Supplementary File 2 The ARRIVE Guidelines Checklist

<table>
<thead>
<tr>
<th>Item</th>
<th>Response to Recommendation</th>
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</thead>
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Abstract

**Background:** This research aimed to evaluate the effect of four homeopathic treatments: Passival (PaV); Passival and Phosphoricum acid Similia (PaV–PhA); Passival and Silicea terra Similia (PaV–SiT); Fator Endecto Aquicultura and Fator Infeccoes Aquicultura (End–Inf) and one control treatment without homeopathy on coccidia in *L. guttatus* juveniles.

**Methods:** Histopathological analyses were performed on gills, liver, intestine and stomach of *L. guttatus* juveniles to determine the presence of coccidia in target tissues; histochemical analyses in liver to quantify carbohydrates and in gills and intestine to quantify mucosal cells; finally, haematological analysis in peripheral blood of *L. guttatus* juveniles to determine the presence of leucocytes.

**Results:** Fish treated with End–Inf recorded the least intestinal coccidia incidence and a higher lymphocyte percentage with respect to the other treatments. Fish treated with PaV–PhA recorded a higher percentage of neutrophils compared to the other treatments. Fish treated with PaV–PhA, PaV–SiT and End–Inf revealed a decrease in lesions, degree of alteration and change in intestine and stomach tissues. The number of mucous cells in gills was greater in those treated with End–Inf (43 ± 0.58 and PaV–SiT (40 ± 2.89) than those in T0 and control groups (19 ± 0.58 and 28 ± 2.31), respectively. Moreover, fish treated with PaV and PaV–SiT accumulated greater carbohydrate reserves in liver (28.4 ± 1.39% and 22.4 ± 0.12%, respectively) compared with T0 and control groups (3.4 ± 0.32 and 5.6 ± 0.66%), respectively. Juvenile fish survival was 97.7 ± 0.16% in fish treated with PaV and End–Inf.

**Conclusion:** The results suggested that the evaluated homeopathic treatments had a positive and statistically significant effect on fish health status; besides the accumulation of metabolic reserves produced in the liver, they promoted mucin increase in gills and stimulated a greater production of lymphocytes and neutrophils in blood, which could act as macrophages against intestinal coccidia.

INTRODUCTION

**Background**

Farming the Spotted rose snapper (*L. guttatus*) has a great potential in marine culture because of its commercial demand and adaptation to zootechnical practices. Nonetheless, the stress caused in fish-culture farms by inadequate and intensive management destabilises the host–pathogen–environment equilibrium, which favours proliferation and development of parasites, bacteria and other pathogens, causing weight, appetite loss and slow growth including high mortality associated with infectious diseases. Coccidian endoparasites generally cause intestinal or extra-intestinal infections in marine and anadromous fish, causing cell degeneration. In natural environments, coccidia do not cause mortality. Nonetheless, in culture conditions, their presence is associated with poor fish performance, which is frequently a product of stress and reflected in mortality increase. The need of controlling parasite outbreaks has brought with it the use and abuse of chemical agents that have unchained negative impacts to the environment. For this reason, new eco-friendly alternatives in animal health, such as homeopathic medicines, which are highly diluted because of their nature, are being studied in veterinary medicine for the treatment and control of infectious and parasitic diseases. In aquaculture, recent studies have shown their efficiency. This background suggests that homeopathy can really offer alternatives for marine fish farming under a productive perspective that involves several aspects in terms of health, safety and eco-sustainability.

**Objective**

The objective of this research was to evaluate the effect of homeopathic medicines on nutrition and health related to coccidia parasite control in juveniles of the marine fish *L. guttatus*. The degree of parasite infestation severity, as well as prevalence and intensity of the lesions caused by them in the affected tissues, such as gills, liver, intestine and stomach, was used as response variables for histopathologic analyses.

**METHODS**

**Ethical statement**

The aquacultural research bioassay was approved to be carried out prospectively in accordance with the 3Rs principles (www.nc3rs.org.uk), under farm-production practices and best zootechnical standards in marine aquaculture to minimise harm, pain, suffering, distress or lasting harm to fish. The fish were born and raised in a commercial laboratory under human management. They were cultivated in the best possible controlled experimental conditions, with continuous flow of clean sea water and aeration, eliminating daily faeces and detritus. The culture tanks used have an internal and non-toxic blue gelcoat cover that is friendly to marine culture, with adequate and intensive management destabilising the host–pathogen–environment equilibrium, which favours proliferation and development of parasites, bacteria and other pathogens, causing weight, appetite loss and slow growth including high mortality associated with infectious diseases. Coccidian endoparasites generally cause intestinal or extra-intestinal infections in marine and anadromous fish, causing cell degeneration. In natural environments, coccidia do not cause mortality. Nonetheless, in culture conditions, their presence is associated with poor fish performance, which is frequently a product of stress and reflected in mortality increase. The need of controlling parasite outbreaks has brought with it the use and abuse of chemical agents that have unchained negative impacts to the environment. For this reason, new eco-friendly alternatives in animal health, such as homeopathic medicines, which are highly diluted because of their nature, are being studied in veterinary medicine for the treatment and control of infectious and parasitic diseases. In aquaculture, recent studies have shown their efficiency. This background suggests that homeopathy can really offer alternatives for marine fish farming under a productive perspective that involves several aspects in terms of health, safety and eco-sustainability.

