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(54) Titre : UTILISATION D'EXTRAITS DE QUILLAJA SAPONARIA EN VUE DE LA PREVENTION ET DU CONTROLE
D'INFECTIONS BACTERIENNES CHEZ LES POISSONS
(54) Title: USE OF QUILLAJA SAPONARIA EXTRACTS FOR THE PREVENTION AND CONTROL OF BACTERIAL
INFECTIONS IN FISH

(57) **Abrégé/Abstract:**

The present invention provides a new use of Quillaja extracts for manufacturing a medicinal composition for the prevention and control of bacterial diseases in fish, which is preferably administered orally in combination with food. Other objects of the invention are related to a medicinal composition and a food composition for the prevention and control of bacterial diseases in fish comprising said Quillaja extracts.

Abstract

The present invention provides a new use of *Quillaja* extracts for manufacturing a medicinal composition for the prevention and control of bacterial diseases in fish, which is preferably administered orally in combination with food. Other objects of the invention are related to a medicinal composition and a food composition for the prevention and control of bacterial diseases in fish comprising said *Quillaja* extracts.

USE OF *QUILLAJA SAPONARIA* EXTRACTS FOR THE PREVENTION AND CONTROL OF BACTERIAL INFECTIONS IN FISH

Technical field

5 The present invention relates to the aquaculture industry, and particularly provides a new use of extracts of *Quillaja saponaria* trees for the prevention and control of bacterial diseases in fish, as well as compositions comprising such extracts.

Background of the invention

10 It is widely known that salmon farming industry has increased significantly worldwide in the last two decades and, particularly in Chile, this economic sector has become one of the most important in the country. However, there are different diseases caused by bacteria that infect fish and significantly affect the production.

 Among bacterial diseases affecting fish, Piscirickettsiosis or septicemic
15 rickettsial salmonid syndrome (SRS) is caused by *Piscirickettsia salmonis*, a facultative intracellular pathogen belonging to Gram negative bacteria that causes one of the most serious diseases that affects the salmon industry in Chile. Piscirickettsiosis affects fish during the seawater production cycle, causing high mortality rates that can reach 75% and economic losses for hundreds of millions of
20 dollars (Henriquez, P., Kaiser, M., Bohle, H., Bustos, P., & Mancilla, M. (2016). Journal of fish Diseases 39 (4) 441-448).

 Symptoms of systemic infection caused by *P. salmonis* in fish include colonization of organs such as kidneys, liver, spleen, intestine, brain, ovary and gill (Rozas, M., & Enríquez, R. (2014). Journal of fish Diseases, 37 (3), 163-188). To
25 control Piscirickettsiosis, various strategies have been developed such as early harvesting of fish, the use of antibiotics and vaccines. Some factors that currently decrease the control of this pathogen are vaccines having limited efficacy and high quantities of antibiotics used in production, which may produce bacterial resistance to these drugs (Pulgar, R., Travisany, D., Zuniga, A. Maass, A., &
30 Cambiazo, V. (2015). Journal of biotechnology, 212, 30-31). In addition, preventive

actions involving the use of vaccines provide fish protection for short periods and are not effective in adult stages of salmon at the final stages of the production cycle (Tobar, JA, Jerez, S., Caruffo, M., Bravo, C., Contreras, F., Bucarey, SA, & Harel, M. (2011). *Vaccine*, 29 (12), 2336-2340). Moreover, it has been shown the
 5 existence of variants of *P. salmonis* resistant to the most commonly used antibiotics such as flumequine, enrofloxacin, erythromycin, amoxicillin and oxolinic acid (Mora, SJP, Farias, RC, Gädick, LHP, Rozas, SM (2011). Thesis veterinary medicine: "Analysis of susceptibility of strains of *Piscirickettsia salmonis* isolated salmon and trout farmed in regions of the Rivers, Los Lagos and Aysen 2007 and
 10 2008" University of Concepción, Faculty of veterinary Science). Due to the low efficacy of treatments and since the level of protection depends on various factors such as the immunobiology of fish, the conditions of administration of treatments and environmental factors, disease control has been difficult to achieve (Tobar, I. et al. (2015). *Frontiers in immunology*, (6) 244). Despite all the efforts, the high
 15 frequency of new epizootic events caused by *P. salmonis* indicate that there is a need for new alternatives to prevent and control this disease.

