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Biocontrol of the vibriosis in the white shrimp (*Litopenaeus vannamei*) using organic acids in the feeding.

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ABSTRACT

In this research 3 formulations were used; pellets were mixed with lactic acid, formic acid, citric acid, sorbic acid and aquaculture binder (M1), pellets were mixed with citric acid, fumaric acid, sorbic acid and aquaculture binder (M2), and pellets were mixed with formic acid (M3). Infected shrimps (*Litopenaeus vannamei*) with Vibriosis were fed on 200 g of pellets mixed with the 3 formulations twice daily for 5 days. Using the mixture 1 (M1) was obtained the lowest mortality of 10.00%, highest percent survival index of 80.00% and also the highest weight of 54.00 g, whereas using the mixture Control (only pellets) was obtained the highest mortality of 50.00%, lowest percent survival index of 0.00% and also the highest weight of 43.00 g. In addition, differences were found between the results of the 3 mixtures tested, infected *Litopenaeus vannamei* with *Vibrios* using the mixture 1 (M1) did not present symptoms of Vibriosis after of the biocontrol test. The mixture 1 (M1) showed the highest content of protein and ash being of 344.20 and 145.70 g.Kg^{-1} respectively. The results showed the use of organic acids is directly related to the control of Vibriosis and the improvement in the growth of juvenile white shrimp *Litopenaeus vannamei*.

INTRODUCTION

Shrimp culture in Ecuador started at the end of 1960´s, between 1968 and 1998 about 180000 ha of land was converted to shrimp farms (**Sonnenholzner and Boyd, 2000; Valenzuela-Cobos and Vargas-Farías, 2020**). The shrimp is the second non-oil product of economic importance, the aquaculture has an average annual growth rate of 8.6% (**Rivera *et al.*, 2018**). The causes of the major economic losses in the shrimp industry are the result of Vibriosis, a disease that kills shrimp due to the bacterial infection caused by the *Vibrio* species (**Adams and Boopathy, 2013**). *Vibrio* spp. are among the most important bacterial pathogens of cultured shrimp responsible for a number of diseases, and mortalities up to 100% have been reported due to Vibriosis (**Lightner, 1983**). For all these reasons, the shrimp industrial field has development different strategies to control the Vibriosis such as: the use of antibiotics and probiotics to combat fish and shrimp pathogens (**Chythanya *et al*., 2002**).

These methods present different disadvantages: antibiotics use in aquaculture may be detrimental to the environment and human health, because them promote the development and transfer of resistance to other bacteria, including human and fish pathogens (**Perez-Sánchez *et al*., 2014**). The application of antibiotics is a partially effective strategy for disease management (**Harikrishnan *et al*., 2011**), the use of antibiotic in shrimp aquaculture is banned in USA (**Graslund and Bengtsson, 2001; Le and Munekage, 2004; Lyle-Fritch *et al*., 2006**). Among the constrains of probiotics are: improper application in host sometime may be negatively treated, probiotics work slowly, advance technology required to prepare probiotics which are the main challenge (Wang et al., 2008). Studies have presented that the use of organic acids like: formic acid is an effective bactericide of Salmonella and various species of *Vibrio*, including *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, and *V. cholerae* (**Adams and Boopathy, 2013**).

Organic acids can be used as growth promoters in aquaculture, aiding in pathogen inhibition in the intestinal tract, and improving the digestibility of dietary nutrients, such as nitrogen and phosphorus (**da Silva *et al*., 2013**). Organic acids and their salts can also contribute in nutritional ways, because they are components in several metabolic pathways for energy generation, for instance, for ATP generation in the citric acid cycle or carboxylic-acids cycle (**Lückstädt, 2008**). However, studies of marine shrimp fed with organic acids or their salts are limited, only some specific acids or salts have been studied such as: formic acid, butyrate, lactate, citrate and potassium diformate (**Anuta *et al.*, 2011; da Silva *et al*., 2016**).

The aim of this study was to determine the effects in the supplementation of mixture of organics acids (fumaric acid, ascorbic acid, citric acid, lactic acid, formic acid) in different dietary inclusions to control the Vibriosis and also improve in the growth of juvenile white shrimp *Litopenaeus vannamei*.

MATERIALS AND METHODS

Composition of the organic acids

Organic acids 1 (VIBRO PLUS): The composition of the mixture is 46% lactic acid, 18% formic acid, 18% citric acid and 18% sorbic acid.