**Study design**

Research was performed in the Experimental Plant of Marine Fish Culture in Centro Regional de Investigación Acuícola y Pesquera (SAGARP-NAPECSA/CRIP La Paz, México) with *L. guttatus* juvenile fish (n = 458) with an initial average total weight and length of 1.9 ± 0.01 g and 4.9 ± 0.03 cm, respectively. A group of 28 specimens was randomly selected for initial sampling (T0) and 430 were distributed in groups of 43 in 10
### Experimental procedures

- **7** A completely randomised design was applied with four treatments in duplicate, constituted by homeopathic medicines for human- (TH1, TH2, TH3) and veterinary use (TH4) and a fifth one (control) without medication. TH1 (PAV) was a complex commercial formulation (Passival 30 cH; Similia, MX) for treating nervousness, stress and lack of sleep. TH2 (PAV-PhA) was a combination of Passival and *Phosphoricum acidum* 30 cH (Similia, MX). TH3 (PaV-SiT) was a combination of Passival and *Silica terra* 30 cH (Similia, MX). Starting from a ‘stock’ dynamisation (30 cH Similia), the respective ‘study’ dynamisation (31 cH) was prepared at Centro de Investigaciones Biológicas del Noroeste (CIBNOR) by diluting/succeeding the homeopathic medicine (30 cH) in a proportion 1:99 using ethanol 87° (Similia, MX) diluted to 30° with distilled water. For their dynamisation, all Similia and control (TH5) treatments were agitated in a vortexer at 3200 rpm (BenchMixer; Edison, New Jersey, USA) for 3 minutes and then sprinkled in balanced feed at a ratio of 5% volume/weight. Once sprinkled, feed was dried at room temperature (23°C) to eliminate alcohol and avoid its potential side effects. To keep freshness, only the amount to be used in a maximum of a 2-day period was prepared at a time. TH4 (End-Inf) was a mixture of two complex homeopathic formulations for veterinary use (Fator Endecto Aquicultura and Fator Infecces Aquicultura) from Arenales Homeopathy (Brazil), which are used for endo- and ecto-parasites and infection control, respectively, in marine and fresh-water fishes. For the application of Arenales complexes, granulated medicines were diluted in drinking water and sprinkled in feed following the producer’s instructions (0.15 g/kg of fish). TH5 (Control) did not contain any type of medicine, only ethanol Similia diluted to 30° with distilled water and then succussed in a vortexer.

### Experimental animals

- **8** *L. guttatus* juveniles (*n* = 458) were evaluated, of which a group of 28 specimens were randomly selected for initial sampling (T₀), and 430 were distributed in groups of 43 in 10 experimental units.

### Housing and husbandry

- **9**
  - **a. Housing** We used 10-fibreglass tanks (43 fish/tank) with an operational volume of 100 L and sea water change of 900 L/day/tank.
  - **b. Husbandry** The 430 fish specimens were acclimated for 15 days and fed daily to satiety with EP1 Otohime (BioKyowa Japan), an extruded 1.7-mm pellet and a minimum of 48% crude protein and 14% lipids. From the start of the experimental period, homeopathic medicines were incorporated in feed (5% volume/weight). Daily food ration was established as 10% of initial fish weight for each treatment and provided in three portions (09:00, 12:00 and 15:00 h) for the first week. The initial amount was adjusted weekly to satisfy the demand to satiety of each fish group in each culture tank. At this time, temperature, dissolved oxygen, salinity and pH values were recorded to get daily average data.

### Sample size

- **10** Twenty-eight individuals were collected for initial (T₀) biometric analysis and 10 individuals/replicate (20/treatment, 100 in total) for final (T₅) biometric analysis.

### Allocating animals to experimental groups

- **11** All tests corresponded to a completely randomised design; 430 specimens were distributed in groups of 43 in 10 experimental units (43 fish/tank).

### Experimental outcomes

- **12** Define primary and secondary experimental outcomes assessed. Growth and survival were assessed. Blood samples were taken, and blood smear slides were prepared for the haematological analyses (T₅). Whole fish were fixed in Davidson AFA solution for 48 hours for the histopathological and histochemical analyses. The response indicator parameters evaluated were as follows: intestinal coccidia, leukocyte cells in blood, median assessed value (MAV) and degree of tissue change (DTC), mucous cells in gills and intestine, carbohydrates in liver, weight-length reason, condition factor (CF) and survival.