An alternative to the use of vaccines and antibiotics is administrating a food composition that allows the improvement of fish health. In this regard, various diets rich in microalgae containing polyunsaturated fatty acids, glycans, carotenoids,
 20 among others have been tested. These ingredients can promote fish welfare while improving intestinal health and by increasing the resistance to diseases (Kousoulaki, K. et al. (2015). *Journal of Nutritional Science*, 4, e24).

Quillaja saponaria Molina (common name Quillay) is a native tree of Chile primarily used as a soap substitute due to the presence of saponins (San Martín, R. (1999). *Economic Botany*, 53 (3), 302-311). Saponins can be obtained
 25 industrially as powder or liquid extracts, and may be in a purified state, partially purified or unpurified. These extracts are marketed by several companies, being one of the most important Natural Response and Desert King (San Martín, R. and Briones, R. (2000). *Journal of the Science of Food and Agriculture*, 80 (14), 2063-
 30 2068).

To date, saponins rich extracts are used as natural emulsifiers in cosmetics, food and beverages. Additionally, these have been used as adjuvants for vaccine

production and pharmaceutical formulations (Maier, C. et al. (2015). Journal of Agricultural and Food Chemistry, 63 (6), 1756-1762). Other uses such as biocide to eliminate nematodes (US 20050074508), mollusks (US 20070196517) and fungi (Elizondo, E. A. M. et al. (2010). Agro south, 38 (2), 87-96) have also been described.

The review of Wang, Y. et al. (2016), International Journal of Molecular Sciences, 17 (3), 325, describes the use of saponins in aquatic animals showing that these can modulate the immune system of shrimp and fish, and also promote the growth of the latter. However, the document states that most saponins are unstable in aqueous conditions and are very toxic to fish at high concentrations.

Prior art analysis regarding the application of *Quillaja* extracts in fish, shows patent application PCT WO2015155293 disclosing an oral food additive for use in the prevention and/or treatment of infections in a fish composition and particularly describes a composition comprising *Quillaja saponaria* saponins for prophylactic treatment against the ectoparasite of the genus *Caligus* in fish. The experimental evidence provided by this document does not include the determination of a beneficial effect against other pathogens.

Patent application WO2015179840 describes combinations or compositions comprising *Yucca schidigera* and *Quillaja saponaria*, and further including antimicrobials, antibiotics and anticoccidial agents, for administration to animals to prevent diseases. As a general disclosure, it describes that can be applied to fish orally.

Patent application WO0151083 discloses an adjuvant composition comprising a saponin and an oligonucleotide comprising at least one CpG unmethylated dinucleotide. Preferably, the composition includes saponins derived from *Quillaja saponaria*, and most preferably, the saponin is chemically modified or substantially pure (QS-7, QS-17, QS-18 or QS-21). No description is done for the use in fish.

Chilean patent application CL200402942 discloses a food additive for fish formulated with a purified extract of *Quillaja saponaria* Molina comprising 15-25% w/w of triterpene saponins obtained from said extract and 75-85% w/w of potato

maltodextrin. This document does not mention that this food promotes the health of the fish.

The PhD thesis of Fernandes, R. N, (2014) Using *Quillaia* saponin (*Quillaia saponaria* Molina) em juvenis of pacu, Universidade Estadual Paulista, Faculty of
 5 Agricultural Sciences and Veterinary Center Aquicultura, Brazil, describes a study wherein the effect of administering *Quillaia* saponins in doses from 100 to 400 mg/Kg in pacu fish (*Piaractus mesopotamicus*). After 15 days of feeding fish with *Quillaia* saponins, 325 fish were inoculated with *Aeromonas hydrophila* and clinical signs were observed. After seven days, the survival of pacu fish against
 10 experimental infection was higher in fish fed with *Quillaia* saponins in a dose of 200 mg/Kg.

On the other hand, Vinay et al. (2014), Veterinary immunology and immunopathology, 158 (1), 73-85, describes an evaluation of the effect of *Quillaia* saponins administered intraperitoneally as vaccine adjuvant in *Paralichthys*
 15 *olivaceus*. This study showed that saponins are a good inducer of inflammation, but are also toxic for the fish. Saponins concentrations of 500, 160, 50, 16 and 5 µg/fish produced 95%, 65%, 20% and 5% mortality rates, respectively, and with a lethal dose (LD50) of 22.4 mg/Kg. The results determined that the toxic effect of saponins depended on the level of purification and the source of the product.
 20 Finally, the authors found that a concentration of 3.4 mg/Kg of fish is toxic when administered intraperitoneally, and it is recommended to use a lower concentration in *Paralichthys olivaceus*.