Organic acids 2 (VIBRO): This mixture is composed by: 40% citric acid, 40% fumaric acid and 20% sorbic acid.

Organic acids 3 (FORMIC): 100% formic acid.

The organic acids were realized at the Research and Development Laboratory of Ecuahidrolizados Industry.

Organoleptic and microbiological parameters of the organic acids

Among the organoleptic parameters determined were: color and texture. The microbiological parameters evaluated were: aerobic mesophilics, yeasts and molds (**AOAC, 2005; Valenzuela-Cobos *et al*., 2020a; Valenzuela-Cobos *et al*., 2020b**).

Preparation of the mixtures

Mixture 1 (M1): 25 kg of pellets were mixed with 2 L of solution S1. Solution 1= To dissolve 250 g of VIBRO PLUS and 200 mL of AQUAPEGA ATUN "LA" on 2 L of water.

Mixture 2 (M2): 25 kg of pellets were mixed with 2 L of solution S2. Solution S2= Dissolving 250 g of VIBRO and 200 mL of AQUAPEGA ATUN "LA" on 2 L of water.

Mixture 3 (M3): 25 kg of pellets were mixed with 2 L of solution S3. Solution S3= Dissolving 250 g of FORMIC and 200 mL of AQUAPEGA ATUN "LA" on 2 L of water.

In addition the shrimps were fed with only pellets (Control). The aquaculture binder AQUAPEGA ATUN "LA" was obtained by (**Valenzuela-Cobos and Vargas-Farías, 2020**). The experimentation was realized at the Shrimp Farm "La Chorrera".

Chemical composition of the mixtures

The mixtures were dried at 60 °C for 24 h and then milled to perform proximal analysis using standard methods. Moisture, ash and crude fat were determined according to the Association of Official Analytical Chemists methods (**AOAC, 2005**). Total nitrogen was evaluated with the micro-kjeldahl method, crude protein was calculated from total nitrogen content by employing the converting factor 6.25, and energy value was estimated according to modified methodology of (**Manzi *et al*., 2004; Manilal *et al*., 2012; Valencia del Toro *et al*., 2018; Valenzuela-Cobos *et al*., 2019**).

Test of biocontrol of Vibriosis

The biocontrol tests were realized using modified methodology of (**Manilal *et al*., 2012**) and were used juvenile shrimps (8 g of weight). Ten shrimps with presence of Vibriosis were treated with 200 g of the mixture 1 twice daily during 5 consecutive days (G1). The second group (G2) was performed for 5 consecutive days, ten shrimps with clinical symptoms of Vibriosis were treated with 200 g of the mixture 2 twice daily. Third group (G3), ten shrimps with incidence of Vibriosis were treated with 200 g of the mixture 3 twice daily during 5 days. Additionally, ten shrimps with manifestation of Vibriosis did not receive treatment, only were fed with 200 g of pellets twice daily during 5 days (Control). Final mortality was evaluated after of the biocontrol test, see Eq. (1)

Eq. (1) **Mortality (%)=**(No of dead shrimps-A)/(Total No of shrimps-A) x 100

where: A= represents the number of dead shrimps on the first day after biocontrol test. Additionally was calculated the percent of survival index (PSI), see Eq. (2)

Eq. (2) **Percent Survival Index (%)=**[1-((% mortality in treated group)/(% mortality in control group))] x 100

To determinate the presence of Vibriosis (PV) after of the biocontrol test was used the following equation, see Eq. (3)

Eq. (3) **Presence of Vibriosis (%)=**((Shrimps with vibriosis)/(Survival shrimps after test)) x 100

Test of growth of shrimps

To determine growth, 4 survival shrimps were randomly taken after the biocontrol test and weighed.

Statistical analysis

In all experiments, a completely randomized design and the results were examined using one-way analysis of variance (ANOVA) to determine the significance of individual differences at $p<0.05$ level, of the microbiological parameters of the organic acids, the chemical composition of the mixtures, the mortality, the percent survival index of the shrimps, the presence of Vibriosis and the growth juvenile white shrimp *Litopenaeus vannamei* after using the mixtures in the biocontrol test, when statistical differences were found, the Duncan Test with $\alpha = 0.05$ was applied. The analyses were carried out using statistical software (Statgraphic ver. 16).

