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Efficacy of natural products and antibiotics in shrimp hatcheries

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Only three natural products effective to control most pathogenic *Vibrio* strains



Shrimp producers can apply relatively simple in vitro analyses, like the ones used in this study, to help make adequate management decisions to reduce the impact of bacterial diseases and increase profits. Photo by Darryl Jory.

The high demand of postlarvae to support the cultured shrimp industry has increased the occurrence of infectious pathogens in this production stage. One of the main concerns in shrimp hatcheries are the bacterial pathogens. *Vibrio* spp, such as *Vibrio harveyi*, *Vibrio alginolyticus* and *Vibrio campbellii* are recurrent pathogens in shrimp hatcheries in America and Asia.

In Ecuador, shrimp hatcheries have suffered from some bacterial diseases caused by pathogens of the *Vibrio* genus, such as Bolitas nigricans syndrome, caused by *V. harveyi*, and Zoa 2 syndrome, caused by *V. harveyi* and *V. alginolyticus*. Therefore, the efficiency of therapeutic products is of vital importance for the control of aquaculture diseases.

Antibiotics (<https://www.aquaculturealliance.org/advocate/researchers-examine-antimicrobial-resistance-potential-aquaculture/>), are extensively used as prophylactics against bacterial pathogens. However, the use of antibiotics carries important disadvantages, these being residues in aquaculture products, development and propagation of resistance between pathogens, including human pathogens. For these reasons, the regulation of antibiotics is rigorously controlled, resulting in few antibiotics authorized for use in aquaculture. In this context, alternative strategies of disease control are necessary to replace antibiotics for use in animal production, which has led to consider the use of natural products to control the growth of pathogens in shrimp hatcheries.

The administration of **probiotics** (<https://www.aquaculturealliance.org/advocate/assessment-supplemental-bacillus-probiotics-whiteleg-shrimp-juveniles/>), is one of the alternative strategies that may be used in aquaculture; their benefits include the potential for colonization in the gastrointestinal tract, selective antagonism against bacterial pathogens, improvement of the shrimp immune system, enhanced shrimp growth and survival, degradation of detritus and maintenance of water quality.



(<https://link.chtbl.com/aquapod>).

The use of organic acids, produced by organisms and used as preservatives and bacterial control in food, agriculture and animal production, is another potential strategy to control bacterial diseases in animal production. Similarly, essential oils have shown to have antimicrobial, antioxidant and antifungal properties, which can be an alternative to the use of additives and drugs in shrimp production. Although the use of natural products like organic acids and essential oils in Ecuadorian hatcheries is relatively new, there is an increasing demand for their application as control strategies of bacterial diseases in shrimp hatcheries.

In general, there are a huge number of products marketed as therapeutic products for shrimp hatcheries worldwide; therefore, producers should take suitable decisions as to which products are effective based on technical information and further tests in their own facilities.

This article – summarized and adapted from the original publication (<https://doi.org/10.1371/journal.pone.0210478> (<https://doi.org/10.1371/journal.pone.0210478>)) – reports on a study with the main objective to determine, through *in vitro* analyses, the antimicrobial effectiveness of antibiotics and some commercial products used in Ecuador as therapeutic agents for shrimp larviculture. To determine this, we first performed a survey to identify the pathogenic bacterial strains circulating in Ecuadorian shrimp hatcheries, confirming their virulence through challenge tests and verifying their molecular similarity with previously reported pathogenic *Vibrio*. Then, we tested the antimicrobial effectivity of some antibiotics and commercial products used in Ecuador as therapeutics against the pathogenic circulating strains through *in vitro* tests.

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Study setup

Thirty-one samples of *Litopenaeus vannamei* larvae [from Nauplii 5 (N5) to 13 days postlarvae (PL13)] were collected from tanks of 10 shrimp hatcheries in Santa Elena, Ecuador, during mortality events. Samples were sent by farmers to the National Center for Aquaculture and Marine Research (Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM, Santa Elena, Ecuador) for the quantification of shrimp bacterial load (microbiologic analysis services performed by CENAIM). Bacterial strains isolated from these samples were used for this study. Larvae presented clinical signs of abnormal swimming behavior, empty digestive tract, low activity and retardation of larval development.

Bacterial strains were isolated and preserved, and challenge tests performed to evaluate the pathogenicity of the presumptive pathogenic strains in brine shrimp. Bacterial strains causing higher mortalities in the artemia challenge test were selected and their pathogenicity was again verified by a challenge test using healthy *vannamei* postlarvae. Then, the presumptive pathogenic strains were identified by 16S rRNA sequence analysis.

