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## **Aquacultural Homoeopathy: A Focus on Marine Species**

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### **Abstract**

Homoeopathy is an alternative medical system proposed by Samuel Hahnemann in the eighteenth century. It uses highly diluted and agitated substances that derived from plants, minerals or animals, which have shown to be effective in human medicine, agronomy, veterinary, and as a novelty, in marine aquaculture. Aquacultural homoeopathy has developed rapidly in recent years, partially motivated by the misuse of powerful drugs (hormones,

antibiotics, disinfectants) that when solving a problem generate undesirable side effects. In the last 10 years, scientific articles have been published on its application in freshwater fish native to Brazil, obtaining beneficial effects on growth, survival, hepatosomatic index, development of muscle fibres and lipid content in muscle. At Centro de Investigaciones Biológicas del Noroeste (CIBNOR, Mexico: [www.cibnor.mx](http://www.cibnor.mx)), we have studied the effects of homeopathy to improve the culture of economically important marine species of molluscs, fish and shrimp. In this chapter, we show a selection of different research with preliminary or advanced results, related to the use of homeopathy and its impact on zootechnic, biochemical, genomic and transcriptomic parameters in marine molluscs, fish and crustaceans. The results obtained suggest that homeopathy is an eco-friendly alternative applicable in aquaculture industry to improve various productive and health aspects.

**Keywords:** homeopathic medicine, marine molluscs, crustaceans and fish, growth, survival and biomass production, physiology and reproduction, genomic, metagenomic and transcriptomic response

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## 1. Introduction

Aquaculture has become a dynamic industry in constant development with the fastest growth food production sector of animal origin, even at a higher rate than human population, which could provide half of the fish consumption worldwide. However, its great development has turned out into great problems and challenges, based mainly on hyper-intensive production systems where the common denominator is high cultivation densities that generally cause physiological stress. Acute or chronic stress acts in a synergistic way to other environmental factors, negatively impacting productive parameters or increasing sensitivity to diseases produced by opportunistic pathogens as bacteria and viruses. Altogether finally translates into great economic losses to producers. Among the solutions for these problems is the use of expensive immunostimulant substances, so producers have opted to use and misuse antibiotics and/or prophylactics to treat stress and its consequences. Antibiotics promote the resistance of target organisms and leave residues in the environment and in tissues, which has become a public health problem in some countries [1, 2]. For this reason, alternatives have been sought such as the use of probiotics [3], phytobiotics [4], and recently homeopathy that has been investigating as a novel alternative to improve various productive aspects in the culture of aquatic organisms [5]. Homeopathy is a branch of universal medicine based on the Law of Similars also expressed as “principle of like” (*Similia Similibus Curentur* = Like cures Like), and it is applied in ultra-diluted and succussed minimal doses. It is assumed that a substance that is applied in high doses (massively) generates a pathological symptomatology, so if it is applied in minimum doses (obtained by serial dilution and agitation), it can in turn cure it [6]. Homeopathy derives from a Hippocratic medical concept proposed by the German physician Samuel Hahneman (1755–1843), who developed decimal (D; 1: 9), centesimal (C; 1: 99) millesimal (M; 1: 999) dilutions and other medicines, which are actually known as homeopathic “dilutions” or “dynamisations.” This process consists of serial dilution of mineral, plant and animal concentrate materials in water-ethanol vehicle, and vigorous agitation or “succussion” [7]. The starting point to obtain a certain dynamisation is a concentrate or Mother Tincture (MT), which is an alcoholic extract prepared from plants, animals, minerals, and even nanoparticulate metals [7]. In spite

of its high dilution, it is possible to detect nanoparticles of the “ponderable active principle” (MT) in the dynamisations, even in high centesimal dilutions despite the fact that according to Avogadro’s theory, they should not have a single molecule of MT [8]. Therefore, re-naming homoeopathy as “Adaptative Network Nanomedicine” has been recently proposed [9].

Homoeopathy has the peculiarity of stimulating the self-recovery of dynamic homeostasis when it has been lost due to exogenous and/or endogenous factors. Therefore, it does not focus on “the disease” but on the manifestations and intrinsic defence mechanisms of the “patient.” It offers “signals” of systemic action to the treated individual to promote its self-regulation to recover homeostasis, and as it uses ultra-diluted minimal doses, it does not leave residues in the organism or in the environment. As a counterpart, the other medicine known as “allopathy” derives from a galenic concept based on the “principle of opposites” and the application of massive doses of various chemotherapeutic agents officially classified as anti-microbial, anti-viral, anti-inflammatory, anti-spasmodic, anti-histaminic, anti-fever and other “anti” drugs. Homoeopathic medicines can be administered to any living being, including terrestrial and aquatic plants, wild animals in captivity and breeding, as well as freshwater and marine species of commercial interest [7]. It has been widely used in human, animal and plant medicine because it induces specific responses and increases immunity, favouring resistance to pathogens under stressing situations, promoting a better post-infection recovery and improving internal dynamic homeostasis [5, 6, 10].

In Mexico, homoeopathy is recognised as a therapy of alternative medicine, and its practice was authorised by presidential decree in July 31, 1895; today its study and practice is officially recognised in the general health law (2015), and only health professionals can prescribe homoeopathic medicines, which must have an official code. This is the way homoeopathic medicines are differentiated from products like herbs for infusion and herbal remedies. In countries such as Brazil, there are homoeopathic medicines exclusively for veterinary use, for marine and freshwater fishes, registered with the Ministry of Agriculture. Important and promising results have been reported in freshwater organisms, mainly Nile Tilapia *Oreochromis niloticus* and Pacu *Piaractus mesopotamicus* [11–13]. Taking into account these antecedents, this chapter compiles not only the experimental results obtained in marine organisms, such as molluscs, fish and crustaceans when treated with commercial homoeopathic medicines for human use and approved by Federal Health Law and Health Ministry of México but also other ones that have been designed and developed at Centro de Investigaciones Biológicas del Noroeste (CIBNOR) in La Paz, Baja California Sur, Mexico.

For the purposes of this chapter, commercial drugs for human use have been utilised in the form of liquid hydro-alcoholic dynamisations (Similia® Laboratories, Mexico), injectable aqueous dynamisation (Rubiopharma®, Mexico) or sugar impregnated with homoeopathic complexes (Arenales Homoeopathy®, Brazil). They were considered “stock dynamisations,” from which the respective “work dynamisations” were obtained through a serial process of dilution-succussion decimal or centesimal. Other non-commercial medicines were designed and developed at CIBNOR, from bacterial products (nosodes). This chapter deals with unitary or complex laboratory treatments used and registered with the Mexican Ministry of Health, such as Similia® and Rubiopharma® and a Cuban drug of Labiofam®, which will be described as follows: *Passiflora incarnata*, *Valeriana officinalis*, *Ignatia amara* and *Zincum valerianicum* of

Similia® (PaV); Cyme-Heel, Gal-Heel, Hepa-Heel, Mucs-Heel and Chol-Heel of Rubiopharma® (INM); Endecto (END) and Infecções (INF) of Arenales Homoeopathy®; *Phosphoric acid* (PhA), *Phosphoric acid* (AcF), *Silicea terra* (SiT), *Sodium methasilicate* (MsS), Scorpion toxin Vidatox® (ViT); *Calcium sulphuricum* (CaS); *Hepar sulphuris* (HeS), *Ferrum phosphoricum* (FeP); *Zincum phosphoricum* (ZiP); *Magnesium phosphoricum* (MaP), *Mercurius solubilis* (MeS). Also, other nosode-type homoeopathic medicines from *Vibrio* compounds (ViP, ViA) were applied. These and other nosode type HOM-products have been designed by CIBNOR that is processing the respective trademark and industrial property titles (Office for industrial protection and technology transfer; OTT-CEPAT/CIBNOR; www.cibor.gob.mx). Ethanol (ET) and no-HOM nor ethanol (NT) were used as control treatments. Homoeopathic medicines (HOM) were sprinkled on balanced food or inert sugar pills or added directly to culture seawater.

## 2. Effects of homoeopathy in marine species

### 2.1. Mollusc

#### 2.1.1. Catarina scallop (*Argopecten ventricosus*)

The production of mollusc bivalves around the world is still challenged every year by the propagation and emergence of new diseases. Scallops are especially susceptible to epizootic pathogenic bacteria in the hatchery, mainly those related to *Vibrio* spp. and *Aeromonas* spp. [14]. Four experiments described below were performed with *A. ventricosus*.

**Trial 1.** To assess the effects of homoeopathic treatments (HOM treatments) in the immune system of Catarina scallop *A. ventricosus*, an experimental design was applied at CIBNOR during nursery management with five homoeopathic treatments and three controls, four replicates each. Juveniles were placed in recirculating upwelling nursery units (40 l), each one with four PVC upwelling cylinders for 21 days. Continuous aeration and a microalgal food mix 1:1 (*Isochrysis galbana* - *Chetoceros calcitrans*; 150,000 cel ml<sup>-1</sup>) were provided. The following HOM treatments and controls were applied: ViP (T1), ViA (T2), PhA Metasilicate (T3), PhA-SiT (T4), ViT (T5), dynamised ET (T6), diluted ET (T7) and NT nor ethanol added (T8).

At the end of the assay, mainly nosodes (ViP, ViA) formulated from pathogenic bacterial compounds, stimulated a significant ( $p < 0.05$ ) growth rate and increased haemocyte count which were counted using three images at 100X from scallops tissue were processed by Image Pro Plus 6.0 to count the number of haemocytes in a tissue area (0.21 mm<sup>2</sup>). Haemocytes count was 1–3 times higher than controls (**Table 1**). These results suggested immune system enhancement by the action of HOM treatments because it is known they stimulate enzymatic activity of superoxide dismutase (SOD) and catalase (CAT) related with antioxidant responses of the organisms during oxidative stress [15]. The proliferation of haemocytes is related to activation of immune response because they are the primary cells responsible to protect organisms against infections [16]. Antioxidant activity increases have also been linked to improving survival when organisms are challenged against stressful conditions [16–18]. These findings make it clear that HOM treatments can activate a quantifiable biological response on the immune and antioxidant system in juvenile scallop *A. ventricosus*.