RESULTS

Organoleptic and microbiological parameters of the organic acids

The organoleptic and microbiological parameters of organic acids are indicated in the Table 1. The VIBRO PLUS composed by 46% lactic acid, 18% formic acid, 18% citric acid and 18% sorbic acid presented texture liquid with suspensions and clear white coloration, the same color and texture were presented by FORMIC. VIBRO composed by 40% citric acid, 40% fumaric acid and 20% sorbic acid showed texture solid with fine particles and white coloration.

On the other hand, the three mixtures of organic acids showed similar microbiological parameters: aerobic mesophilic values of 1.00×10^0 UFC/g, yeasts and molds values $<1.00 \times 10^1$ UP/g. Diets with salts of organic acids improve feed extrusion, increase stability, and reduce the expansion of the pellet (**Morken *et al.*, 2012**). The use of organic acids (citric and lactic acids) has attractive effects and palatability (**Xie *et al.*, 2003**).

Table 1. Organoleptic and microbiological parameters of organic acids

Organic Acids	Color	Texture	Aerobic mesophilic (UFC/g)	Yeasts and molds (UP/g)
VIBRO PLUS	Clear white	Liquid with suspensions	1.00×10^{0a}	$<1.00 \times 10^{1a}$
VIBRO	White	Solid with fine particles	1.00×10^{0a}	$<1.00 \times 10^{1a}$
FORMIC	Clear white	Liquid with suspensions	1.00×10^{0a}	$<1.00 \times 10^{1a}$

*Different letters in each column indicated significant difference among the presence of aerobic mesophilic, yeasts and molds of organic acids at level p < 0:05, according to Duncan's test, n = 3.

Chemical composition of the mixtures

Chemical composition of the mixtures based on addition organic acids is presented in Table 2. The mixture 1 showed the highest content of protein (344.20 g.Kg^{-1}), whereas the mixture (Control) presented the lowest protein content (335.80 g.Kg^{-1}). For otherwise, the highest fat content was presented by the mixture 2 (120.40 g.Kg^{-1}) and mixture 3 (102.50 g.Kg^{-1}), while the mixture (Control) presented the lowest content of fat (99.10 g.Kg^{-1}). Mixture 1 showed the highest ash content (145.70 g.Kg^{-1}), whereas the mixture (Control) presented the lowest ash content (142.50 g.Kg^{-1}). The highest content of moisture was presented by mixture (Control) being of 8.60%, where the mixture 2 presented the lowest moisture (7.10 %). The highest value of energy was presented by the mixture 1 (4368.00 cal.g^{-1}) and the mixture 2 (4364.00 cal.g^{-1}), while the lowest value of energy was presented by the mixture (Control) (4272.00 cal.g^{-1}). Diets supplemented with 3% citric acid exhibited an increase in protein and phosphorus digestibility (**Khajepour and Hosseini, 2012**). The addition of 1% sodium formate (NADF) in the diet increased the digestibility of lipids, ash, and proteins, including all essential and nonessential amino acids except phenylalanine (**Morken *et al.*, 2011**). The mixture 1 showed the highest protein content, the mixture 1 and 2 presented the highest energy value. Mixture Control presented the lowest content of fat, ash and protein providing the lowest energy value.

Table 2. Proximate composition of the mixtures used in the feeding of infected *Litopenaeus vannamei* with Vibriosis.

Mixtures	Moisture (%)	Ash (g.Kg^{-1})	Fat (g.Kg^{-1})	Protein (g.Kg^{-1})	Energy value (cal.g^{-1})
Control	8.60±0.02^{a}	142.50±3.46^{d}	99.10±1.85^{c}	335.80±2.93^{d}	4272.00±4.15^{c}
M1	7.60±0.01^{b}	145.70±1.13^{a}	100.90±1.03^{b}	344.20±2.41^{a}	4368.00±3.96^{a}
M2	7.10±0.07^{c}	144.80±3.48^{b}	102.40±2.40^{a}	340.70±0.64^{b}	4364.00±5.37^{a}
M3	7.40±0.05^{b}	143.60±2.17^{c}	102.50±0.86^{a}	338.50±0.75^{c}	4332.00±4.19^{b}

*Control: only pellets, mixture 1 (M1): pellets were mixed with 2 L of solution S1, mixture 2 (M2): pellets were mixed with 2 L of solution S2, and mixture 3 (M3): pellets were mixed with 2 L of solution S3.