Regarding disclosures of saponins from sources other than *Quillaia saponaria*, Krogdahl et al. (2015), *Journal of Agricultural and Food Chemistry*, 63
 25 (15), 3887-3902 discloses that soybean saponins administered orally as feed additive in doses of 2-10 g/Kg produced intestinal inflammation in Atlantic salmon (*Salmo salar*), and the severity is dose-dependent.

This analysis of prior art shows that, although *Quillaia saponaria* saponins have been described for various uses and applications, they are not associated
 30 with treatment of pathogens in commercial fish.

Summary of the Invention

The present invention discloses a new use of *Quillaja* extracts for the prevention and control of bacterial diseases in fish. An object of the present invention relates to the use of a *Quillaja saponaria* extract useful for manufacturing a medicinal composition for prevention and control of bacterial disease in fish. Such medicinal preparation for fish is administered orally in a dose range of 0.9 to 12 mg of saponins/Kg live weight of fish. Administration to fish is performed orally in combination with a food. In a preferred embodiment, the bacterial disease is caused by a Gram negative bacterium, wherein the bacteria selected is from the group consisting of *Piscirickettsia*, *Aeromonas* and *Vibrio*, and in a preferred embodiment, the bacteria belonging to genus *Piscirickettsia* is *Piscirickettsia salmonis*.

In another preferred embodiment, the bacterial disease is caused by a Gram positive, preferably bacteria belonging to the genus *Renibacterium*.

The present invention is intended preferably for salmonid fish, where these are from the group consisting of *Salmo salar*, *Salmo trutta*, *Salmo gairdnerii*, *Oncorhynchus mykiss* or *Oncorhynchus kisutch* species.

Another object of the present invention is a medicinal composition for the prevention and control of bacterial disease in fish comprising a *Quillaja saponaria* extract and an appropriate excipient. *Quillaja saponaria* extract is in a ratio of 2 to 90% w/w of the total weight of the composition.

The present invention also provides a food composition for the prevention and control of bacterial disease in fish, comprising a *Quillaja saponaria* extract and an appropriate additive. *Quillaja saponaria* extract is in a proportion of less than 6% w/w of the total weight of the composition.

Brief description of figures

Figure 1 is the chromatographic profile of a complete *Quillaja saponaria* Molina extract measured by HPLC, where the main saponins QS7, QS17, QS18 and QS21 are indicated.

- 5 Figure 2 is an image obtained by optical microscopy of an ASK salmonid cell line in the presence of different concentrations of *Quillaja* extracts UD100-Q (Ultra Dry® 100-Q) and QD100 (Quillaja Dry® 100).

Detailed description of the invention

- 10 The present invention describes a novel use of plant extracts of *Quillaja saponaria* for the prevention and control of bacterial infections that affect fish. The inventors have found that different extracts of *Quillaja saponaria* Molina saponins, a Chilean endemic tree, have a protective effect against bacterial infections affecting salmonid farming. The inventors have tested and acquired appropriate
15 dose concentrations of *Quillaja* extracts to be used safely in salmon, without altering pathophysiological treated individuals.

The present invention relates to the use of *Quillaja* extracts, which have a specific profile of saponins. In all cases, the profiles of these extracts are saponins own exclusive of *Quillaja*, either in purified extracts, partially purified or unpurified.

- 20 Entire or unfractionated extracts of *Quillaja saponaria* Molina have a distinctive profile and own saponins containing over 100 types of chemically different saponins. The main saponins are QS7, QS17, QS18 and QS21, as seen in the chromatographic profile of Figure 1. The relative concentrations of these saponins depend on the source of the raw material. Additionally, the partially
25 purified extracts (from 2 to 90% w/w or w/v of saponins depending on powder or liquid product) contain non-saponin compounds, which mainly include a mixture of polyphenols and, in smaller amounts, other sugars.

Extracts of *Quillaja* saponins can be obtained industrially as powder or liquid extracts, with varying degrees of purification. For example, for the purposes

of the present invention various commercial extracts of *Quillaja saponaria* may be used, such as those shown in Table 1 below.