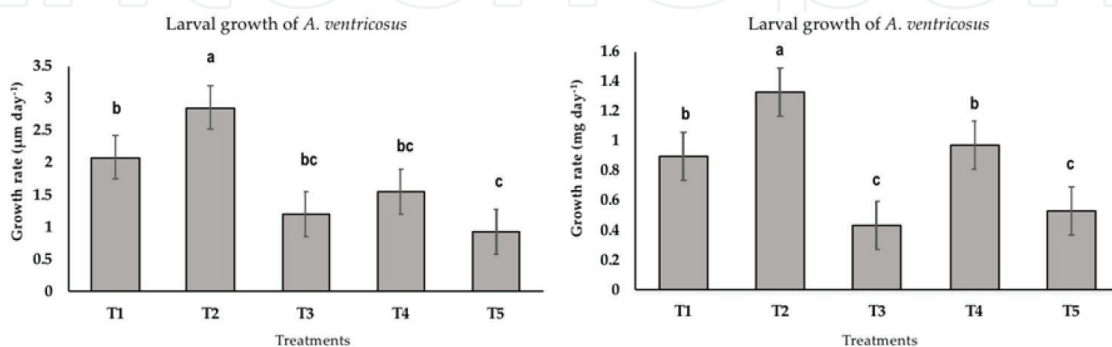
	Haemocyte count	Growth rate ( $\mu\text{m day}^{-1}$ )
Initial	$19 \pm 0.38_e$	
T1	$44 \pm 0.54_a$	$117 \pm 1.32_a$
T2	$41 \pm 0.57_b$	$108 \pm 0.97_a$
T3	$34 \pm 0.75_c$	$53 \pm 6.31_c$
T4	$24 \pm 0.40_d$	$94 \pm 1.38_b$
T5	$31 \pm 0.32_c$	$36 \pm 5.83_d$
T6	$21 \pm 0.87_e$	$14 \pm 0.40_e$
T7	$22 \pm 1.13_{de}$	$34 \pm 0.06_d$
T8	$16 \pm 0.60_f$	$20 \pm 0.14_e$

Numbers show mean  $\pm$  standard error. Identical lowercase letters denote lack of significant differences between treatments at  $p < 0.05$ .

**Table 1.** Haemocyte count and growth rate in *Argopecten ventricosus* spat treated with homoeopathic medicines for 21 days.

**Trial 2.** To compare between homoeopathy and antibiotic efficiency in *A. ventricosus* juveniles, an experimental design was performed by triplicate (6 treatments and 18 replicates, 120 seeds each replicate) at CIBNOR for 21 days. Juveniles ( $4.14 \pm 0.06$  mm;  $13.33 \pm 0.03$ ) received PaV-Pha (T1), PaV-Sit (T2) as HOM treatments, ampicillin AMP (T3) as antibiotic treatment, and ethanol ET (T4) and NT (T5) as control treatment. Liquid treatments were applied ( $100 \mu\text{l l}^{-1}$  for homoeopathy and 10 ppm for antibiotic) after seawater exchange every 48 h and before feeding scallops. Evaluations were performed for growth in height (mm), total wet weight of the shell (mg), biochemical flesh composition ( $\text{mg g}^{-1}$ ) and SOD activity. Thirty juveniles were taken at random from each replica at 7, 14 and 21 days to measure size and total wet weight and to determine absolute growth in height and weight (mm, mg) for each treatment.

Juveniles grew significantly more in size with HOM T1 ( $6.22 \pm 0.11$  mm;  $0.05 \text{ mm d}^{-1}$ ) and T2 ( $6.99 \pm 0.09$  mm;  $0.08 \text{ mm d}^{-1}$ ) compared with NM (T5) ( $5 \pm 0.02$  mm;  $0.02 \text{ mm d}^{-1}$ ). A significant increase in total wet weight was recorded with HOM T2 ( $41.16 \pm 0.35\text{mg}$ ;  $1.3 \text{ mg d}^{-1}$ ) compared to NT group T5 ( $24.33 \pm 0.10$  mg;  $0.5 \text{ mg d}^{-1}$ ). Survival was 100% in all treatments and their replicates (**Figure 1**).



**Figure 1.** Growth in size ( $\mu\text{m day}^{-1}$ ) (left) and weight ( $\text{mg day}^{-1}$ ) (right) of juvenile Catarina scallop *Argopecten ventricosus* treated with homoeopathic medicines.

**Trial 3.** As a continuation of Trial 2, once the previous experiment (21 days) was completed, a pathogen challenge was performed at CIBNOR with those treated juvenile scallops. About 30 juveniles were randomly selected from each previous treatment (in duplicate) and challenged with a pathogenic strain of *Vibrio alginolyticus* (CAIM57: www.ciad.mx). An initial single dose ( $1 \times 10^7$  CFU ml<sup>-1</sup>) was provided as based on the mean lethal dose (LD<sub>50</sub>) determined by the Probit method and based on the dose–response model described by Finney [19]. To obtain greater clarity in the results, in addition to the groups previously treated PaV-PhA (T1), PaV-SiT (T2), antibiotic AMP (T3) and ET (T4), two new groups that did not receive any previous treatment were included. First, a new group was infected with CAIM57 and defined as positive control (CTRL +), and another new group was not infected and defined as negative control (CTRL -). Survival (%) of juveniles was evaluated at 0, 24, 48, 72 and 120 h after infection, and the activity of SOD before infection at 48, 72 and 96 h post-infection was determined. For each treatment, soft tissues (100 mg) from six juveniles were weighted and 500 µl phosphate buffer (pH 7.5) were added. The tissues were homogenised and centrifuged at  $9327 \times g$  for 10 min at 4°C, recovering the supernatant and storing it at -20°C until further analysis. SOD activity was determined with a commercial kit (SOD Assay Kit #19160, Sigma-Aldrich). Results were expressed as an indirect measure of SOD activity as a per cent of the water-soluble tetrazolium salt formazan complex inhibition. During the challenge, no water changes were made. All juvenile scallops not treated but challenged (CTRL +) died at 72 h while untreated and unchallenged scallops attained the highest survival (95%). The HOM-treated scallops also survived the challenge; T1 scallops attained 85 versus 40% survival in those treated with antibiotic (T3). Finally, the SOD activity increased significantly with respect to the other treatments and controls in the juveniles of the HOM T1 (81%), 72 h post-infection.

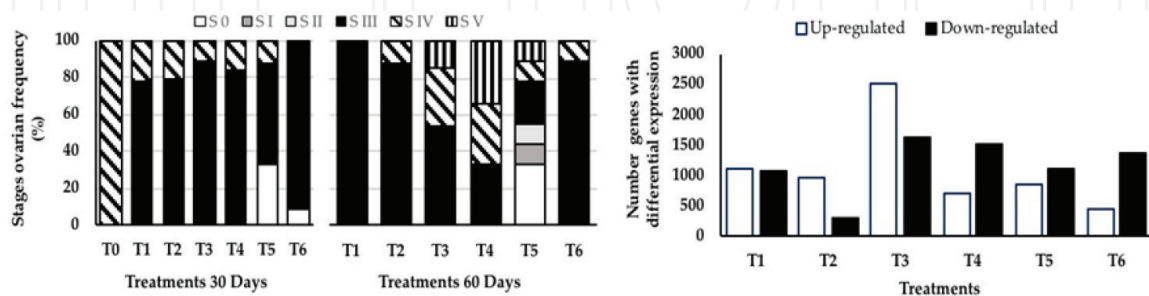
**Trial 4.** To assess the effects of HOM treatments on the microbial communities of the gastrointestinal tract (GIT) of juvenile *A. ventricosus* an experimental design was applied at CIBNOR during nursery management with five homoeopathic treatments and three controls, four replicates each. Juveniles were placed in recirculating upwelling nursery units (40 l) each one with four PVC upwelling cylinders for 21 days; the following HOM treatments and controls were applied: ViP-ViA/a (T1), ViP-ViA/b (T2), AcF-MsS (T3) PhA-SiT (T4), ViT (T5), ET (T6), and NT (T7). At the end of the experiment, eight scallops were randomly taken from each replica and washed, removed fouling organisms of external sides of shell and sprayed with ethanol and dried. Immediately one of the shells was removed and soft tissues dissected to isolate the gastrointestinal tract (GIT) of each scallop, which were fixed in RNAlater® (Thermo Fisher Scientific, Waltham, MA, USA) and preserved at -20°C. The technique of massive DNA sequencing was applied, which is widely used in the study of microbial communities associated with biological systems. The bacterial 16S rDNA was extracted according to Garcia-Bernal *et al.* [3], amplified for sequencing in the Illumina MiSeq Platform (Illumina, San Diego, CA, USA) in a certified Genomic Services Laboratory (www.langebio.cinvestav.mx; Irapuato, Guanajuato, México). Afterward, a bioinformatic and statistical analysis of the generated database was carried out. Initially, significant differences were detected ( $p < 0.05$ ) in growth rate of shell length ( $\mu\text{m d}^{-1}$ ) ( $p < 0.05$ ), with the best results ( $140 \mu\text{m d}^{-1}$ ) in the HOM T2. The groups that received T3 and T5 showed a significantly higher survival rate ( $p < 0.05$ ) than the other groups. Moreover, the dominant phylum was *Proteobacteria*, followed by *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*. The prevalent genera in GIT were *Microbacterium*, *Bacillus*,

*Symbiobacterium* and *Burkholderia*. In general terms, phyla *Proteobacteria* and *Actinobacteria* play an essential role in immunity and nutrition of invertebrates, and both were dominant in juvenile scallop *A. ventricosus* in all groups treated with HOM.

