify TRPV1 receptors. The method was previously described by Vercelli et al. [10], applying some minor modifications. Briefly, sections on Menzel Glaser Superfrost Plus slides (Thermo Scientific, Waltham, MA, USA) with samples of brain, eye, gill, heart, stomach, liver, intestine, spleen and kidney from all trout belonging to the 5 treatment groups were processed with a standard IHC protocol using the Dako REAL™ EnVision™ Detection System, Peroxidase/DAB + Rabbit/Mouse (cat. K 5007, Dako Agilent, Santa Clara, CA, USA). Sections were rehydrated, and endogenous peroxidase activity was blocked using 2.5% H_2O_2 in methanol (Sigma Aldrich, Milan, Italy) for 30 min at room temperature. The slides were then rinsed with distilled water for 5 min. To facilitate the immunostaining, an antigen retrieval was performed by incubating in a thermostatic bath the slides at 98 °C for 30 min in citrate buffer (pH = 6).

After 15 min at room temperature, the slides were rinsed with distilled water, then washed twice for 5 min in PBS and once in a 0.03% Tween-20 solution in PBS. Finally, the sections were incubated with 1.5% BSA in PBS for 30 min to block non-specific binding and incubated over-night at 4 °C with antibody to detect TRPV1 (sc-398417 Santa Cruz Biotech Inc., Dallas, TX, USA) using 1:100 dilution. The slides were then washed twice for 5 min in PBS and once in a 0.03% Tween-20 solution in PBS and then processed using the DAKO Real EnVision kit.

Negative controls underwent to the same procedure, omitting the primary antibody, and consistently yielded negative results.

Samples of different organs collected from mammals have been enrolled as positive controls.

2.3. Development of ICC and IHC Reaction and Image Acquisition

2.3.1. Development of Colorimetric Reactions of ICC and IHC

After a faint counterstaining with Mayer's hematoxylin (Merck, Darmstadt, Germany), the specimens were dehydrated using alcoholic solutions with a progressively increased alcohol concentration, cleared in xylene (Sigma Aldrich, Milan, Italy), and mounted with DPX (Sigma Aldrich, Milan, Italy). All samples were examined using a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) equipped with a Nikon Digital Sight DS-Fi1 camera (Nikon Corporation, Tokyo, Japan).

2.3.2. Image Acquisition, Software Processing and Statistical Analysis

The images were acquired with NIS-Elements F2.30 software (Nikon Corporation, Tokyo, Japan) and processed using Image Pro Plus software (Media Cybernetics Inc., Rockville, MD, USA). The pictures were used to calculate the total area of the section of the organ collected on the slide and to measure the percentage of positivity of the IHC

assay. Values are expressed as percentages. Normality and homoscedasticity of data were assessed using the Shapiro–Wilk and Levene tests, respectively. As the data were normally distributed, a one-way ANOVA was used to compare fish total weight at the two time points (T1 and T2) between treatment groups (A: control; B: 0.5%; C: 1%; D: 2%; E: 3% *w*/*w* F1-BEO). To evaluate the different interactions due to the treatments at each time point on TRPV1 receptor modulation, a two-way ANOVA was performed after assessing the normality and homoscedasticity of data using the Shapiro–Wilk and Levene tests, respectively. A one-way ANOVA and Tukey multiple tests were used to compare ratios between the total area and the area of positivity calculated with Image Pro Plus software at each fixed time point (T1 = 15 and T2 = 30) across treatment groups (A: control; B: 0.5%; CL 1%; D: 2%; E: 3% *w*/*w* F1-BEO). Unpaired Student *t*-tests were used to compare the ratios at the two time points (T1 and T2) between each pairwise comparison of the treatment groups (B: 0.5%; C: 1%; D: 2%; E: 3% *w*/*w* F1-BEO). The statistical analyses have been run in RStudio (Version 1.2.1335) and R (version 3.6.3).

3. Results

3.1. Cell Culture and ICC

RTgill-W1 cells were successfully cultivated despite their slow growth in comparison to the doubling time reported in the manufacturer's datasheet.

Immunocytochemistry permitted us to identify TRPV1 receptors in untreated RT gill-W1 cells (Figure 1). Cells incubated with different percentages of F1-BEO showed similar expressions of TRPV1, lacking any significant difference between the control and the different concentrations of F1-BEO (Figure 1B,C).

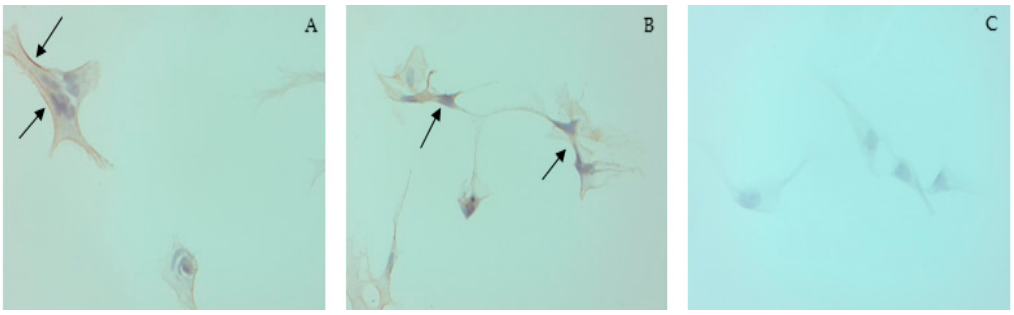


Figure 1. Results of immunohistochemistry performed on RTgill-W1 cells. The pictures are referring to the control (**A**), concentration 0.5 *w*/*w* F1-BEO (**B**) and the negative control (**C**). Black arrows indicate the positivity to TRPV1. Magnification: 40×.