Table 1. Commercial extracts (Desert King Chile) of *Quillaja saponaria* useful for the present invention.

Product name	Description
Ultra Dry® 100-Q	<i>Quillaja saponaria</i> Molina powder extract, mainly containing triterpene saponins up to 65% w/w.
Quillaja Dry® 100	<i>Quillaja saponaria</i> Molina powder extract, mainly containing triterpene saponins up to 25% w/w.
Vax Sap ®	Highly purified <i>Quillaja saponaria</i> Molina powder extract mainly containing triterpene saponins > 90% w/w.
QL 1000 ®	Liquid extract mainly of <i>Quillaja saponaria</i> Molina at a concentration of 8% w/v of saponins.
QL Perm ®	Liquid extract mainly of <i>Quillaja saponaria</i> Molina at a concentration of 2% w/v of saponins.

5

The present invention discloses the use of *Quillaja saponaria* extracts to prepare a medicinal composition and a food composition for the prevention and/or control for undesired medical conditions related to bacterial diseases in fish. By means of the new use of *Quillaja saponaria* extracts described in the present invention, drugs can be obtained intended to prevent, control, cure, treat or alleviate bacterial diseases in fish; and also can be obtained for medicinal use in food products that have therapeutic properties with the same purposes.

The present invention encompasses the use of *Quillaja* extracts against bacteria that affect fish, including Gram negative bacteria such as *Piscirickettsia salmonis*, *Aeromonas salmonicida* and *Vibrio* spp; and Gram positive bacteria such as *Renibacterium salmoninarum*, or any other bacteria that affect the health of fish, either in freshwater or seawater. The most susceptible species of fish are salmonid fish such as rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), Arctic char (*Arctic char*), Atlantic

salmon (*Salmo salar*) Pacific salmon (*Oncorhynchus spp.*), chum salmon (*Oncorhynchus keta*), chinook salmon (*Oncorhynchus tshawytscha*), among others.

5 *Quillaja saponaria* extracts of the present invention are used to prepare a medicinal composition or a food product for the prevention or control of post-bacterial infection in fish. Said products are administered orally in a solid or liquid form.

10 For the use of *Quillaja saponaria* extracts in the preparation of a composition or medicinal preparation, medicine or veterinary composition, different formulations containing such extracts as active ingredient can be used. *Quillaja saponaria* extracts are in a proportion between 2% and 90% w/w of the total weight of the composition. The pharmaceutical form can be powder, oil suspension or solid as part of a food pellet. In a preferred embodiment, the medicinal composition comprises veterinary suitable excipients for oral
15 administration in salmonids, such as lactose, corn starch, silicon dioxide, among others well known by experts of this area.

For the use of *Quillaja saponaria* extracts in the obtention of a product or food composition or dietary supplement, said extracts are mixed with fish food in a pellet form, which is mixed with an equivalent to 2% of oil/weight of the pellet to
20 impregnate, to adhere the extract to the food. Examples of fish diets widely known in the industry are produced by Ewos, BioMar, Salmofood, among others. In a preferred embodiment, the food composition comprises food additives appropriate for oral administration in salmonids, such as fishmeal, fish and/or vegetable oil, vitamins, minerals, among others well known by experts of the area.

25 Examples have been included for the purpose of illustrating the invention, with the preferred embodiments and comparative examples, but in no case to be considered as a restriction to the scope of the patent application, which is only delimited by the content of the claims appended hereto.

EXAMPLES

EXAMPLE 1: Evaluation of *in vitro* and *in vivo* toxicity of *Quillaja* extracts.

In vitro cytotoxicity assay in salmon cell lines.

Assays with *Quillaja* extracts products were tested on cell monolayers
 5 derived from salmon in order to assess the cytotoxicity. The cell lines used were
 SHK-1 and ASK. SHK-1 line, described as macrophage-like cells (*Salmo salar*;
 ECACC 97111106 Number, European Collection of Cell Culture, Salisbury, Wilts,
 SP4 0JG, UK) was cultured at 15 ° C in Leibovitz 15 medium (L- 15, Gibco,
 Invitrogen, Carlsbad, CA, USA) supplemented with 10% v/v fetal bovine serum
 10 (Hyclone, Thermo Fisher Scientific, Logan, Utah, USA), 4 mM L-glutamine
 (Gibco), 1% v/v 2-mercaptoethanol (2-ME, Gibco) and 50 µg/mL gentamicin (US
 Biological, Swampscott, MA, USA). The cell line ASK (Atlantic Salmon Kidney,
 ATCC®™ CRL2747) was cultured at 16 ° C in Leibovitz (L-15, Hyclone, Thermo
 Scientific), supplemented with gentamicin (50 µg/mL), L-glutamine (4mM) (Gibco,
 15 Thermo Scientific), 2-mercaptoethanol 1% (v / v) (2-ME, Gibco) and 10% fetal
 bovine serum (v / v) (FBS, Hyclone).