### 2.1.2. Horse-mussel (*Modiolus capax*)

The Horse mussel *Modiolus capax* is a native species from the Gulf of California with aquaculture potential, but scientific knowledge must be generated to achieve a sustainable production [20]. A study was developed at CIBNOR to assess the effect of HOM treatments on gonadal, physiological and transcriptomic maturation in *M. capax*. Microalga and wheat meal were used as food for broodstock mussels. The experiment was designed in triplicate (482 adult mussels; 60 days) and applied to evaluate three HOM treatments: SiT-CaS-HeS (T1), PhA-FeP-ZiP (T2), ViP-ViA-ViT (T3), ethanol as positive control (T4), and NT as negative control (T5). A sequential sampling in time (days) was made ( $t_0$ ,  $t_{30}$  and  $t_{60}$ ). The histological analyses showed that mussels treated with T1 and T3 attained ( $t_{30}$ ) the best results ( $p < 0.05$ ) in total weight increase, gonadal maturation of 100% females (**Figure 2** left), highest frequency of vitellogenic and postvitellogenic oocytes and highest oocyte quality according to the total area, theoretical diameter and % of ovoplasm. Also, mussels treated with T3 attained the best reproductive condition of the females (gonadal coverage area, ovarian maturity index, gonadal development index and reproductive potential); however, those receiving T2, increased oogonia proliferation and bioenergetic quality of the oocytes (amount of lipids and neutral carbohydrates). Histochemical and biochemical analyses revealed that HOM treatments (T1, T2 and T3) contributed to increase the overall energy reserves (lipids, carbohydrates and proteins) in the ovary, digestive gland and adductor muscle. López-Carvallo *et al.* [20] using wheat enriched di-algal diet, barely reaching gonad to maturity ~ 25% of the *M. capax* broodstock. Thus, we considered that better results in reproductive condition and oocyte quality of the species were attained with HOM treatments with respect to control.

On the other hand, a *de novo* transcriptome characterisation of the ovarian tissue treated with homoeopathy was performed using RNAseq. *In silico* analysis of differential gene expression revealed that mussels treated with T3 showed the highest number of differentially expressed transcripts (**Figure 2** right), and some of them were related to genes that encoded oestrogen



**Figure 2.** Frequency of ovarian developmental stages (left) determined by histology and number of genes (right) with significant differential expression ( $p < 0.01$ ) in the ovary transcriptome of broodstock mussels (*Modiolus capax*) conditioned with homoeopathy for gonad maturation. Stage S0 undifferentiated; stage S1 Previtellogenesis; stage SII Vitellogenesis; stage SIII Postvitellogenic; stage SIV partial spawning; stage SV post spawning;  $n = 482$  mussels. Over-expressed genes = black bars; under-expressed genes = Grey bars.



receptors ER and ERR2- like ( $p < 0.01$ ). Estradiol levels have been reported to have a direct influence on gonadic development and oocyte quality in marine bivalves [21] that allowed explaining the high reproductive condition found in the mussels that received T3.

This work has provided a *de novo* transcriptome characterisation for *M. capax* for the first time and together with the evaluation of physiological variables, it constitutes the first research about the beneficial effect of homoeopathy in gonadal maturation of the species, which has a clear technological applicability during broodstock gonadic conditioning for seed spawning and production of the species. It will likely be applicable to other bivalve molluscs with commercial interest.

### 2.1.3. American oyster (*Crassostrea virginica*)

The American oyster *Crassostrea virginica* is an important fishery and aquaculture resource in the Atlantic coast in the Caribbean and in the Gulf of Mexico. A commercial oyster laboratory is already operating in Centro Ostrícola Tecnológico de Tabasco (COTET), Mexico, and homoeopathic medicines were assessed to improve the species culture. HOM treatments were assessed in larval culture and settlement and early nursery of on-cultch and cultch-less seed. Also, a transcriptomic focus study was developed in adult broodstock oyster. The results obtained in six experiments conducted with *C. virginica* are detailed below.

**Trial 1.** A study was conducted at CIBNOR to analyse the transcriptomic response to five HOM treatments in broodstock oyster *C. virginica* to understand the response mechanisms that are activated by these treatments, thus helping to characterise its mode of action. Adult oysters (120 g; 10–12 cm) from a homogeneous population were conditioned in 80-l plastic boxes provided with continuous microalgal food at COTET for 45 days. An experimental design (3 replicates/treatment; 25 oyster/replicate) was applied with four HOM treatments: ViP-ViA (T1), PhA-SiT (T2), END (T3), INF (T4) and two controls ET (T5) and NT (T6). As treatment vehicle, fully impregnated inert homoeopathic pills were provided one daily per oyster. Routine techniques of the oyster hatchery COTET were applied for conditioning broodstock oyster, and during the trial period, two samplings ( $S_1$  and  $S_2$ ) were performed for histological analyses at CIBNOR. As a general rule, at the end of the conditioning assay, all conditioned groups were capable of sexually maturing and spawning in greater or lesser percentage, but specific details were observed that allowed to distinguish effects associated to different treatments and controls. Female oysters treated with HOM T1 attained the highest frequency of gonad in vitellogenesis (48%) and postvitellogenesis (30.2%) stages at first sampling (T1- $S_1$ ) and 16.7% in the second sampling (T1- $S_2$ ). In T2- $S_2$  oysters, the highest frequency (31.6%) showed at resting (undifferentiated) stage and 30.8% post-spawning stage. In ET control T5- $S_2$ , 38.5% of the female oysters were at resting stage and 30.8% at post-spawning stage. Oysters of NT control group T6- $S_2$  recorded the highest frequency (37.5%) in postvitellogenic stage, 31.3% in partial spawning stage and 25% in post-spawning stage. In the veterinary HOM T3- $S_1$ , oysters showed the highest frequency in partial spawning stage (71.4%), and in T4- $S_1$ , the highest frequency (57.1%) was recorded in post-spawning stage. It is important to highlight that even when T1, T2, T3 and T6 promoted maturation and spawning of the male oysters, there were differences between them related to gamete quality since the presence of atretic oocytes was seen in some samples of T5 and T6 control treatments. The veterinary HOM T4 seemed to have promoted damage to gametes because abundant degenerative

oocytes were observed. To evaluate the quality of the oocytes matured under different treatments, the Sudan Black histochemical technique was used staining the lipid components of the cells. The triglyceride lipid index (TLI) was calculated as described by Rodríguez-Jaramillo *et al.* [22], and significant differences were found in oocytes from different treatments and sampling times. Lipid content (TLI) was significantly higher ( $p < 0.0001$ ) in oysters receiving HOM T2 in a short conditioning time ( $S_1$ ). The rest of the treatments and replicates recorded significantly lower TLI values, including Veterinary HOM T3 and T4. A difference between HOM treatments lies in the fact that some of them seem to trigger a constant production of new generations of oocytes, which may be useful for hatchery purposes because it could be associated to the possibility of several partial spawning events that could derive into several larval batches and more opportunities for seed production.

**Trial 2.** A study was conducted at CIBNOR to analyse the transcriptomic response to five HOM treatments in broodstock oyster *C. virginica* in order to understand the response mechanisms that are activated by these nanomedicines, thus, helping to characterise their mode-of-action. About 25 groups of 25 adult oysters (120 g;  $10 \pm 12$  cm), each from a homogeneous population, were conditioned in 80-l plastic boxes provided with continuous microalgal food at COTET for 45 days. An experimental design with seven different treatments, each one with three replicates, was developed to determine the transcriptomic effect of an *Actinomyces* strain ( $1 \times 10^6$  CFU ml<sup>-1</sup> = RL8) and four homoeopathic drug complexes, alone and in combination: RL8 (T1), ViP-ViA (T2) PhA-SiT (T3), ViP-ViA + RL8 (T4), PhA-SiT+RL8 (T5) and two control groups: ethanol as a positive control (T6) and NT negative control (T7). Five oysters of each homoeopathic and control treatments were initially ( $t_0$ ) collected and dissected. The rest of the oysters were collected and dissected at the end of the experiment ( $t_{45}$ ). Several tissues, including mantle, gills, gonad, muscle and digestive gland (DG), were separately placed on snap-frozen tubes in RNA later<sup>®</sup> (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and stored at  $-80^\circ\text{C}$ . The rest of tissue portion were fixed in Davidson solution for histological examination. DG tissues fixed in RNA later<sup>®</sup> (Thermo Fisher Scientific, Waltham, MA, U.S.A.) from five individuals of each experimental group were subjected to RNA extraction, for the transcriptomic analysis, using Illumina Hiseq 2000 platform (Illumina, San Diego, CA, U.S.A.). The comparison of the transcriptome data with the KEGG database indicated that the treatments influenced associated metabolic pathways in gonadal development and maturation including “Developmental process involved in reproduction”, “Meiotic cell cycle process”, “Steroid metabolic process”, “Response to oestrogen “and” Regulation of reproductive process “. These pathways were assigned to the KEGG categories of “Developmental process involved in reproduction” which was among the most important category, indicating the significance of signal transduction systems and endocrine regulation of gonad development and function in *C. virginica*.