Respecting the manufacturer's instructions, it was not possible to reach a confluence below 70%. Several attempts have been made to improve growing conditions (for example, changing the medium with Minimum Essential Medium Eagle—MEM—or varying the percentage of FBS) without reaching an optimal condition. For this reason, the authors decided to not proceed with the proliferation assay.

3.2. Basil Extract Composition

The chemical composition of F1-BEO has been evaluated using a spectrophotometric assay which permitted us to measure the bioactive compounds, represented by a total polyphenol content and total flavan-3-ol content of 32.97 ± 1.63 mmol gallic acid equivalent (GAE) per 100 g of fresh weight and 21.21 ± 1.04 mmol A2-type proanthocyanidin content equivalent (A2-PACE) per 100 g of fresh weight, respectively. Several polyphenolic compounds were identified by HPLC-ESI-MS/MS in the F1-BEO: flavones (scolymoside, isomyricetin, myricetin diglucoside, cynaroside, myricetin and luteolin), flavonols (nicotiflorin, isoquercitrin, astragalin, kaempferol, quercetin and rutin), flavanols (aro-madendrin, arthromerin B and taxifolin), and eight polyphenolic acids (one of which is a derivative of hydroxycinnamic acid [chicoric acid] and seven are in the salvianolic acid family). The F1-

BEO also contained several VOCs (GC-MS; mg per 100 g of fresh weight): 1,8-cineole (9.33), linalool (25.29), estragol (18.79), eugenol (4.49), methylcinnamylate (8.71), methyleugenol (6.58), b-caryophyllene (7.47) and bergamotene (19.34). The F1-BEO fraction was composed of about 10% of fats; palmitic acid, linoleic acid, and oleic acid accounted for 77% of the total content of fatty acids (GC-MS and GC-FID analysis). More detailed information can be found in the papers of Pastorino et al. and Magara et al. [34,38].

3.3. Rainbow Trout

No mortality was observed during the entire period of administering the fish diet supplemented with F1-BEO. All the fish were healthy, as confirmed by the absence of internal and external lesions at necropsy and histological analysis. Parasitological, bacteriological and virological examination tested negative in all treatments.

There were no significant differences in the weight of fish between the 5 experimental groups at 15 days (T1) and at 30 days (T2) (one-way ANOVA; $p > 0.05$). The mean fish weight ranged from 249.15 g (E) to 252.23 g (A) at T0, from 279.89 g (B) to 282.20 g (A) at T1, and from 310.75 g (E) to 313.15 g (A) at T2.

3.4. Histology

Samples of the liver and kidneys did not show any signs of alteration. The morphology of both organs was considered normal in all specimens, for all trout belonging to different treatment groups at both experimental time points. Therefore, no differences have been highlighted, as shown in Figures 2 and 3, where pictures of the control, 0.5 and 2% specimens of the liver and kidneys, respectively, are presented. A characteristic find is represented by melano-macrophages (blackish spots) which are present in the fish's hematopoietic organs and are involved in the catabolic processes of red blood cells [39].

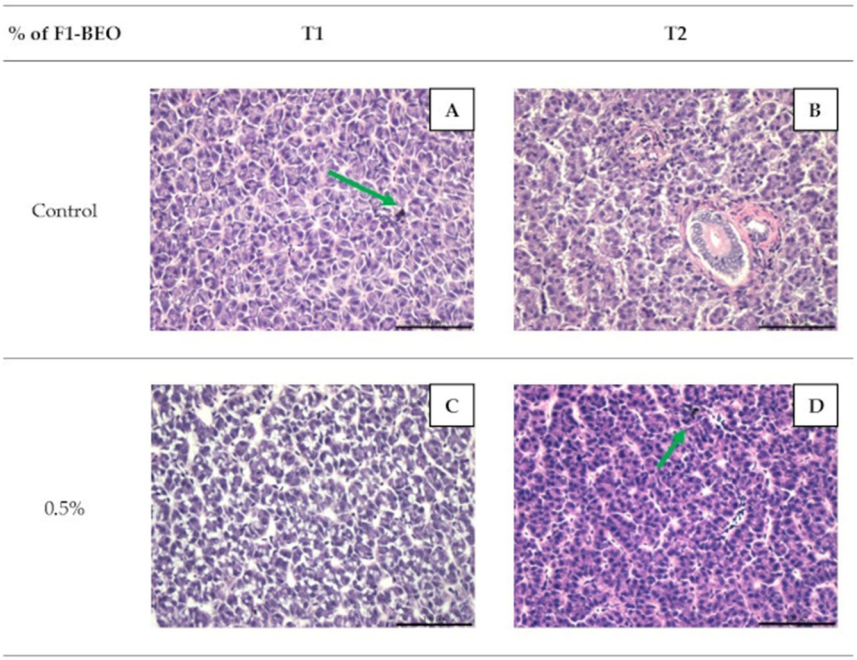


Figure 2. *Cont.*

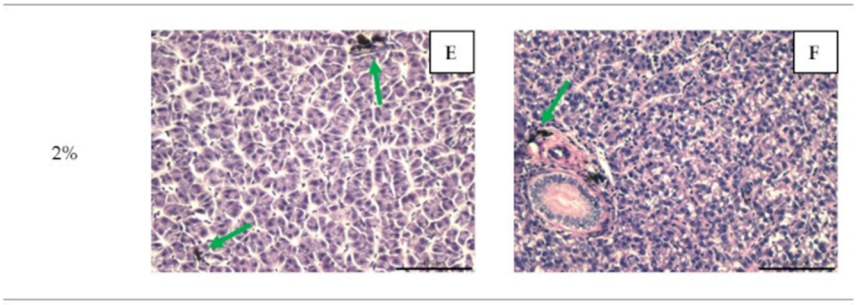


Figure 2. Histology using hematoxylin-eosin staining, evaluating the morphology of liver specimens belonging to the control groups (**A**,**B**), 0.5% groups (**C**,**D**) and 2% groups (**E**,**F**). Green arrows indicate the melano-macrophages. Magnification: 20×.