*Solution S1= to dissolve 250 g of VIBRO PLUS and 200 mL of AQUAPEGA ATUN “LA” on 2 L of water, solution S2= dissolving 250 g of VIBRO and 200 mL of AQUAPEGA ATUN “LA” on 2 L of water, and solution S3= dissolving 250 g of FORMIC and 200 mL of AQUAPEGA ATUN “LA” on 2 L of water.

* Different letters in each column indicated significant difference among the chemical composition of the mixtures used in the feeding of infected *Litopenaeus vannamei* with Vibriosis at level $P<0.05$, according to Duncan's test, n = 10.

Effect of mixtures based on acid organics in infected *Litopenaeus vannamei* with Vibriosis

Table 3 shows the effect of mixtures based on acid organics in infected juvenile shrimps with presence of Vibriosis after using the biocontrol test.

The shrimps after biocontrol test by using the mixture (Control) presented the highest mortality being of 50.00%, while the shrimps by using the mixture 1 showed the lowest mortality being of 10.00%. *Litopenaeus vannamei* after biocontrol test by using the mixture 1 exhibited the highest percent survival index being of 80.00%, whereas shrimps by using the mixture (Control) presented the lowest percent survival index being of 0.00%. *L. vannamei* treated with hot-water extract of *Gracilaria tenuistipitata* via injection displayed resistance against *V. alginolyticus* (**Hou and Chen, 2005**). *Vibrios* are continued to be a significant cause of mortality in shrimp aquaculture, and they are frequent isolates causing Vibriosis (**Manilal *et al*., 2012**). Studies have verified that organic acids such as: acetic, butyric, propionic, formic and valeric acids that can be used in the inhibition of *Vibrios* (**Defoirdt *et al*., 2006; da silva *et al*., 2013**).

Litopenaeus vannamei (4 survival shrimps) after biocontrol test using the mixture 1 showed highest weight being of 51.00 g, whereas 4 survival shrimps after biocontrol test by using the mixture (Control) exhibited the lowest weight being of 43.00 g. Shrimps infected with Vibrios using the mixture 1 did not show presence of Vibriosis after biocontrol test. The oral administration with medicated feed provided complete recovery of clinical signs in infected shrimp with *Vibrio* species (**Manilal *et al*., 2009**). The success of the mixture 1 may be due to the sufficient administration and bioavailability.

Table 3. Efficacy of medicated feed on infected *Litopenaeus vannamei* with Vibriosis.

Treatments	M (%)	PSI (%)	Presence of vibriosis (%)	Weight (g)
Control	50.00^{a}	0.00^{c}	100.00^{a}	43.00 ± 2.16^{d}
G1	10.00^{c}	80.00^{a}	0.00^{d}	54.00 ± 1.95^{a}
G2	20.00^{b}	60.00^{b}	25.00^{b}	51.00 ± 0.64^{b}
G3	22.00^{b}	56.00^{b}	43.00^{c}	48.00 ± 0.17^{c}

*Control= ten shrimps with manifestation of Vibriosis did not receive treatment, only were fed with 200 g of pellets during 5 days, G1= ten shrimps with presence of Vibriosis were treated with 200 g of the mixture 1 during 5 days, G2= ten shrimps with clinical symptoms of Vibriosis were treated with 200 g of the mixture 2 and G3= ten shrimps with incidence of Vibriosis were treated with 200 g of the mixture 3 during 5 days.

*Mixture 1: pellets were mixed with solution S1, mixture 2 (M2): pellets were mixed with solution S2, and mixture 3 (M3): pellets were mixed with solution S3.

*Solution 1= To dissolve 250 g of VIBRO PLUS and 200 mL of AQUAPEGA ATUN “LA” on 2 L of water, solution S2= Dissolving 250 g of VIBRO and 200 mL of AQUAPEGA ATUN “LA” on 2 L of water, and solution S3= Dissolving 250 g of FORMIC and 200 mL of AQUAPEGA ATUN “LA” on 2 L of water.

* Different letters in each column indicated significant difference among mortality (M), percent survival index (PSI), presence of Vibriosis and weight of infected *Litopenaeus vannamei* with Vibriosis after using the treatments at level $P<0.05$, according to Duncan's test, n = 10.

CONCLUSION

The mixture 1 based on lactic acid, formic acid, citric acid and sorbic acid showed the highest content of protein and ash.

The use of organic acids in the feeding is related to the biocontrol of Vibriosis, reducing mortality, and also improves the growth of juvenile white shrimp *Litopenaeus vannamei*.

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