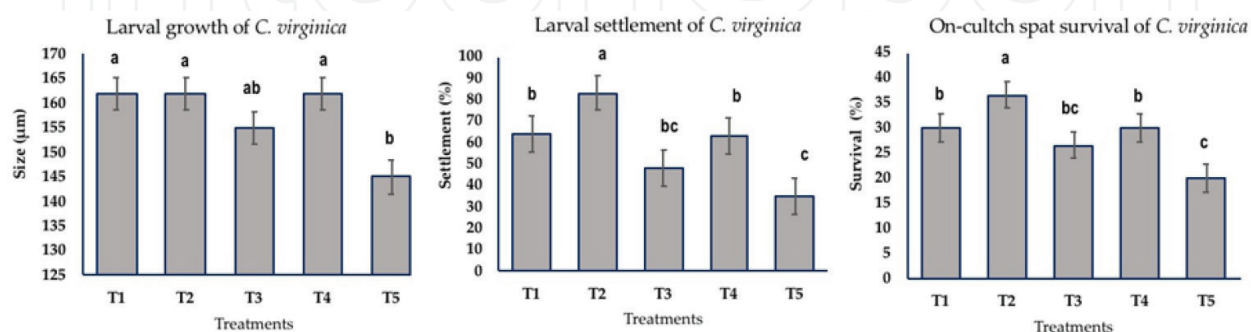
The transcriptome of T2 and T3 had 998 and 881 genes with a significantly increased level of expression, respectively, compared to the control ( $p < 0.05$ ). In T4 and T5, there were 748 and 789 genes with an increased level of expression, respectively, compared to a separate control ( $p < 0.05$ ). No genes were significantly differentially expressed under T1 compared to a separate T2, T3, T4, T5 and controls ( $p < 0.05$ ). Genes with increased expression following T2 and T3 were associated with biological processes, including metabolic pathways, ribosomal biogenesis, and transport of nitrogen compounds and anions. Following T4 and T5, genes with increased expression were involved in metabolism processes related with response to

stress, maintaining protein expression. Genes with increased expression following T1 were associated with protein kinase A (PKA) signalling that regulates stress responses and mitochondrion degradation. The transcriptomic results obtained allowed us to determine that homoeopathic treatments expressed transcripts associated with complex biological processes, such as reproduction, stress response, cell growth and metabolism of the *C. virginica* oyster.

**Trial 3.** An experimental design was applied at COTET for oyster larval culture assessment including HOM treatments: SiT (T1), ViT (T2), FeP (T3), MaP (T4) and NT (T5) as a control treatment. The experimental larviculture was performed in 20-l plastic buckets three replicates per treatment with filtered seawater, gentle and continuous aeration and the microalgae *I. galbana* and *C. calcitrans* (1:1) as food. Three-day old veliger larvae from a mass production fibreglass tank (25 t) were placed in buckets at initial density of 2 larvae ml<sup>-1</sup>. Liquid HOM treatments were added daily to culture water (0.01%). The larviculture test finalised when the first pediveliger larva with a retractable foot was detected but without reaching the final stage of eyed and fully developed pediveliger. Analyses were based on initial and three sequential biometrical data from 30 larvae for each treatment replicate. The HOM larval groups showed better development, survival and setting efficiency in comparison with untreated groups. As seen in **Figure 3**, *C. virginica* larvae that received T1 (162 ± 3.08 µm), T2 (162 ± 2.84 µm) and T4 (162 ± 3.13 µm) reached the largest sizes but T5 (145 ± 2.97 µm) reached the lowest sizes, while those larvae receiving T2 reached the highest setting efficiency (83 ± 7.98%) and the highest seed survival (36.5 ± 4.9%).

**Trial 4.** To assess larval settlement, mature eyed pediveliger larvae were obtained by selective screening of the same bulk culture tank; then, they were placed in 20-l buckets at a seeding density of 0.25 larvae ml<sup>-1</sup>. The same HOM treatments: SiT (T1), ViT (T2), FeP (T3), MaP (T4) and NT (T5) were applied at COTET during the on-cultch setting process. Seawater change, aeration and microalgae were provided as food, and 50 clean oyster shells/buckets were placed as a natural settlement (cultch) substrate. Liquid homoeopathic treatments were added daily to the culture water (0.02% v/v). The results achieved are shown in **Figure 3**.

**Trial 5.** A pilot experimental design in triplicate was applied at COTET for larval culture: MaP–FeP (T1), SiT–ViT (T2) and NT (T3) as control. Four-day old larvae were placed in nine-conical fibreglass tanks (750 l) at initial density of 5.33 larvae ml<sup>-1</sup> and the microalgae *I. galbana* and *C. calcitrans* (1:1) was provided as food. Larval growth and harvest of pediveliger larval

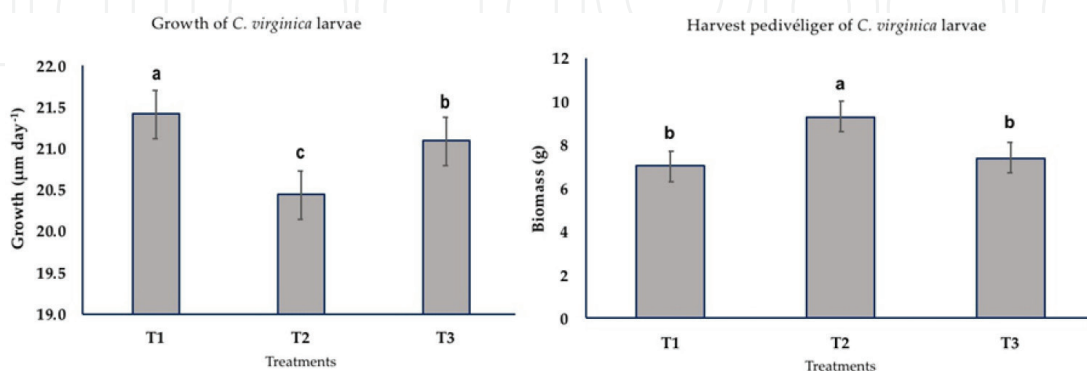


**Figure 3.** Larval growth (left), larval setting efficiency (Centre) and seed survival in 20-l plastic buckets (right) of American oyster *Crassostrea virginica*, treated with homoeopathic drugs in COTET oyster hatchery.

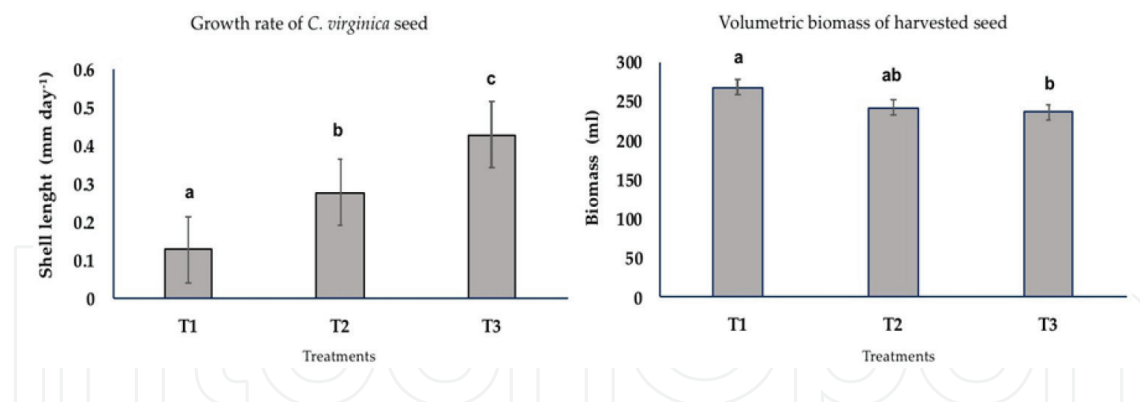
biomass was evaluated. Temperature and salinity were maintained in the range of  $27 \pm 3^\circ\text{C}$  and  $22 \pm 7$  psu. Liquid HOM treatments were added daily to culture water (0.01%). Based on biometrical data of 30 larvae for each replicate every 48 h, the best results in larval growth were obtained with HOM T1, followed by control (T3) and finally by T2 (Figure 4). The highest yield in biomass of mature pediveliger larva (9.29 g) was obtained with HOM T2, followed by control T3 (7.42 g) and the lowest production (7.04 g) was obtained with T1 (Figure 4). Differential results obtained in this study were attributable to better survival of larvae because homoeopathy favours nutrition and assimilation of nutrients, increases stress resistance because of high culture density and strengthens the immune system and resistance to attack by pathogens [5, 23]. A better survival associated to small sized larvae has been observed in other species treated with homoeopathy, such as *Panopea globosa* and *Crassostrea sikamea* (Mazón-Suástegui *et al.*, unpublished).

**Trial 6.** An experimental design was applied at COTET for cultch-less spat culture with two homoeopathic treatments and one control (four replicates each) in recirculating upwelling nursery units (40 l) each one allocating four upwellers of PVC pipe 4" and airlift devices. As HOM treatments, MaP–FeP (T1) SiT–ViT (T2) and NT control (T3) were assessed. Initial seed density was set on 4750 spat/upweller and *I. galbana* and *C. calcitrans* (1:1) was used as food. The total harvest of seed per treatments and replicates (volume) were measured weekly with a graduated cylinder. Temperature and salinity were maintained in the range of  $27 \pm 3^\circ\text{C}$  and  $24 \pm 3$  psu. Liquid dynamisation treatments were added daily to culture water (0.02%). Based on initial and weekly biometrical data of 30 spat for each replicate, the best growth results were obtained in the control T3, followed by HOM T2 and finally T1 (Figure 5). In contrast, the best results in oyster seed biomass were attained with HOM T1 (268 ml) and T2 (242 ml) and the lowest production (236 ml) with control T3 (Figure 5).

Oyster seed receiving HOM treatments grew less in regard to individual size, but an overall greater volume of marketable juveniles was harvested, which means that survival was greater in HOM treatments. From a commercial hatchery perspective, it is more important to produce live seed biomass even if small sized than dead ones. In real production and profitability terms, the hatchery could produce and sell more seeds if homoeopathic drugs, such as those evaluated in this study were routinely applied. The results in larval settlement and juvenile



**Figure 4.** Growth in size ( $\mu\text{m}$ ) of veliger larvae (left) and harvest of pediveliger larvae (right) of American oyster *Crassostrea virginica* treated with HOM in 750-l conical fibreglass tanks in COTET hatchery.