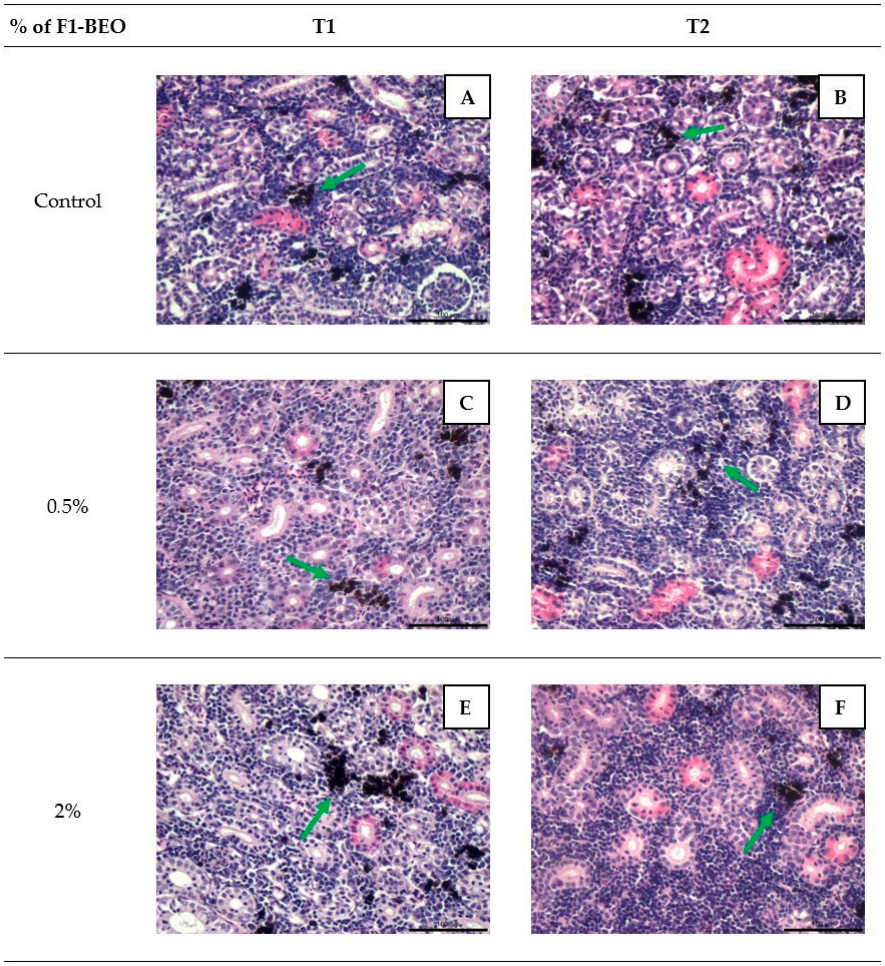


Figure 3. Histology using hematoxylin-eosin staining, evaluating the morphology of kidney specimens belonging to the control groups (**A**,**B**), 0.5% groups (**C**,**D**) and 2% groups (**E**,**F**). Green arrows indicate the melano-macrophages. Magnification: 20×.

3.5. Immunohistochemistry

The immunohistochemistry permitted us to identify the TRPV1 in all organs used for the present investigation. The positive IHC staining was visible as a brownish and brilliant color in all slides derived from each organ that was collected from all trout belonging to all treatment groups and at both experimental time points.

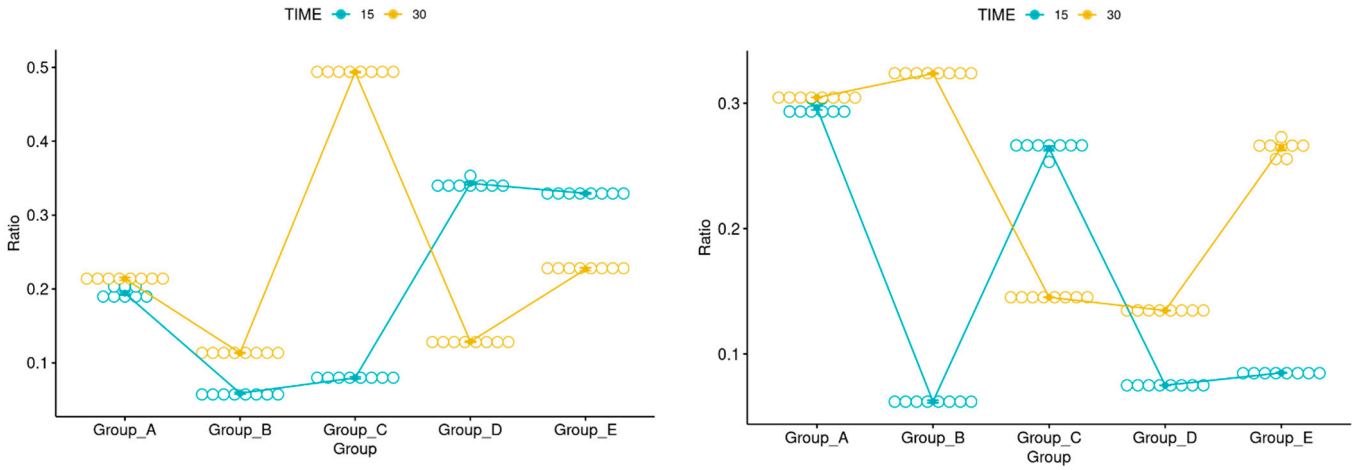
A strong positivity was appreciated in the pallium (cerebral cortex), in the striated muscle of the eye, in the myocardium (the bulb was negative), and in the spleen (red pulp)

without a statistically significant difference among treatments and compared with controls at both time points.