### Statistical methods

- **13** Data normality was assessed with Kolmogorov–Smimov test, and variance homoscedasticity was verified with Cochran C test. A one-way variance analysis and a posteriori Tukey’s test with a statistical significance of *p* = 0.05 were performed; multiple comparison of mean rank test was used for all groups, and differences were determined between treatments versus the control group. The results were expressed in function of the means and their standard error of the mean using StatSoft.Statistica (v.10) software.

### RESULTS

- **14** Fish were donated by Earth Ocean Farms Company and transferred to the Experimental Plant of Marine Fish Culture in Centro Regional de Investigación Acuicola y Pesquera (SAGARPA-INAPESCA/CRIAP La Paz, México). Fish were acclimated for 15 days and fed with EP1 Otohime (BioKyowa Japan), an extruded 1.7-mm pellet and a minimum of 48% crude protein and 14% lipids to satiety daily. From the start of the experimental period, homeopathic medicines were incorporated in feed (5% volume/weight). Daily food ration was established as 10% of initial fish weight for each treatment and provided in three portions (09:00, 12:00 and 15:00 h) for the first week. The initial amount was adjusted weekly to satisfy the demand to satiety of each fish group in each culture tank. At this time, temperature, dissolved oxygen, salinity and pH values were recorded to get daily average data.
Supplementary File 2 (Continued)

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<tr>
<td>Numbers analysed</td>
<td>15 Twenty-eight individuals were used for initial (T0) biometric analysis, and 10 individuals per replicate (20 per treatment, 100 in total) for the final (T45) biometric analysis.</td>
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Outcomes and estimation

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Figure 1 Degree of severity of intestinal coccidian infection in *L. guttatus* juveniles treated with different homeopathic medicines recorded at the end of the experiment (T45) versus non-treated fish at the start (T0) and end (T45) of the experiment. Average values ± standard error of the mean. Different letters indicate significant differences (Tukey; *p* = 0.05).

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Figure 2 Average number (±standard error of the mean) of mucous cells produced in gills of *L. guttatus* juveniles at the beginning (T0) and at end of the experiment (T45) with different homeopathic medicines and the control group (without homeopathic medicine). The area per image capture of mucous cells in gills is equal to 0.135 mm². Different letters show significant differences (Tukey; *p* = 0.05).

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Figure 3 Average percentage (±standard error of the mean) of carbohydrates in *L. guttatus* liver at the beginning (T0) and at end of the experiment (T45) with different homeopathic medicines and a control group. Different letters show significant differences (Tukey, *p* = 0.05).

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**Supplementary File 2 (Continued)**

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<td><strong>Adverse events</strong></td>
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<td>Give details of all-important adverse events in each experimental group. Describe any modifications to the experimental protocols made to reduce adverse events.</td>
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**DISCUSSION**

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<td>Throughout the experimental bioassay, it was possible to distinguish and associate different effects and similarities with respect to the results that derived from the application of the homeopathic treatments evaluated in juvenile <em>L. guttatus</em>. For example: (1) the lowest incidence of intestinal coccidia occurred in fish treated with PaV and End−Inf; (2) the best survival results were obtained (97.7 ± 0.16%) in these same treatments; (3) the highest values in CF were obtained with PaV−PhA, PaV−SiT and End−Inf; (4) in these same treatments the lowest values of tissue damage (MAV) and the highest production of mucus in the gills were obtained; and (5) the highest CF value (1.66 ± 0.013) was obtained in the fish treated with PaV−PhA. By making a final integration of these and other results previously reported, it is totally feasible to conclude that the complex treatments evaluated, which were formulated with homeopathic medicines for human or veterinary use, are definitely appropriate to maintain an optimal state of health in <em>L. guttatus</em> juveniles under experimental hatchery culture conditions.</td>
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<th>Generalisability/ translation</th>
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<td>The complex treatments evaluated, which were formulated with homeopathic medicines for human or veterinary use, are definitely appropriate to perform or maintain an optimal state of health in <em>L. guttatus</em> juveniles under experimental hatchery culture conditions; therefore, aquaculture homeopathy has potential applications in the culture of this and other marine fish.</td>
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<th>Funding</th>
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<td>This study was financed by Public Sectorial Research Fund for Education, projects BASIC SCIENCE CONACYT No. 258282 ‘Experimental evaluation of homeopathy and new probiotics in the culture of molluscs, crustaceans and fish of commercial interest’, and R&amp;D+i PROINNOVA CONACYT/PEASA-241777, under the academic responsibility of the corresponding author JMMS. The authors are grateful to institutional incentives for research and development projects IPN-SIP 20150142, IPN-SIP 20160503 and COFAA-IPN; to scholarships and incentives granted to APRG to carry out Master studies under the direction of SD and JMMS; to INAPESCA (CRIAP-LP) and histological and histopathological laboratories at CIBNOR-La Paz and CIAD-Mazatlán; Earth Ocean Farms (EOF) for the donation of <em>L. guttatus</em> juveniles.</td>
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