**Figure 5.** Growth rate in shell length ( $\mu\text{m day}^{-1}$ ) (left) and volumetric biomass of harvested seed (right) of American oyster *Crassostrea virginica* seed, receiving HOM treatments in recirculating upwelling nursery units (40 l) at COTET hatchery.

nursery confirmed a great potential for aquacultural homoeopathy to strengthen the state-of-the-art technology in oyster seed production and increase actual productivity and economic profitability of the commercial production of the species in the hatchery.

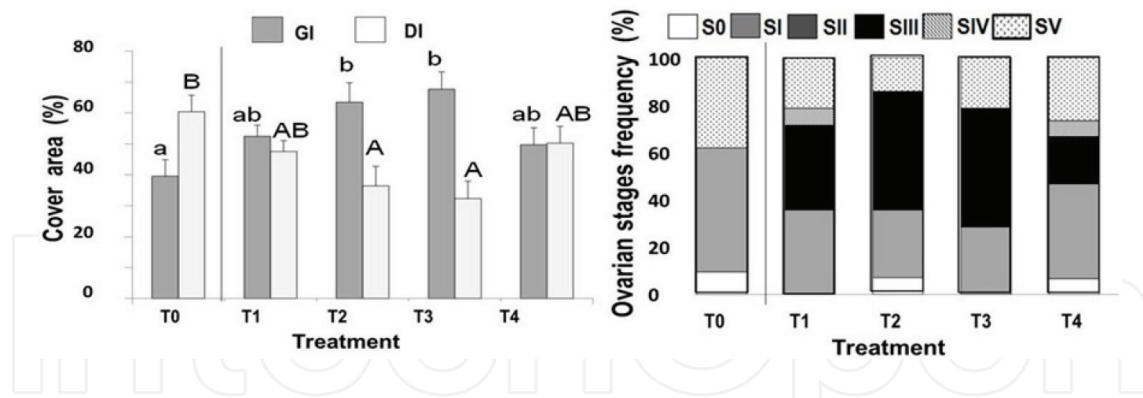
#### 2.1.4. Kumamoto oyster (*Crassostrea sikamea*)

Kumamoto oyster *Crassostrea sikamea* is a highly valuable mollusc species and cultivated in several countries, so it is important to generate new knowledge for its culture and alternative treatments for its management in the laboratory. Four experiments with *C. sikamea* were performed as described below.

**Trial 1.** An experimental design was applied at CIBNOR for conditioning broodstock with two replicates (25 oysters each) in eight plastic boxes (60 l), in which the following treatments were tested: ViP-ViA (T1) and PhA-SiT (T2) as HOM treatments, and ET (T3) and NT (T4). As treatment vehicle, fully impregnated inert homoeopathic pills were used, one-a-day per oyster.

Oysters were dissected (30 at  $t_0$  and 15 from each replicate at  $t_{42}$ ) and soft tissues histologically processed by haematoxylin and eosin staining to determine gonadic (GI) and digestive gland (DG) indexes, reproductive stages and theoretical diameter in oocytes (DT) according to Rodriguez-Jaramillo *et al.* [22] and Barber and Blake [24]. Moreover, significant differences were observed ( $p < 0.05$ ) in GI in broodstock oysters with respect to the initial value (39%), and the best results were observed in oysters treated with T2 and T3 (63 and 67%). The highest DI was recorded at the beginning of the experiment (60%) and the lowest ( $p < 0.05$ ) for T2 and T3 (36 and 32%), which suggested a greater energy storage in the gonad for oocyte maturation [24] (**Figure 6**). A high frequency of organisms in post-spawning and gonadic development stage (38 and 52%) was observed at  $t_0$ . At  $t_{42}$ , the oysters of T2 and T3 showed the highest percentage of full sexual maturity (50%) while the greatest number (35 and 40%) of organisms in gonadic development stage was observed in T3 and T4 (**Figure 6**).

Relative to the final DT (35  $\mu\text{m}$ ), which was 28  $\mu\text{m}$  at  $t_0$ , no significant differences were recorded between HOM-treated and control oysters. However, maturity and undifferentiated stages were simultaneously observed in T3 oysters. Since *C. sikamea* matures and partially spawns several times during the same reproductive season, this result suggested a positive effect by



**Figure 6.** Gonadic index (GI) and digestive index (DI) (left) and gonad development (right) in broodstock oyster *Crassostrea sikamea* conditioned in the laboratory for 42 days. Different letters show significant differences between treatments ( $p < 0.05$ ). Stage 0: Undifferentiated; stage I: Previtellogenesis; stage II: Vitellogenesis; stage III: Postvitellogenesis; stage IV: Partial spawning; stage V: Post spawning.

the activation of a new oocyte production cycle associated to HOM T2 (PhA-SiT). In bivalve molluscs, an expenditure of digestive gland reserves is associated to gonad development and maturation of gametes [24], and T2 was associated to a greater number of sexually mature oysters with higher GI and lower ID values. It could be applicable to gonadic conditioning for sexual maturation of broodstock oyster at the hatchery.

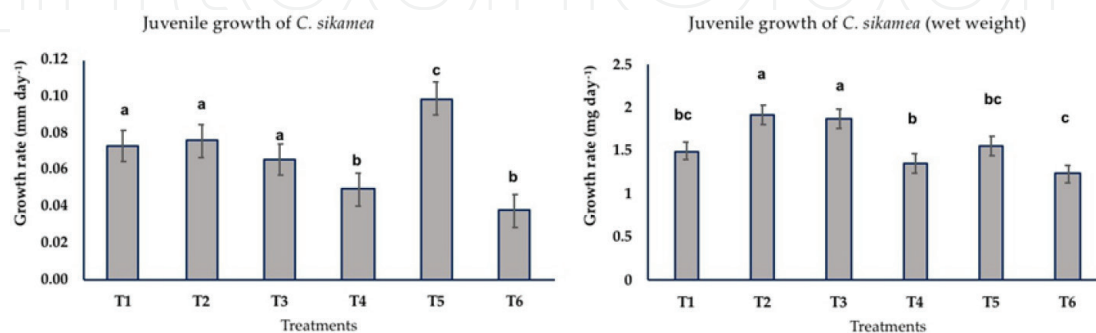
**Trial 2.** Once the broodstock assay finished, a subsequent second bioassay was developed to evaluate larval performance in two different larval cohorts from HOM-treated spawners (T1, T2, T3, T4; three replicates each) and NT spawners (T5, T6, T7, T8; three replicates each). The greatest growth in length was recorded in T5 and T6 (217 and 212  $\mu\text{m}$ ) and the lowest in T3 and T4 (197 and 190  $\mu\text{m}$ ). A similar growth pattern was observed in height: the greatest in T5–T8 and the lowest in T1–T4. In general, larvae from untreated broodstock grew more. Nevertheless, as a counterpart, the general mean survival of larvae from HOM-treated spawners (T1–T4) was 32.3% higher than those from NT broodstock, and it could be very important to improve hatchery seed production. Those differences in larval survival suggested a favourable effect of HOM treatments in oocyte quality and then a potential effect in the progeny.

**Trial 3.** A 35-day experimental design in triplicate (six treatments and 18 replicates, 120 seed each replicate) was applied at CIBNOR to compare efficiency of antibiotics against homoeopathy in juvenile *C. sikamea*. Juveniles ( $6.13 \pm 0.16$  mm) were placed in 2-l plastic containers with filtered (1  $\mu\text{m}$ ) and UV-sterilised seawater ( $23 \pm 1^\circ\text{C}$ ; 38 psu), continuous aeration and microalgal food. Juvenile oysters received PaV-Pha (T1), PaV-Sit (T2), Pav-MeS (T3) as HOM treatments, ampicillin AMP (T4) as antibiotic treatment, and ethanol ET (T5) and NT (T6) as control treatments. Liquid treatments were applied 100  $\mu\text{l l}^{-1}$  for homoeopathy and 10 ppm for antibiotic after exchange of seawater every 48 h and before feeding oysters. Evaluations were performed on growth in height (mm), and total wet weight of the shell (mg), biochemical flesh composition ( $\text{mg g}^{-1}$ ) and SOD activity. The biochemical composition was determined by triplicate sampling at the beginning and end of the trial. Samples were stored at  $-80^\circ\text{C}$  and then lyophilized, rehydrated in 3 ml cold saline solution (35%) and homogenised to obtain crude extracts. Crude extracts were processed at CIBNOR laboratory applying traditional and certified techniques, which are described by López-Carvalho *et al.* [20] and Mazón-Suástegui *et al.* [23]. For SOD analyses, after deep cleaning of shell, soft tissues were dissected and fixed individually in RNAlater<sup>®</sup>

(Thermo Fisher Scientific, Waltham, MA, USA) at a 1:5 ratio (100 mg tissue: 500  $\mu$ l RNA-Later<sup>®</sup>) and then preserve it at  $-20^{\circ}\text{C}$ . SOD analyses activity was determined with a commercial kit (SOD Assay Kit #19160, Sigma-Aldrich). Results were expressed as an indirect measure of SOD activity as a per cent of the water soluble tetrazolium salt formazan complex inhibition. The juveniles grew significantly more with HOM T1 ( $9.27 \pm 0.18$  mm,  $0.073$  mm day<sup>-1</sup>) and T3 ( $9.36 \pm 0.18$  mm,  $0.076$  mm day<sup>-1</sup>) compared with NT (T6) ( $8.02 \pm 0.23$  mm;  $0.053$  mm day<sup>-1</sup>), but of all treatments, the ET T5 group was the best ( $10.17 \pm 0.31$  mm;  $0.105$  mm day<sup>-1</sup>). A significant increase in total wet weight was recorded with HOM T2 ( $107.33 \pm 6.9$  mg,  $2.0$  mg day<sup>-1</sup>) compared to NT group T6 ( $76.11 \pm 2.8$  mg,  $1.1$  mg day<sup>-1</sup>) (Figure 7). Survival was 100% in all treatments and their replicates. Moreover, the biochemical composition of the juveniles showed significant differences in carbohydrates and lipids but not in proteins. The highest amount of lipids was obtained with HOM T2 ( $96.32 \pm 1.18$  mg g<sup>-1</sup>) and that of carbohydrates in antibiotic T4 ( $27.48 \pm 2.27$  mg g<sup>-1</sup>) compared with the NT (T6) ( $6.96 \pm 1.47$  mg g<sup>-1</sup>). At the end of this trial, SOD activity was higher in HOM T1 (92%) than control T6 (88%) and antibiotic T4 (84%).