The gills showed positivity in the basal and lateral epithelium and in mucoid cells, and sparse positivity was evident in cartilage. Additionally, for the gills, no differences have been appreciated between the control and treated samples. Considering that no differences have been found, in Figure 4, some examples of positivity are proposed to readers.

Figure 4. Pictures show the positivity (brownish color) of TRPV1 receptors in the brain (**A**), eye (**B**), gill (**C**), myocardium (**D**) and spleen (**E**). Black arrows indicate the melano-macrophages. Magnification: (**A**) 2.5×, (**B**) 10× (**C**,**D**) 5×, (**E**) 40×.

An intense signal of positivity was also appreciated in the stomach and intestine (particularly in the muscular layer), in the liver, and in the kidneys. The percentage of positivity, measured with Image-Pro Plus, showed statistical differences: the two-way ANOVA test was used to study the interaction effect of the treatment and the time on the modulation of TRPV1 in the different organs. For all organs, all three null hypotheses were rejected, highlighting not only the statistical difference of the time factor and the group factor (defined by essential oil concentration), but also the interaction between the two factors (p-value lesser than 2×10^{-16}). This result permits us to analyze in more detail the differences among the groups at fixed experimental time points and the differences between the experimental time points for defined groups. A one-way ANOVA was used to compare ratios at each of the fixed time points (T1 = 15 and T2 = 30) across treatment groups (A: control; B: 0.5%; C: 1%; D: 2%; E: 3% w/w F1-BEO) highlighting a significant difference in each pairwise comparison of each organ, assessed by the Tukey multiple comparisons test. Unpaired Student t-tests were used to compare ratios at the two time points (T1 and T2) between each pairwise comparison of the treatment groups (B: 0.5%; C: 1%; D: 2%; E: 3% w/w F1-BEO), highlighting significant differences in all organs (Figure 5).



Kidney Bowel

Figure 5. *Cont.*

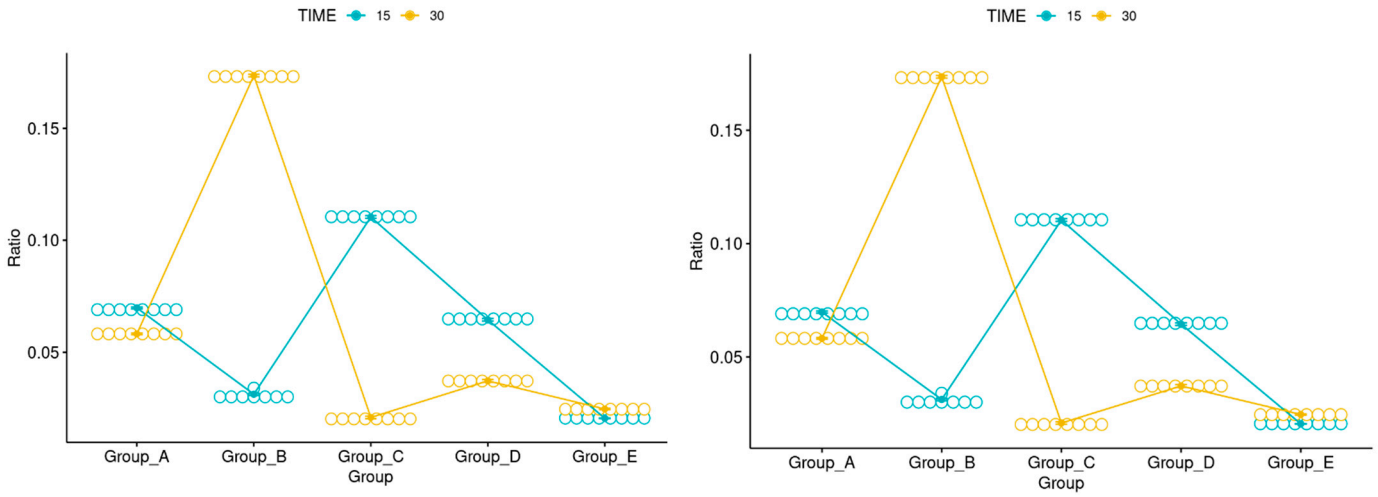


Figure 5. Ratios of the total area and the area positive for TRPV1 after immunohistochemistry assay and picture evaluation with Image Pro Plus software. Data were analyzed with a two-way ANOVA to evaluate interactions between treatment and time, while a one-way ANOVA with Tukey multiple comparisons test or with unpaired Student *t*-test were used to evaluate differences among groups' fixing treatments and time, respectively.

The expression of TRPV1 reached maximum values in the kidneys in the treatment group corresponding to 1% F1-BEO, after 30 days of administration. In the stomach, bowel and liver, the maximum expression of TRPV1 was appreciated in the treatment group receiving the diet supplemented with 0.5% of F1- BEO for a 30-day period.

3.6. Morphological Evaluation and IHC of Liver and Kidney

According to the results presented by Magara et al., 2022 [38], special attention was given to the liver and kidneys. Immunohistochemistry was performed to identify TRPV1 receptors, and this gives us the opportunity to appreciate its specific localization in these two organs both in controls and in samples derived from fish receiving the experimental administration of F1-BEO (Figures 6 and 7).

The positivity was appreciated on the membrane of hepatocytes, mainly on the edge of contact between cells. This draws on a specific pattern that was appreciated in all samples. The strongest positivity was highlighted in livers collected from trout fed with 0.5% *w*/*w* F1-BEO and with 1% *w*/*w* F1-BEO, even if the latter was less intense than the former. The weakest positivity was highlighted in liver samples taken from of trout fed with 3% *w*/*w* F1-BEO (Figure 6).

