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The effects of supplemented diets with a phytopharmaceutical preparation from herbal and macroalgal origin on disease resistance in rainbow trout against *Piscirickettsia salmonis*

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ABSTRACT

The present study aimed to evaluate the effects of a commercial phytopharmaceutical preparation from herbal and macroalgal origin on the growth and immune response of rainbow trout adapted to seawater and its susceptibility to *Piscirickettsia salmonis* infection. Preliminary in vitro trials, evaluated the effects of the commercial product Futerpenol® on the expression levels of selected immune-regulatory genes and its protective effect in a challenge against *Piscirickettsia salmonis* (LF89). Subsequent in vivo feeding trials were conducted to corroborate fish protection against *Piscirickettsia salmonis*. Control and treatment diets (with or without the commercial product Futerpenol® at a concentration of 1 kg/ton) were fed to triplicate groups of 50 fish (average weight: 100.1 ± 11.1 g) during 30 days. Fish from all dietary groups were equally redistributed in three tanks and challenged by cohabitation with fish infected with *P. salmonis* (shedders) at the end of the feeding treatment, and mortalities were recorded over 80 days post-infection. A cumulative mortality of 35.0 ± 4.3 and 15.0 ± 6.0% was registered when challenged fish were previously fed during 30 days with the control and treatment diets respectively. Futerpenol® dietary administration preceding the cohabitation challenge gave significant protection from *P. salmonis* as suggested by the Relative Percent Survival (RPS) values of 62.3 and 57.1% after 60 and 80 days post-infection, respectively. These results suggest that dietary supplementation with Futerpenol® at 1 kg/ton do not affect growth performance and strengthen immunity of *Oncorhynchus mykiss* against *P. salmonis* under the experimental conditions applied during this study.

Statement of relevance: This study demonstrates that dietary supplementation with a phytopharmaceutical preparation, rich in fucoidans and labdane diterpenes strengthen immunity of *O. mykiss* against *P. salmonis*. The use of feed supplemented with this kind of phytopharmaceutical preparations in anticipation to potential infections or vulnerable stages of the production could be useful as an alternative method to prevent and limit the outbreak of salmonid rickettsial septicaemia (SRS) which constitutes one of the main infectious diseases causing production problems and substantial loss in salmonids and marine fish aquaculture worldwide.

1. Introduction

World aquaculture production continues to grow and play a key role in improving human nutrition since it provides almost half of all fish addressed for human consumption (FAO, 2014). The importance of this industry for food security is widely recognized, and also constitutes a main source of income and employment in many countries (Bórquez and Hernández, 2009; HLPE, 2014). While the significance of aquaculture production is accepted and encouraged, it also requires the generation of specific strategies ensuring sustainability and reduction of any possible constraints.

Diseases are substantial deterrents to aquaculture production worldwide (Sindermann, 1984; Zaccone et al., 2009a,b). The industry has suffered important economic losses mainly due to bacterial and viral diseases affecting the production efficiency (Smith et al., 2003; Wiens, 2009). Therefore it is necessary to develop adequate methods to prevent and limit the outbreak of infectious diseases that may compromise the sustainability of this vital industry.

Among the practices for containing some of the most common aquaculture diseases outbreaks the use of countermeasures, such as vaccines, chemicals or antibiotics, has been widely extended (Burridge et al., 2010; Sekkin and Kum, 2011; Newaj-Fyzul et al., 2014). However, limitations to use such products have arisen due to public health concerns in

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relation to evidence on food contamination, bacterial drug resistance and negative effects over the environment (Manzetti and Ghisi, 2014; Menz et al., 2015; Robert et al., 2015). Furthermore, sometimes the effectiveness of these practices is marginal, the application is costly, stressful for the animals and in some cases the use is banned or strictly regulated (Hanson, 2000; Schachte, 2000; Smith et al., 2003; Gudmundsdóttir and Björnsdóttir, 2007; Dhayanithi et al., 2015). Infectious fish diseases can be caused by pathogens that take advantage of immunocompromised fish (Magnadottir, 2010; Thanigaivel et al., 2015). Consequently, much attention has given for the development of new approaches that allow improving health condition of cultured fish.

One of many approaches to improve fish health and performance in aquaculture is by including feed additives to produce functional feeds (Castillo et al., 2014). Such diets provide physiological benefits beyond basic nutritional requirements, like improving health status and reducing incidence of diseases (Cencic and Chingwaru, 2010; Jensen et al., 2014). Feeding diets rich in bioactive ingredients is being considered as a potentially beneficial way to significantly prevent and control disease incidence. The use of different additives to boost health condition has been evaluated in diets for salmonids and other cultured aquatic species with varied results (Lazado and Caipang, 2014; Hauton et al., 2015). An important number of in vitro and in vivo studies have confirmed a wide range of activities of phytobiotics in human and animals (Allen et al., 1997; Panda et al., 2006; Cespedes et al., 2008; Suwalskya et al., 2008; Grashorn, 2010; Silva and Fernandes-Júnior, 2010; Sen and Chatterjee, 2011; Severino and Ambrosio, 2012; Wink, 2012; Pan et al., 2015). The use of phytobiotics with pharmacological effects has gained attention in aquaculture since the variety and availability of plants with potential bioactive properties is very high. Additionally, the use of plant products as immunostimulants in aquaculture systems may also be of environmental value due to their biodegradability (Vaseeharan and Thaya, 2014). Terrestrial plants have been studied for the bioactivities of their extracts and in the same way, plants from marine origin (seaweeds or macroalgae) are also considered to be a rich source of bioactive molecules (Reverter et al., 2014; Thanigaivel et al., 2015). However, even if the dietary use of phytobiotics products as immunostimulant has revealed that they increase the immune responses, survival and growth rate of numerous cultured aquatic species (Newaj-Fyzul and Austin, 2014; Vaseeharan and Thaya, 2014; Bulfon et al., 2015; Valladão et al., 2015) a wide range of these compounds and their effect are still unknown. Several plants contain bioactive compounds that may have different effects, either positive or negative, on aquatic animals (Gatlin et al., 2007). Therefore, to assess effectiveness and efficiency of these novel pharmaceutical alternatives in target aquatic species is necessary.