**Trial 4.** Once the previous experiment was completed, a pathogen challenge was made with treated seeds. About 30 juveniles were randomly selected from each previous treatment (in duplicate) and challenged at CIBNOR with a pathogenic strain of *V. alginolyticus* (CAIM57: www.ciad.mx). An initial single dose ( $1 \times 10^6$  CFU ml<sup>-1</sup>) was given as based on the mean lethal dose (LD<sub>50</sub>) determined by the Probit method based on the dose–response model described by Finney [19].

To obtain greater clarity in the results, in addition to the groups treated with homoeopathy, antibiotic and ethanol, two new groups were included, which did not receive any previous treatment; one of which was infected with the pathogen (CTRL +) and another one that was not infected with the pathogen (CTRL -). Survival (%) of juveniles was evaluated at 0, 24, 48, 72 and 120 h after infection and the SOD activity (using a commercial kit; SOD Assay Kit #19160, Sigma-Aldrich) of before infection and at 2, 24, 48 and 72 post-infection was determined. After being infected with the pathogen, all the juveniles survived and no significant differences were observed between treatments and controls with respect to SOD activity. Up to date, that result has no coherent explanation yet; unless the species is highly resistant, the pathogenic strain has not got sufficient virulence or a wrong (lower) dose was applied because  $1 \times 10^7$  CFU ml<sup>-1</sup> was applied to juvenile scallop *A. ventricosus* by Mazón-Suástegui [23].



**Figure 7.** Growth in size (mm day<sup>-1</sup>) (left) and total wet weight (mg day<sup>-1</sup>) (right) in juveniles of the oyster Kumamoto *Crassostrea sikamea* treated with homoeopathic medicines.

### 2.1.5. Geoduck clam (*Panopea globosa*)

The geoduck clam *Panopea globosa* is an important marine resource distributed on both coasts of the peninsula of Baja California, Mexico. Its cultivation is still in experimental stage and totally depending on juveniles produced in the laboratory. As with other bivalves, there are limitations in their production due to mortalities of larvae and seeds, associated with the presence of pathogens. This problem has led to the search for new eco-friendly alternatives such as aquaculture homoeopathy, which has a positive effect on nutrition, health and immune response of bivalve molluscs, shrimp and marine fish [5, 23]. Our study evaluated the effect of various HOM treatments on growth, survival and microbiota of the gastrointestinal tract (GIT) of the species. Juvenile (spat) *P. globosa* with an average length of  $1.98 \pm 0.1$  cm were produced in the laboratory and provided by the company Acuacultura Robles, a commercial mollusc hatchery located in La Paz, B.C.S. Mexico. Clams with an average length of  $1.98 \pm 0.1$  cm were produced and provided by the company Acuacultura Robles, acclimatised at CIBNOR and then cultured (21 days) in nursery units previously described for *A. ventricosus*. About 24 upwelling units were used, each one with 52 clams and 13 clams per upweller cylinder; the following HOM treatments and controls were applied: ViP-ViA/a (T1), ViP-ViA/b (T2), AcF-MsS (T3) PhA-SiT (T4), ViT (T5), ET (T6) and NT (T7). Samples were taken at the beginning ( $t_0$ ) and end of the experiment ( $t_1$ ) by randomly selecting eight clams per replica, accounting for a total number of 216. After external deep cleaning, soft tissues were dissected to isolate GIT of each clam to fix individually in RNAlater® (Thermo Fisher Scientific, Waltham, MA, USA) at a 1:5 ratio (100 mg tissue: 500 µl RNA-Later®) and then preserve it at  $-20^\circ\text{C}$ . The bacterial 16S rDNA was extracted according to Garcia-Bernal *et al.* [3] and amplified using Illumina MiSeq Platform (Illumina, San Diego, CA) in a certified Genomic Services Laboratory ([www.langebio.cinvestav.mx](http://www.langebio.cinvestav.mx); Irapuato, Guanajuato, México). Afterward, a bioinformatic and statistical analysis of the generated database was carried out. Taking as reference the microbial diversity in the GIT of *P. globosa* juveniles, the best results were obtained with HOM T1, a nosode product developed at CIBNOR. T1 favoured dominant abundance of the Proteobacteria phylum and some of its classes as  $\gamma$ -Proteobacteria. In that sense, similarities were observed with the microbiota of other marine species, and that part of the microbiota found in *P. globosa* is associated with stimulation of the immune system. Overall, the results indicated that the HOM treatments modified the abundance of the microbial communities of the species, mainly in the phylotypes related to nutritional processes. On the other hand, significant differences were recorded with respect to growth in weight and length ( $p < 0.0001$ ) between the clams that received HOM treatment and the control groups. The highest growth in weight was recorded in T3, T4 and T5. The difference in growth in length was smaller but equally superior to these HOM treatments. Significant differences ( $p = 0.019$ ) in survival were also observed. The highest value (95%) was recorded in clams with HOM T3 followed by the NT control group T7 (93%) without homoeopathy or ethanol. In contrast, the lowest survival (76%) was observed in T2 and also in the ET control group T6.

### 2.1.6. Octopus (*Octopus bimaculoides*)

An experimental design in triplicate (10 juveniles/replicate) was developed in 60-l fibreglass units at CIBNOR laboratory to assess growth and survival in juvenile octopus during a 28-day



period. This assay was intended to study and compare the effects of fresh crab *Callinectes bellicosus* and squid *Dosidicus gigas* meat, as raw or thermally processed food (35°C and 60°C). Also, a HOM treatment was added to culture water, as a digestive system enhancer to *Octopus bimaculoides*. Two processed food treatments (35°C and 60°C), two HOM treatments (HOM-35 and HOM-60), and a positive control treatment (unprocessed raw food) were assessed. As HOM treatment, PhA-SiT was added directly to culture water alternating each medicine every day from Monday to Saturday. Food was provided *ad libitum* once a day. Wet weight (day 0 and 17) and survival percentage (day 17) were recorded for all 15 groups (Table 2). As expected for a positive control with a traditionally used raw food, the highest survival was attained in juvenile octopus fed on raw meat (97%) but also with HOM-35 (93%) and HOM-60 (86%) the lowest survival was seen in octopuses fed processed food 60°C without giving them HOM treatments (Table 2).

Knowledge concerning octopus culture is recent and scarce even when completing the life cycle successfully in captivity which is possible with some species [25]. One of the main obstacles to achieve production level is the lack of an industrialised food to be physiologically and economically viable since to date the only efficient food is fresh flesh or live preys [26]. In meal production, raw material goes through aggressive thermal processes that induce protein denaturalisation, carbonylation, hydrophobicity and aggregation [27]. Octopus digestive enzymes are sensitive to these effects; therefore, they cannot hydrolyse their substrates, reducing food digestibility and octopus growth [28, 29].

Heat treatment to raw crab and squid meat to obtain meal ingredients to formulate a balanced diet is not traditionally preferred because this process denaturalises proteins, reduces digestibility and assimilation [28], and lipids can oxidise [30]. Experimental results suggested an enhancement of enzymatic function in *O. bimaculoides* promoted by HOM (PhA-SiT) treatment dissolved into culture water when food was not processed. HOM treatment seemed to have increased the digestive capability in juvenile octopus and the assimilability of processed food (35°C), but the loss of quality in crab and squid meals in 60°C food could not be compensated by the HOM treatment.

## 2.2. Crustaceans

### 2.2.1. White shrimp (*Litopenaus vannamei*)

The White shrimp *L. vannamei* is a species with rapid growth, high survival and price in the market, which makes this crustacean one of the most important resources at worldwide level.

Item.	HOM-60°C	60°C	HOM-35°C	35 °C	No HOM
W Day 1 (g)	0.11 ± 0.05	0.10 ± 0.03	0.10 ± 0.04	0.12 ± 0.04	0.12 ± 0.04
W Day 28 (g)	0.11 ± 0.06	0.11 ± 0.06	0.15 ± 0.06	0.20 ± 0.06	0.20 ± 0.07
Survival (%)	28	46	87	54	93

Data are presented as Mean value ± SD. W = Wet weight.

**Table 2.** *Octopus bimaculoides* juvenile growth and survival during food and HOM treatment assessment.

Nonetheless, the production of this important resource has been hindered by recurrent epizootic outbreaks and sudden mortalities caused by pathogen microorganisms. To face the challenge, several chemical and antibiotic products have usually been applied whose prophylactic application was initially an effective strategy. However, they have caused the development of resistant bacteria making it necessary to reduce their application. These problems have led the shrimp industry to explore and develop new and more strategies, as effective as or better than antibiotics, eco-friendly and with long-term sustainability. Previous studies have indicated that homoeopathic medicines stimulated the immune system and caused specific organic responses [31, 32]. Therefore, we evaluated the effect of homoeopathic medicines in growth and survival of *L. vannamei* postlarvae under controlled laboratory conditions (Trial 1); the survival and antioxidant response through superoxide dismutase (SOD) activity in juveniles when challenged ( $1 \times 10^6$  CFU ml<sup>-1</sup>) with a pathogenic strain of *V. parahaemolyticus* (CAIM-170) at CIBNOR, in a biosecurity laboratory (Trial 2); during the production of postlarvae in a commercial hatchery, focusing on dynamics of the bacterial populations (Trial 3); zootechnical (growth) results and gene expression (Trial 4); and growing-out to marketable size in a commercial farm (Trial 5).