The positivity in kidney samples was appreciated in tubule epithelium, in the interstitial part around the collecting duct. Samples collected from trout fed with 1% *w*/*w* F1-BEO for 30 days and with 2% and 3% for 15 days showed the highest positivity compared to controls.

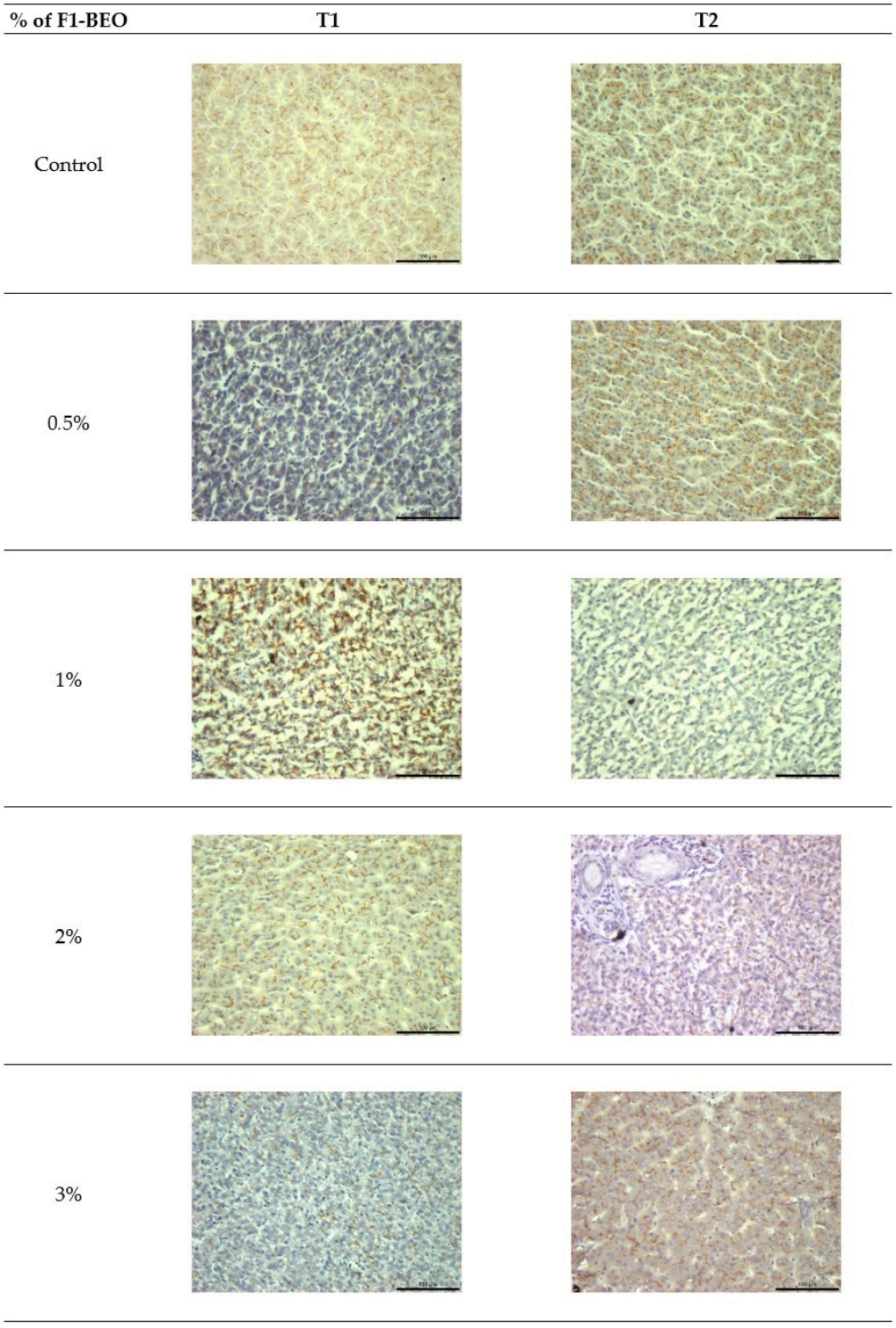


Figure 6. Immunohistochemistry assays to identify the TRPV1 receptor in liver specimens. The positivity is comprehensible due to the brownish color. T1 = 15 days. T2 = 30 days. Magnification: 20×.