The antimicrobial effectivity of 16 natural products (five probiotics, nine organic acids and two essential oils) used in Ecuador as therapeutic agents against shrimp bacterial diseases and eleven antibiotics was screened in terms of the susceptibility of the pathogenic circulating strains to these products through antibiogram and minimal inhibitory concentration tests. We denominated as pathogenic circulating strains those strains that caused high mortality (>50 percent) in the artemia and shrimp postlarvae challenge tests and presented molecular similarity to species previously reported as *Vibrio* pathogens.

For detailed information on sample collection and processing; isolation and preservation of bacterial strains; challenge tests; bacterial characterization by 16S rRNA sequence analysis; antimicrobial effectiveness; susceptibility of pathogenic *Vibrio* strains to antibiotics; susceptibility of pathogenic *Vibrio* strains to probiotics; cell toxicity of selected products; and data analysis, please refer to the original publication.

Results and discussion

The efficiency of therapeutic products is of vital importance for the control of aquaculture diseases. In the present study, we investigate through some in vitro analyses the antimicrobial effectiveness of antibiotics and commercially available therapeutic products used in Ecuador to control pathogenic bacterial strains of shrimp larvae. To perform these analyses, we isolated the strains that were circulating in the shrimp hatcheries, verified their virulence through

challenge tests and identified their molecular similarity with previously reported pathogenic *Vibrio*. By doing this, we confirmed that we were working with the circulating strains that cause real problems at the production level. The results were dependent on the product, concentration of the product and bacterial strain.

Sotomayor, probiotics, Table 1

Product code	Declared composition	Declared dosage/dosage used by produceres	Presentation/form	Manufactured in
P1	Probiotic microorganisms: total aerobesa. Concentration: > 4 × 10 ⁹ CFU g ⁻¹	2-10 µg mL ⁻¹	Powder	USA
P2	Probiotic microorganisms: total aerobesa. Concentration: 2 × 10 ⁹ CFU g ⁻¹	5 µg mL ⁻¹	Powder	USA
P3	Strains of <i>Bacillus subtilis</i> , <i>Bacillus licheniformis</i> and <i>Bacillus pumilus</i> . Concentration: minimum 2 × 10 ¹⁰ CFU g ⁻¹	1 to 5 g kg ⁻¹	Powder	USA
P4	Mixture of strains of <i>Bacillus</i> spp. Concentration: 5 × 10 ¹⁰ CFU g ⁻¹	100-200 g ha ⁻¹	Powder	USA
P5	<i>Vibrio alginolyticus</i> . Concentration: 1 × 10 ⁸ CFU mL ⁻¹	10 mL t ⁻¹	Liquid	Ecuador

OA1	Calcium formate, calcium propionate, premix carvacrol and thymol, premix allicin,	1-7 kg t-1 of feed	Powder	Ecuador
OA2	Formic acid, propionic acid, ammonium formate, acetic acid, silic acid and vermiculite	0.6 kg t-1 of feed	Powder	Austria
OA3	Calcium propionate 16%, calcium formate 18% and calcium carbonate 66%	1-2 kg t-1	Powder	Ecuador
OA4	Propionic acid 25%, formic acid 25% and formaldehyde 15%	1-3 kg ha-1	Powder	Ecuador
OA5	Formic acid, and its salts, mixture of flavors (essences and plant extracts: <i>Allium sativum</i> , <i>Origanum vulgare</i> , <i>Cinnamomum zeylanicum</i> , <i>Eugenia caryophyllata</i>), propionic acid and its salts, citric acid, malic acid, anti-caking agent	2-3 kg t-1 of feed	Powder	Spain
OA6	Lactic acid 23%, fumaric acid 20%, citric acid 20%, malic acid 25% and succinic acid 10%	2-4 µg mL-1	Powder	Ecuador
OA7	Acid formic 35.4%, formate 34.6% and potassium 30.0%	2-5 kg t-1 of feed	Powder	Germany
OA8	Formaldehyde 35%: 28.6%, propionic acid 10%, bentonite 39% and silicic acid 22.4%	1 kg t-1 of feed	Powder	Spain
OA9	Mixture of short chain organic acids, acetic acid, propionic acid, formic acid and formaldehyde	0.5-2 kg t-1 of feed	Powder	Spain
E01	Oregano oil extract	1-5 mL t-1	Liquid	USA
E02	Highly concentrated mix of essential oils	1-10 mL t-1	Liquid	Spain

Table 1. Description of the products marketed in Ecuador as therapeutic agents against shrimp bacterial disease. Probiotics: P1, P2, P3, P4 and P5; Organic acids: OA1, OA2, OA3, OA4, OA5, OA6, OA7, OA8 and OA9. Essential oils: E01 and E02. a Specific bacterial strain are not declared in the product. <https://doi.org/10.1371/journal.pone.0210478.t001>

Antibiotics were the most efficient therapeutic agents against the growth of pathogenic bacteria. Eight of the antibiotics inhibited the growth of all or most of the pathogenic bacterial strains, but most of these products are not authorized for use in aquaculture. We included several antibiotics in our evaluation because we wanted to investigate if the pathogenic circulating strains exhibited patterns of

multiple antibiotic resistance, which could be associated with antimicrobial use. In our study, the multiple antibiotic resistance, (MAR), index was on average 0.23, showing some level of resistance to antibiotics.