**Trial 1.** To evaluate growth and survival of postlarvae, an experimental design was applied at CIBNOR with three HOM treatments: ViP-ViA (T1), PhA-SiT (T2), ViP-ViA + PhA-SiT (T3) and ET (T4) as control. HOM treatments were applied for 30 days, spraying liquid dynamisations in commercial pelleted food, administered *ad-libitum*. In general, the best results were obtained in T3 (T1 + T2), showing a clear synergy between T1 and T2 (Table 3).

**Trial 2.** To assess survival and SOD activity in juveniles, an experimental design with four HOM treatments: INM (T1), PaV (T2), INM-PaV (T3), ViT (T4) and NT (T5) was applied at CIBNOR. HOM treatments were applied to juveniles 7 days prior to challenge and 5 days during challenge. Liquid dynamisations were sprayed in commercial pelleted food, which was supplied *ad-libitum*, 7 days prior to and during challenge. At 70 h after the start of the challenge, SOD was determined in shrimp tissue. At the end of the challenge (120 h post-infection), the shrimp treated with T2, T3 and T4, exhibited significantly higher average survival ( $p < 0.05$ ) than the control group T5. Juveniles treated with T3 and T4 showed the highest

Parameter	Treatments			
	T1	T2	T3	T4
LI (cm)	4.64 ± 0.94 b	4.61 ± 0.94 a	3.92 ± 0.93 a	4.59 ± 0.70 a
WI (g)	2.82 ± 0.60 a	2.92 ± 0.65 b	3.15 ± 0.74 b	2.62 ± 0.53 a
DWI (g day <sup>-1</sup> )	0.018 ± 0.007 ab	0.02 ± 0.009 ab	0.052 ± 0.018 c	0.016 ± 0.007 a
GR	0.094 ± 0.020 a	0.097 ± 0.021 b	0.105 ± 0.024 c	0.087 ± 0.017 a
S (%)	99.30	97.33	99.33	98.00

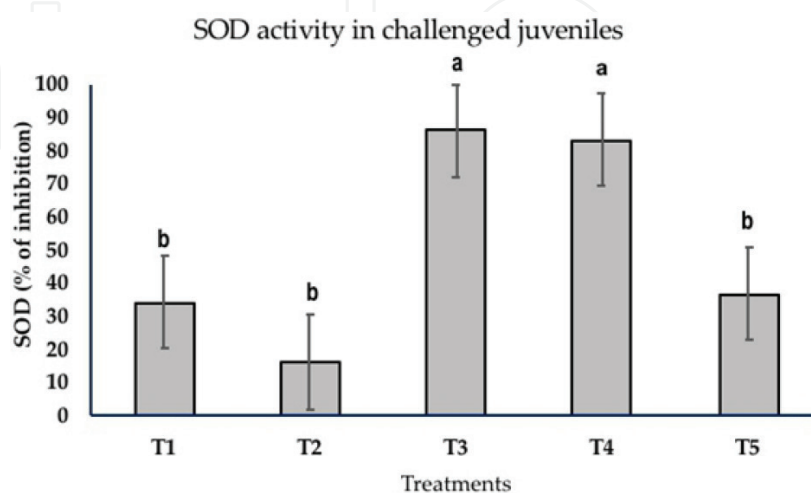
Different letters in the same column, indicate significant difference ( $p < 0.05$ ). Length increase (LI), weight increase (WI), daily weight increase (DWI), growth rate (GR), survival (S).

**Table 3.** Growth and survival of *Litopenaeus vannamei* postlarvae treated with homoeopathic treatments during an experimental assay at CIBNOR laboratory.

survival with 64.43 and 56%, respectively, while all those treated with T1 and T5 died. These results suggested that the greatest survival of HOM-treated shrimp could have been related to a stimulation of their immune system, and consequently, to a greater resistance to acute infectious diseases associated with the genus *Vibrio* [32]. Regarding SOD activity, at 70 h after the start of the challenge, the groups treated with T1 and T2 did not show significant differences ( $34.48 \pm 1.87$  and  $16.32 \pm 1.22$ , respectively) in relation to the control group ( $41.63 \pm 2.59$ ) while T3 and T4 with a SOD activity of ( $86.43 \pm 1.02$  and  $83.47 \pm 5.54$ , respectively) exhibited significantly higher values than the control group ( $p < 0.05$ ) ( $41.63 \pm 2.59$ ) (Figure 8).

Treatment T3 contained PaV used in human medicine as a tranquilliser to reduce stress and improve sleep, in addition to Heel-Mix (Rubiopharma®, Mexico) that contains medicines used for the treatment of enzymatic disorders, infectious diseases and stimulation of the body defences in human beings, whereas T4 is a homoeopathic medicine whose active principle is the venom of the scorpion *Rhopalurus junceus* which is an endemic species of Cuba. These results suggested a potential applicability of the homoeopathic medicines studied as prophylactic treatments to reduce stress and to improve shrimp immune response, which could help reduce the incidence of epizootic diseases and massive mortalities that have been a great problem for the industry due to economic losses associated with *V. parahaemolitycus* in shrimp culture.

**Trial 3.** A third experiment consisted of the evaluation of HOM treatments during the commercial production of *L. vannamei* postlarvae in the facilities of the commercial hatchery Aquacultura Mahr in square concrete tanks with a capacity of 20 t. An experimental design was applied with four tanks with three HOM treatments: MaP-CaP-Fep-Zip-PhA (T1) Hes-Sit-Cap-Pha (T2), INM-PaV-ViT-PhA (T3), and NT as the control group (T4). Liquid homoeopathic dynamisations were added to commercially pulverised food given periodically for a 24-h period (day-night). Shrimp farming has been affected by viral and bacterial diseases principally those associated to a highly virulent strain of *V. parahaemolyticus*; this problem is of primary interest concern [33], so this assay was intended to determine the population dynamics of the bacterial populations mainly *Vibrio* spp. Bacteriological examination of isolated vibrio species depend mainly on using TCBS agar as a selective media to differentiate

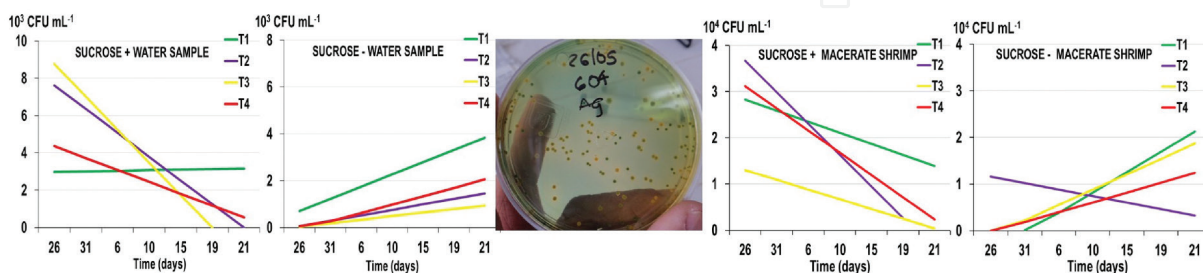


**Figure 8.** SOD activity in HOM-treated juvenile shrimp *Litopenaeus vannamei* treated with homoeopathy and then challenged with *Vibrio parahaemolitycus*.

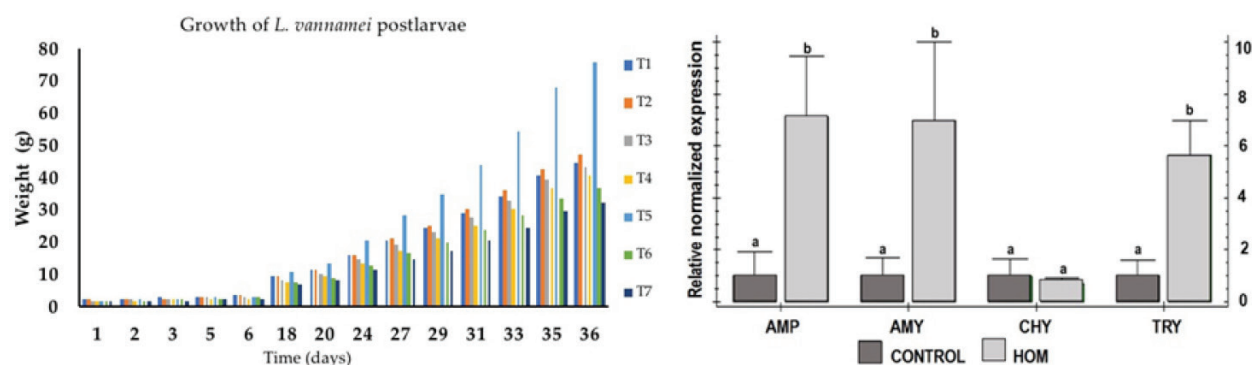
between sucrose and non-sucrose fermented colonies; *V. alginolyticus* is sucrose fermenter and shows yellow-coloured colonies while *V. parahaemolyticus* and *V. vulnificus* are non-sucrose fermenters and have green colonies [34]. Although no statistical significant ( $p > 0.05$ ) differences were found in HOM-treated and non-treated PLs, positive effects were apparently attained with HOM treatments (Figure 9). Research must continue not only with controlled and multi-replicate experimental designs in the laboratory but also efforts should continue in commercial hatcheries even if obvious difficulties exist because production is at most the first priority and not necessarily compatible with a strict and traditional scientific research.