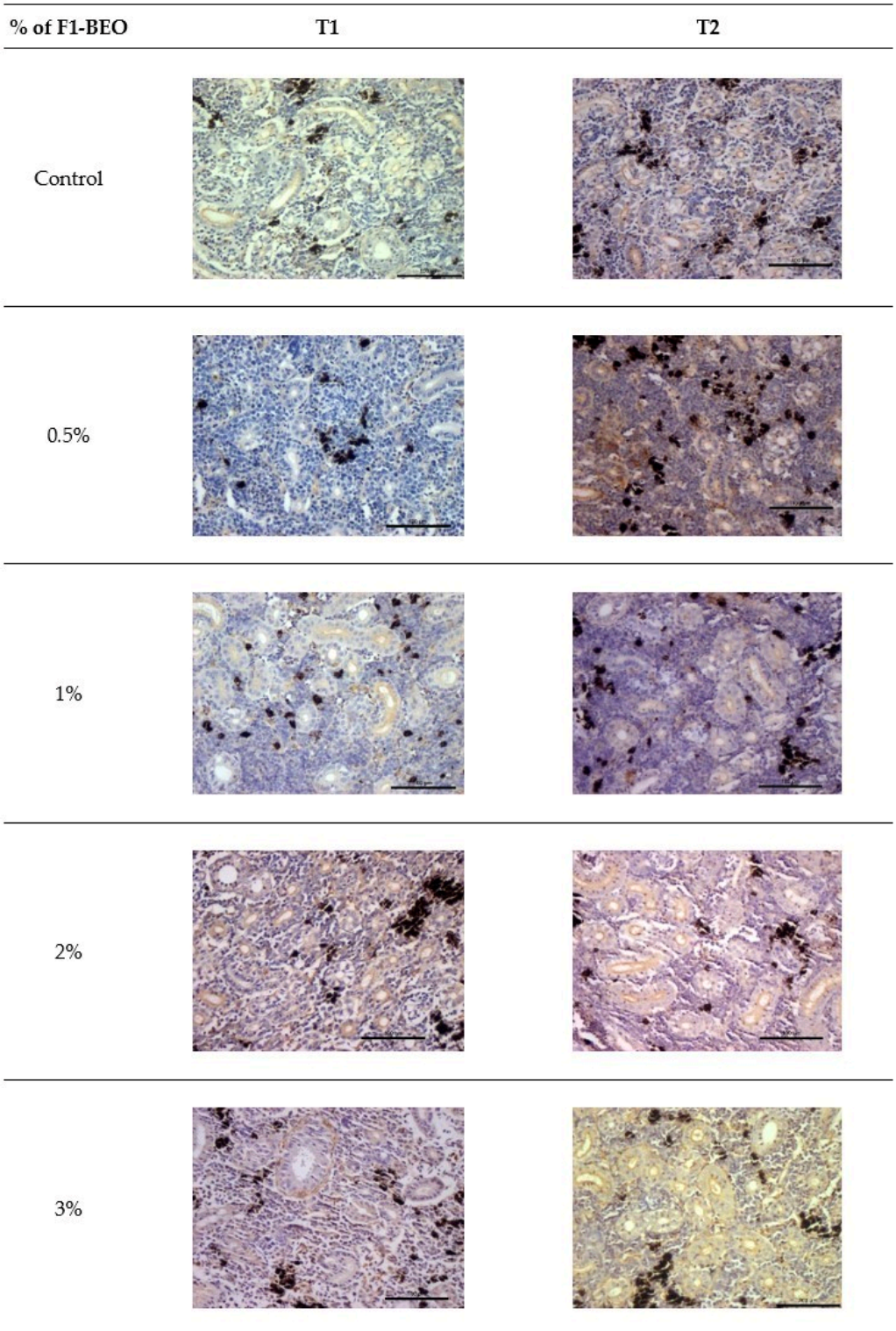


Figure 7. Immunohistochemistry assays to identify TRPV1 receptor in kidney specimens. The positivity is comprehensible due to the brownish color. T1 = 15 days. T2 = 30 days. Magnification: 20×.

4. Discussion

The present paper resumes the procedures and the results of the last part of a huge project that was designed to investigate the different properties exerted by the final product of a supercritical fluid extraction of basil, F1-BEO, in rainbow trout. In order to reach this goal, different in vitro and in vivo experiments have been set up.

Regarding the present experimental session, the first aim was to confirm the identification of the TRPV1 receptor in trout cells, organs and tissues using an immunohistochemistry technique. RTgill-W1 cells were successfully cultivated, and the ICC technique permitted us to identify TRPV1 receptors on cell membranes. Nevertheless, it was not possible to perform the proliferation analysis and other experiments on cells. The authors carefully followed all manufacturer's instructions present on the datasheet, but the cells grew slowly and never reached 100% of confluence. Moreover, it was not possible to set up a functionality assay using specific TRPV1 agonist and antagonist drugs (for example capsaicin and capsazepine, respectively) to understand the biological behavior of rainbow trout cells. Authors decided to present these unsuccessful results in order to provide information to other researchers that are dealing with this cell line and might face similar situations.

Considering the results of in vivo experiments, the TRPV1 receptor was successfully identified in all organs collected in the control groups, thus permitting visualization of the localization of this receptor in cell membranes in the brain, eyes, gill, heart, stomach, liver, intestine, spleen and kidneys. In all samples, the presence of the receptor was localized on the membrane, in accordance with other studies performed in mammals and present in the literature [6–8,10]. Different expressions have been appreciated among the different organs in the control groups. This is not surprising, since receptor expression can vary significantly according to the organ under investigation, both in physiological and pathological conditions. Moreover, our results concerning the expression of TRPV1 in rainbow trout are in accordance with those reported in the literature, stating that the expression of TRPV1 receptors is two-folds greater in the intestine and kidneys compared to other tissues, such as the retina, brain, pineal organ, spleen, heart and blood cells, and ever greater when compared to the liver [24].

Authors decided to focus their attention on: (i) the gastrointestinal tract, given the fact that F1-BEO was administered through feeding and (ii) the liver and kidneys, according to the results obtained in the previous step of the project and reported by Magara et al., [38] who evaluated the antioxidant pathways in rainbow trout fed with F1-BEO at the same concentrations reported in the present study. It was interesting to appreciate the different influence of F1-BEO in TRPV1 expression in different organs according to the different treatment and the time. In the stomach, bowel and liver, the highest receptor expression was appreciated in the treatment group receiving 0.5% F1-BEO supplementation through diet. These three organs demonstrated decreased levels of receptor expression in treatment groups receiving higher concentrations. This trend might be motivated by a physiological adaptation to the essential oil supplementation, which has been previously reported in the literature [39,40].