High antibiotic resistance has been found in hatcheries worldwide, as well as higher MAR indexes in hatcheries rather than in shrimp farms. For instance, MAR index ranges from 0.21 to 0.38 have been reported for bacteria isolated from shrimp hatcheries, whereas, MAR indexes in shrimp farms range from 0.11 and 0.32. The average MAR index determined in this study is low compared to values reported in other shrimp hatcheries. However, all strains were resistant at the same time to penicillin and oxytetracycline, both antibiotics used in human medicine. Most of the sampled hatcheries are in a region of multiple anthropogenic activities, without wastewater treatment, which could be a source of antibiotic pollution.

Producers must consider alternative strategies for the control of pathogenic bacteria. In our study, only one commercial probiotic (P5) exhibited a high antagonistic capacity against the bacterial strains (85 percent of the strains). The rest of the probiotics could inhibit the growth of 15 to 30 percent of the strains, which showed intermediate effects, and therefore could be considered as functional for the growth control of some pathogenic bacterial strains. Of the two strains that were not completely inhibited by probiotic P5, neither inhibited the other probiotics.

Only one organic acid (OA9) showed inhibition of growth of most of the strains, at low concentrations. This product is a mixture of acetic acid, propionic acid and formic acid. These acids, as well as butyric acid, are efficient for the control of aquatic and shrimp pathogenic *Vibrio*. OA6 was the second most efficient organic acid, and contains lactic, fumaric, citric, malic and succinic acids.

OA4 contains propionic acid and formic acid, whereas OA7 contains formic acid. Three of these four organic acids contain formic acid, which is considered to be particularly effective against pathogenic *Vibrio*. In addition, OA9 contains three of the four organic acids reported as good bacterial inhibitors, including acetic acid, which is a good disinfectant of *V. parahaemolyticus*. Possibly the five organic acids whose MIC were not determined, could be effective at higher concentrations than tested in this study, which makes them inefficient products.

Essential oils are effective for inhibiting bacterial growth; in our study the essential oil EO1, whose declared composition includes extract of oregano oil, efficiently inhibited the growth of all evaluated bacterial strains, with MIC values equal to or lower than 3 mg per liter. The efficacy for the bacterial inhibition of EO1 might be related to the presence of thymol and carvacrol, two of the compounds of oregano essential oil that decrease the bacterial counts of *V. vulnificus*, *V. parahaemolyticus* and *V. cholera* in muscle and hepatopancreas of juvenile *P. vannamei*.

Other authors have mentioned the ability of essential oils to interrupt bacterial communication, decreasing bacterial virulence and pathogenicity. Therefore, it would be advisable to evaluate these properties of oregano essential oils and at the same time its toxic effect.

Perspectives

The tests performed in this work are designed to analyze whether commercially available natural products inhibit the growth or kill the pathogenic bacteria, but the natural products evaluated in this work could exhibit other modes of action not studied in this work. Therefore, further studies will be necessary to evaluate their efficacies, in terms of others mode of action, such as: capacity to disrupt bacterial communication, improvement of the shrimp immune system, colonization of the gastrointestinal tract, enhanced shrimp growth and survival, among others.

Ninety-five percent of the isolated strains (19 of 20) belong to the *Harveyi* clade, known to be the pathogenic clade for shrimp. This was consistent with the results of the challenge tests, thus verifying that the isolated strains were pathogenic. Most of the strains were identified as *V. harveyi* and *V. alginolyticus* (12/20 strains), which have been a continual problem for the Ecuadorian hatcheries since 1988–1989.

This study, however, has identified the fact that new pathogenic strains have appeared (*V. campbellii*, *V. owensii*, *V. inhibens* and *V. natriegens*) in the Ecuadorian hatcheries, diversifying the circulating bacteria and making it crucial to study the effectiveness of treatments for each pathogenic strain. Periodic surveys at regional level and further challenge tests could be performed to identify the pathogenic circulating bacterial strains and to focus on the bacteria of concern.

At the same time, shrimp producers can apply relatively simple *in vitro* and *in vivo* analyses, such as those employed in this study and take adequate management decisions based on these results, which in turn could reduce the impact of bacterial diseases and increase profits.

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