**Trial 4.** A fourth assessment was made also at Aquacultura Mahr hatchery. An experimental design was applied in six concrete tanks (20 t) with five HOM treatments: BaC-INM, Sit-INM (T2), HeS-INM (T3), PhA-INM (T4), PaV-INM (T5), and two NT control groups (T6 and T7). Liquid homoeopathic dynamisations were provided with pulverised food as vehicle. Growth in weight of the shrimp postlarvae was exponential with a correlation coefficient ( $r^2$ )  $> 0.95$ . The best treatment was T5 (PaV-INM). The NT control group had the lowest growth rate and the lowest survival (20.2%), while in the HOM-treated groups, it was  $25.6 \pm 5.38\%$  (21–34.1%) (Figure 10, Left). At the end of the production cycle, gene expression analyses were made at CIBNOR to compare HOM-treated with non-treated postlarvae. The results of gene expression related to the activities of the aminopeptidase (AMP), amylase (AMY), chymotrypsin (CHY) and trypsin (TRY) enzymes showed clear and statistically significant differences ( $p < 0.05$ ;  $n = 30$ ) between the HOM-treated and NT postlarvae (Figure 10, Right). These results confirmed a positive impact of the use of homoeopathic medicines in the commercial production of *L. vannamei* postlarvae.

**Trial 5.** A fourth experiment consisted of the evaluation of HOM treatments during the mass cultivation of *L. vannamei* from postlarvae to adult size in commercial facilities of the company BCS Camarón, a commercial shrimp farm. The evaluation was performed in six earthen ponds of 10 ha each, initially seeded at a density of 8 PL  $m^{-2}$ . An experimental design was applied with six earthen ponds 10 ha, four ponds with HOM treatment and two NT ponds without homoeopathy: PhA-SiT (T1), PaV-ViT (T2), and NT as negative control group (T3). Liquid homoeopathic dynamisations were sprayed in commercially balanced food and applied in the culture ponds, each treatment component on alternate days for 130 days. Three samplings were made: after seeding and 7 days for acclimatisation ( $t_1$ ); after 52 days ( $t_{52}$ ) and after 130 days post seeding ( $t_{130}$ ). Growth parameters and biomass production as body weight (BW), total length (TL) and weight gain average (WGA) were determined. As physiological health indicators, the hepatopancreatic coverage index (HCI) was



**Figure 9.** Straight regression showing the increasing trends in the bacterial count of *Vibrio* spp. in culture seawater and macerate of *Litopenaeus vannamei* postlarvae. Petri dish with TCBS culture medium, which was previously inoculated, showing the presence of sucrose positive (yellow) and sucrose negative (green) colonies of the genus *Vibrio*.



**Figure 10.** Growth in weight (left) and gene expression (right) in *Litopenaeus vannamei* postlarvae or adult treated and with homoeopathic medicines in a commercial shrimp hatchery (Acuacultura Mahr). Aminopeptidase (AMP), amylase (AMY), chymotrypsin (CHY), trypsin (TRY).

evaluated as a morpho-histological index variable [HCI (%) = hepatopancreatic coverage area/cephalothorax coverage area  $\times$  100]. From an initial time characterised by no significant differences in BW and TL after 130 days of culture both HOM (T1 and T2) produced the best growth results in BW, TL and WGA compared to NT ponds (**Table 4**).

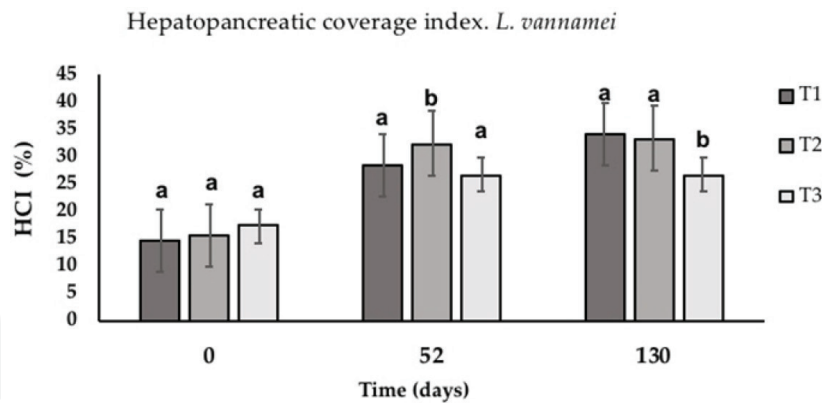
The shrimp treated with PaV-VIT after 52 days of treatment (T1) achieved the highest HCI ( $32.32 \pm 0.61\%$ ) and differed from PhA – SIT and un-treated control ponds. ( $28.34 \pm 0.87\%$ ;  $26.58 \pm 0.64\%$ , respectively). At the end of the experiment (130 days), both homoeopathic treatments showed better HCI; in relation to the negative control group (PhA – SIT =  $33.87 \pm 1.02\%$ ; PaV – VIT =  $33.31 \pm 0.77\%$  control T3 =  $26.54 \pm 0.56\%$ ) as shown in **Figure 11**.

The positive effect of the HOM treatments evaluated in growth and morpho-histological index of *L. vannamei* could be attributable to smaller micelle and higher activity in water with

Growth variables	Experimental groups (Two 10 h ponds each)		
	T1	T3	T2
BW T <sub>0</sub> (7 days)	$2.47 \pm 0.06^a$	$2.43 \pm 0.06^a$	$2.52 \pm 0.07^a$
BW T <sub>1</sub> (52 days)	$16.79 \pm 0.22^a$	$16.02 \pm 0.19^a$	$18.83 \pm 0.21^b$
BW T <sub>2</sub> (130 days)	$20.08 \pm 0.18^a$	$18.59 \pm 0.17^b$	$23.90 \pm 0.29^c$
TL T <sub>0</sub> (7 days)	$7.13 \pm 0.05^a$	$7.08 \pm 0.05^a$	$7.06 \pm 0.06^a$
TL T <sub>1</sub> (52 days)	$13.89 \pm 0.05^a$	$13.71 \pm 0.06^a$	$14.31 \pm 0.06^b$
TL T <sub>2</sub> (130 days)	$15.02 \pm 0.05^a$	$14.66 \pm 0.05^b$	$15.81 \pm 0.06^c$
WGA T <sub>1</sub> (52 days)	$14.37 \pm 0.27^{ab}$	$13.59 \pm 0.05^a$	$15.72 \pm 0.78^b$
WGA T <sub>2</sub> (130 days)	$17.58 \pm 0.26^a$	$16.41 \pm 0.14^b$	$21.08 \pm 0.64^c$

Body weight (BW), Total length (TL), weight gain average (WGA). Values within the same row with different letters represent significant differences ( $p < 0.05$ ).

**Table 4.** Growth of *Litopenaeus vannamei* treated with homoeopathy while cultured in semi-intensive system in a commercial shrimp farm (BCS Camarón Farm; six earthen ponds, 10 ha).



**Figure 11.** Hepatopancreatic coverage index (HCI %) of adult shrimp in a commercial shrimp farm (earth ponds 10 h) treated with homoeopathic medicines.

nanoparticle content [35]. The presence of nanoparticles has been demonstrated in highly homoeopathic ultra-diluted medicines [8]. On the other hand, the same homoeopathic medicines used in shrimp ponds, (PaV, PhA, SIT and ViT) outperformed antibiotics in juvenile scallop *A. ventricosus* [23] These results enhanced the evidences that aquacultural homoeopathy has applicability in shrimp commercial culture to improve the productivity of the shrimp industry.

### 3. Conclusion

One of the most promising and novel fields that strengthen the immune system in marine organisms is the use of immunostimulants that are natural compounds modulating the immune system and increasing resistance of the host against disease mainly those caused by bacteria [18]. Regarding immunostimulants, homoeopathy has been proposed as a novel alternative in aquaculture practices to improve health and strengthen the organism's immune response [5, 23]. The production of marine mollusc, shrimp and fish around the world still challenged every year by the propagation and emergence of new diseases, mainly those related to viruses and bacteria such as *Vibrio spp.* and *Aeromonas spp.*, which are treated with conventional methods as antibiotics [2, 14]. The findings in our research suggested that homoeopathic medicines have a great potential to increase health and performance in marine mollusc including bivalves and octopus, shrimp and marine fish.

Findings in the scallop *A. ventricosus* support the fact that homoeopathic medicines do not act directly over the disease-cause *per-se*, killing the bacteria or removing the stressing agent but enhancing the capacity of the HOM-treated organisms to resist the infection or overpass stressful conditions. Some results in molluscs have shown a greater effect of homoeopathic medicines on survival than on growth of larvae and seeds. However, it is necessary to consider that greater survival implies maintaining a higher density in larval culture and that not only larvae of larger size are able to settle and become marketable seeds with a good performance in the field. From a commercial point of view, it is more important to attain bigger biomass of successfully setting larvae even if small sized because independently of their

size, all larvae are capable and competent for setting process and seed sales. This work has also contributed to the knowledge of octopus for aquaculture purposes since this organism is an important fishery in Mexico with precocious development and high growth rates. For this and all other species, homoeopathic medicines are not intended as food additives; on the contrary, they contribute to acquire a better internal homeostasis, and as a consequence, a better digestive enzyme function and nutrition, and an enhanced immune system, despite of lacking differences in growth parameters. Regarding fish culture, this industry is increasing with time in Mexico but especially marine fish, which represents a great opportunity to improve fish culture by using homoeopathy; as it was demonstrated in this work, it participates at diverse developmental stages, enhancing fish health and growth performance.

To date, overall results are positive and suggest that homoeopathy is a natural, viable and eco-friendly treatment to reduce the use of disinfectants and chemotherapeutics, including antibiotics, in mollusc, shrimp and marine fish industries, to reduce stress, improve nutrition and immune response, to increase their resistance to any of the various pathogenic strains of bacteria and viruses that have come to hatcheries and farms and will continue to reach them worldwide. Future experiments are being planned at CIBNOR to elucidate the role that homoeopathic medications could play in these organisms.

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