The results of our experiment permit us to state that the most evident identification is in the muscle layer of organs such the stomach, bowel and kidneys. The positivity in the gills is particular, since they consist of different cell populations, and the positivity was appreciated in wall vessels perfusing gills, primary and secondary axes of the *lamellae* and mucoid epithelium. Sparse positivity was also appreciated in cartilage. The positivity of myocardium and blood vessels is in accordance with the results proposed in dogs and bovine species [10]. The positivity of liver and kidney of rainbow trout was never described in the literature using IHC techniques, and this is the first report carrying these data, since no information is currently available about the possible modulation of this receptor in these organs. The most interesting part in the visualization of the TRPV1 receptor in the liver of rainbow trout was the localization in the membranes of adjacent cells. This seems to suggest a possible role of the TRPV1 receptor in cell junctions or communicating mechanisms among cells. It has been reported in the literature that TRPV1 is involved in hepatic glucose metabolism in rats and its loss seems to be associated with increased gluconeogenesis [41]. Moreover, it was proven that capsaicin, an agonist of TRPV1, is able to modulate the receptor activity, achieving the conversion of cholesterol to bile acids and improving the lipid metabolism in rats [42]. The change in the composition of bile acids was mainly promoted by the fact that capsaicin is able to increase the expression of cytochrome

(Cyp) 7a1 in liver and Cyp71b in the liver and colon, reducing fat accumulation [42]. No similar reports are available for fishes, but the role of endocannabinoid system has been investigated to improve the food intake in rainbow trout [30]. It was demonstrated that TRPV1 mRNA levels decrease in the hypothalamus of fish fed a high fat diet and increase in fish fed with a control diet. Moreover, the entire endocannabinoid receptor system decreases in the presence of a high fat diet, due to a feedback counter-regulatory mechanism. It seems that the hedonistic response to food intake is similar to those previously reported in mammals and mediated by TRPV1 [30].

TRPV1 has been identified in the kidneys of rats and it was demonstrated that capsaicin could exert a protective effect toward renal toxicity caused by methotrexate [43]. This effect is associated with a concurrent increase of superoxide dismutase (SOD) and renal glutathione peroxidase (GPx) [43]. Similarly, it was demonstrated that capsaicin is able to limit renal fibrosis in a rat model, mainly acting through the inhibition of TGFβ_1 Smad 2/3 signaling [44]. The results obtained in our study are coherent with other data reported in the literature on the identification of TRPV1 in the kidneys of mammals and fishes. They are also in line with the results reported by Magara et al. [38] and, even if a major expression of the antioxidant pathway exists, it is not associated with parenchymal damages and might be a prospective future candidate for therapeutic purposes.

The use of essential oils and polyphenol-enriched extracts is also rapidly increasing in the aquaculture sector as a means of greater industrial and environmental sustainability [34]. The sensitivity of TRPA1 and TRPV1 in various animals to polyphenols can be significantly different. It was recently reported that HEK293T cells expressing TRPA1 or TRPV1 from mammals, birds, reptiles, amphibians and fish have diversity in polyphenol sensitivity since mammalian subjects showed relatively higher sensitivity to polyphenols, but reptiles were insensitive [45]. Moreover, the polyphenol-sensitivity of zebrafish TRPA1 and TRPV1 was quite different from that of medaka (*Oryzias latipes*) TRP channels. Since many polyphenols are present in plants and the sensing of polyphenols using TRP channels in the oral cavity might cause astringent taste, the observed diversity of the polyphenol-sensitivity of TRP channels might be involved in the diverging food habits of various animals [45,46]. The protective role of essential oils from oxidative stress damage has been previously demonstrated, and they can affect the health condition and the immunomodulation of fishes [47]. The data presented by Magara et al. [38] jointly with those reported in the present paper, can support the scientific hypothesis that essential oil of basil can improve the immune system of rainbow trout, decreasing the activation of oxidative mechanisms and inducing a potentiated expression of TRPV1. This consideration finds support given that other authors have proved that the essential oil of coriander is a potent immunomodulation and can enhance the innate immune parameters of tilapia (*Oreochromis niloticus*) in experimental induced infection if administered through diet [47]. An anti-inflammatory effect was also recorded in silver catfish treated with *Melaleuca alternifolia* essential oil during experimental infection caused by *Aeromonas hydrophilia* [48]. In contrast, an opposite effect was demonstrated in koi carp receiving a diet containing oregano essential oil [49]. This underlines the importance of a careful evaluation of the employment of different essential oils related to the final effect that can be induced in the modulation of immune system and inflammatory pathways.

In the rainbow trout, the presence of melano-macrophages in the haematopoietic organs has previously been described [50]. In the present study, it was possible to observe these macrophages, which appear black pigmented, as they contributed to the catabolism of red blood cells in the kidneys, spleen and liver. The phagocytic activity may vary as innate and adaptative immune response [40]. Agius and Roberts showed an increase in the number of melano-macrophages in relation to the age of the trout and the presence of diseases [50]. The trout enrolled in this project were all young and no differences emerged between the controls and the groups treated with the different concentrations of F1-BEO, so it would be interesting to assess the expression of melano-macrophages in relation to TRPV1. In fact, the relationship between TRPV1 and its possible modulation with essential oils

has not been previously evaluated and reported elsewhere. This is absolutely innovative and might lead to the acquisition of knowledge about a possible use of essential oils as anti-inflammatory agents, acting specifically on TRPV1 receptors. It has been established that TRPV1 is strictly correlated to the response to pathological stimuli or pathogenic agents (such as viruses and bacteria) in Salmo salar, and it is responsible for organic responses, such as behavioral fever and cytokines release [31]. A similar situation can be shown in other fishes, and modulation of TRPV1 activities can be used to improve treatment protocols and fish welfare. Moreover, it has been proposed to synergically use probiotics and essential oils to improve fish gut flora and prevent the overgrowth of pathogenic bacteria [47]. In the present study, we demonstrated the presence of TRPV1 receptors in the stomach and bowel in the control group and a maximum increased expression of the receptor after a long administration of 2% and 0.5% for the stomach and bowel, respectively. It would be interesting to evaluate the use of F1-BEO on bacterial populations collected from the gastrointestinal tract of trout receiving F1-BEO supplementation.