A wide variety of bioactive components have been identified and extracted from some plant species of the family Acanthaceae and labdane diterpenes are some of the most distinctive. A broad spectrum of significant biological activities have been encountered in labdane diterpenes such as antibacterial, antiviral, antifungal, antiprotozoal, enzyme inducing, anti-inflammatory activities and modulation of immune cell functions (Demetzos and Dimas, 2001; Chinou, 2005; De Las Heras et al., 2007; Mahaira et al., 2011; Kulkarni et al., 2013). Seaweeds are also rich in bioactive metabolites with different pharmacological activities, such as antioxidants, soluble dietary fibers, proteins, minerals, vitamins, phytochemicals, and polyunsaturated fatty acids (Mohamed et al., 2012). Numerous studies have reported the beneficial effects of the use of seaweed meal in the diet of fish including an improvement of stress response and in disease resistance (Fleurence et al., 2012). The fucose-containing sulfated polysaccharides, which are widely found in the cell walls of brown seaweed, but not in other algae or higher plants, exhibit a wide range of bioactivities (Berteau and Mulloy, 2003; Løvstad-Holdt and Kraan, 2011; Ehrig and Alban, 2015).

Piscirickettsiosis or salmonid rickettsial septicaemia (SRS) caused by *Piscirickettsia salmonis* constitutes one of the main infectious diseases causing production problems and substantial loss in salmonids and marine fish aquaculture worldwide (Tapia-Cammas et al., 2011; Yañez et al., 2012; Yañez et al., 2013; Avendaño-Herrera et al., 2014). This fastidious pathogen was first observed in Coho salmon (*Oncorhynchus kisutch*) at southern Chile (Bravo and Campos, 1989). Nowadays, the cost associated to the control and treatment of this disease, in countries like Chile where it affects all cultured salmonid species (*Salmo salar*, *Oncorhynchus mykiss*, *Oncorhynchus kisutch* and *Oncorhynchus tshawytscha*), is very high and the limited effects of the traditional chemotherapy applied indicate that the bacterium has developed drug resistance (Mauel and Miller, 2002; Gómez et al., 2011; Tacchi et al., 2011; Yáñez et al., 2014; Rozas and Enriquez, 2014; Jakob et al., 2014; Rees et al., 2014, Valenzuela et al., 2015). According to the National Fisheries and Aquaculture Service, SRS is the main infectious cause of mortality affecting sea-farmed rainbow trout in Chile (SERNAPESCA, Servicio Nacional de Pesca y Acuicultura, 2015).

The innate immune system constitutes a powerful defense in the protection of fish against the enormous array of pathogens to which they are intimately exposed and innate immune cells that interact with pathogens secrete cytokines that enhance antimicrobial activity (Ooi et al., 2008). Remarkable progress has been achieved in isolating and characterizing immunological genes from fish in recent years (Feng et al., 2009; Zaccone et al., 2009b, Overturf, 2009; Mohanty and Sahoo, 2010). In particular, expression of cytokines genes are commonly accepted as an effective method of measuring the immune response in different fish species (Secombes et al., 2001; Lindenstrøm et al., 2004; Martin et al., 2007; Mulder et al., 2007; Panigrahi et al., 2007; Gioacchini et al., 2008; Awad et al., 2011).

Futerpenol® is a commercial phytopharmaceutical product, especially formulated for aquaculture, which is described to contain as main active principles fucoidans and labdane diterpenes. Thus, the aim of this study was to examine the effects of Futerpenol® as a bioactive phytogenic feed additive for rainbow trout. A first in vitro trial, evaluated the effects of Futerpenol® on the SHK-1 cell line derived from salmon head kidney by quantifying the expression levels of selected immune-regulatory genes at different concentrations and incubation times. A second in vitro trial evaluated the protective effect of Futerpenol® over cellular mortality in a challenge with *P. salmonis*. Subsequently, an in vivo feeding experiment was set up to assess its protective effect on rainbow trout, *O. mykiss*, juveniles challenged with a pathogenic strain of *P. salmonis*.

2. Materials and methods

A commercial phytopharmaceutical product branded as Futerpenol® and manufactured by Maqui New Life Inc. (Santiago, Chile) was used to conduct consecutive in vitro and in vivo assays. This product has been especially formulated for aquaculture to contain fucoidans and labdane diterpenes as main active compounds deriving from an Acanthaceae family herb and different brown seaweeds.

2.1. In vitro experiments

2.1.1. Cell cultures and treatments

SHK-1 cells line were cultured without antibiotics in L-15 medium supplemented with 10% fetal calf serum at 20 °C in 75 cm^2 flasks (Costar, Fisher Scientific, Ottawa, ON, Canada). Cells used in this study were passage between 61 and 63 times. For the time and functional doses estimation, the cells were dispensed into 24-well plates at a concentration of 5×10^5 cell/well 24 h at 20 °C before stimulation. Later, the medium was removed and the cells were incubated with different concentrations of Futerpenol® 1 μg/mL, 0,1 μg/mL and 0,01 μg/mL diluted in fresh medium. Expression analysis was carried out through a kinetic study in cells that were incubated at 20 °C for 2, 4, 6, 24 and 48 h, followed by washing and total RNA extraction and cDNA synthesis as described in Romero et al. (2012). Poly I:C at a concentration of