The identification of TRPV1 in the brain and eye in the present study is in line with what was previously reported for rainbow trout [24–26,30]. The first report of TRPV1 identification in rainbow trout was performed by Nisembaum et al., [24] who were focused on identifying both TRPV1 and TRPV4 receptors in pineal photoreceptor cells and to understand the thermoregulatory processes. Moreover, they described a ubiquitous localization performing a real-time quantitative PCR (qPCR), using samples collected in different organs, but no immunohistochemistry assay was performed to visualize the receptors.

TRPV1 presence in the brain and eye was previously reported also in zebrafish (*Danio rerio*): in this case, the presence of this receptor in the central nervous system has been correlated with vision and movement control [51]. The identification of TRPV1 receptors in zebrafish was highlighted even at the early stages of neuron formation in larvae [26]. TRPV1 is already expressed in the first wave of somatosensory neuron development, suggesting a vital importance of thermal sensation in early larval survival. TRPV1 in zebrafish acts as a molecular sensor of environmental heat with temperatures higher than 25 °C (much lower than the 42 °C threshold required for mammals). For zebrafish, it was proven that a direct derivation from precursor tetrapod exists, even if a single TRPV1-2 orthologue has been identified [26].

TRPV1 and TRPV4 identification have been described in rainbow trout retinal cells and in pineal photoreceptor cells, with a direct correlation with melatonin secretion and thermomodulation [24]. This highlights the importance of these receptors and their involvement in food intake and circadian cycle with specific relationships to environmental temperature and with modulation related to the season. TRPV1 is expressed in the pineal glands of mammals as well, but they lack direct the neuronal transmission that is still present in fish and allows direct conveyance of light information to the brain [26]. The localization to pineal glands is also commonly shared with zebrafish and goldfish, in which TRPV1 has been identified in ribbons of retinal receptors [52].

The response of TRPV1 to ligands can be dramatically different among mammals and, in fishes, can hide singular and inedited aspects [7,53]. The pharmacological classification of agonist and antagonist substances is based on mammalian species and thus do not guarantee the same applicability in fishes. Moreover, the temperature of water, the environment and the season could strongly influence the expression of TRPV1 receptors that can undergo annual or seasonal variations [24]. This latter aspect was further understood when the TRPV1 receptor was identified, and the role of TRPV1 has also been investigated in the sperm of freshwater teleost fish (*Labeo rohita*) [52]. Capsaicin was administered to evoke a receptor response: no alteration in sperm motility was seen and seemed that cells were not responsive to capsaicin at all. These results highlighted that TRPV1 may have implications in fertilization process of *Labeo rohita* breeding and in cryopreservation of fish sperms [48] and is an example of a species-specific response of TRPV1 to ligands that can be different among mammals and fishes [7,53].

These data might seem surprising since the functions of TRP channels appear to be conserved in fishes [54,55]. In fact, most of the mammalian thermos TRP genes are conserved in teleosts [56], but some differences about temperature-gated TRPV1 in zebrafish and TRPA1 paralogs in zebrafish, medaka and pufferfish have been demonstrated in vitro [23].

Genes encoding for TRPV1 are present in teleost fish and originated by duplication events after the separation of fishes from the last common ancestor of tetrapods and was successfully proven by the most recent molecular analysis [57]. Recently, the paper of Nisembaum et al. [25] demonstrated the presence of TRPV1 and TRPV4 receptors in *Salmo salar*. In this case, mRNA for TRPV1 was identified in all tissues and organs that were used for experimental purposes and TRPV1 was defined as ubiquitous also in this species. In that study, the identification was performed using in situ hybridization and immunohistochemistry, giving particular attention to the pineal gland and retina. These two localizations were put in correlation with melatonin production and seasonal changes, and migratory routes and climate variations are believed to be responsible for the fluctuation of TRPV1 expression [25]. In the same study, the high presence of TRPV1 in kidney and intestine specimens was demonstrated to be higher in July, and was lower in February. Considering global warming, the receptor expression could be influenced further [25].

The presence of TRPV1 was also demonstrated in Antarctic fishes: notothenioid fish genome contains 23 out of 29 TRP channels genes, similar to teleosts. The involvement of TRPV1, TRPA1, TRPM4 and TRPM7 in thermoregulation has been proven in these fishes [23]. Moreover, two subtypes of TRPV1 receptors have been identified in Antarctic fishes: TRPV1 *a* and *b*. For both of them, the involvement in thermoreception has been proven [23].

The complement of TRP channel genes found in notothenioid was similar to other teleosts and supported recent analyses of TRP evolutionary history. All notothenioid species studied had two TRPV1 paralogs, due to the teleost ancestral genome duplication. All teleosts lack TRPV2, TRPV3, and TRPM8, while presenting a single TRPV5/6 ortholog similar to teleost, confirming an ancestral origin [56,57].

5. Conclusions

According to the most recent literature, this is the first report on the identification of TRPV1 receptor in rainbow trout using immunohistochemistry. This simple but precious technique permitted us to appreciate the localization in different organs, layers and on the surface of cell membranes. The quantitative evaluation of receptor expression permitted us to appreciate a strong positivity in the kidneys and the muscle layer of the stomach and bowel. The administration of the supercritical fluid extraction of basil, F1-BEO, influenced the receptor expression in specific organs such as the liver and kidneys, but did not damage these organs and no morphological alterations have been appreciated. Further studies will be necessary to evaluate the modulation and the functionality using specific TRPV1 ligand to explore similarities and differences among TRPV1 in fishes and mammals.

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