All *Quillaja* extracts products were prepared in MEM or 15 Leibovitz medium
 at a concentration of 1 mg/mL, being dissolved at 37 °C for 3 hours with gentle
 stirring. All prepared solutions of these extracts were filtered through a 0.22 µm
 20 nitrocellulose membrane to avoid contamination in cell cultures. The evaluated
 dilutions were prepared by serial dilutions from the standard solution.

To assess the cytotoxicity of *Quillaja* extracts in salmonid cell lines, 5x10⁵
 cells/well were seeded in 6-well plates and incubated in 2 mL of culture medium as
 final volume for 72 hours at 15 °C. After this time the culture medium was replaced
 25 with fresh medium and the confluency was verified. After 24 hours, cells were
 incubated with the different *Quillaja* extracts in 1mL of culture medium. Cytotoxicity
 assessment was made after 24 hours incubation with *Quillaja* extracts. For this,
 the cells were washed twice with cold PBS and then disrupted using a solution
 with 0.05% trypsin and 0.02% EDTA. Cells were analyzed by flow cytometry
 30 (FACS Canto II (Becton Dickinson) and cytosol incorporation of propidium iodide

was determined as a marker for dead cells. Cells were incubated with a solution of ethanol as a positive control of cell death. As negative control, cells were incubated without *Quillaja* extracts, but were subjected to the same conditions. Additionally, cytotoxicity was assessed by visualizing cells by light microscopy.

5 Results indicated that the concentration that exhibited a 50% of cell death (CC₅₀) varied between 3.5 and 83.4 µg/mL and CC₉₀ varied between 4.7 and 92.6 µg/mL depending on the product used as indicated in Table 2. Figure 2 shows representative results with products QD 100 (Quillaja Dry® 100) and UD 100Q (Ultra Dry® 100-Q) by viewing the cell monolayer through optical microscopy.

10 Table 2. Cell citotoxicity (CC₅₀) by flow citometry using propidium iodide.

Product	CC ₅₀ in SHK-1 (µg/mL)	CC ₉₀ in SHK-1 (µg/mL)
Vax Sap ®	20,4	25,3
Ultra Dry® 100-Q	22,1	29,2
Quillaja Dry® 100	83,4	92,6
QL 1000 ®	3,7	6,5
QL Perm ®	3,5	4,7

In vivo oral citotoxicity assay in fish.

To determine the short term oral toxicity (60 days) in fish, 550 Atlantic salmon (*Salmo salar*) fish clinically healthy were used, with an average weight of 9.5 g. Prior to the experiment, fish were acclimated for 8 weeks, during which 50 fish were randomly examined to check health condition through necropsy and microbiological tests to verify the absence of pathogens such as viruses, bacteria and parasites (Thoesen J. (1994) Suggested procedures for the detection and identification of finfish and shellfish Certain pathogens, 4th edn. Fish Health Section, *American Fisheries Society*, Bethesda, MD; . OIE (Office International des

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Epizootics) (2000) Diagnostic Manual for aquatic animals diseases, 3rd edn. OIE, Paris).

Fish were held in 1,000 L capacity fiberglass tanks, each with independent water supply. The level of dissolved oxygen in the water was 10 mg/L. Water
5 temperature, and oxygen levels of nitrogen compounds were controlled daily.

Extruded feed pellets (Micro 10 prepared by Ewos) were used to prepare five diets with 0, 100, 200, 300 and 600 ppm of saponins/Kg of food. These doses are equivalent to 0, 2, 4, 6 and 12 mg of saponins/Kg of live weight of fish, respectively. Fish were divided into 10 individual tanks with 50 fish each (5 groups
10 with duplicate).

Fishes were hand fed twice a day with diets according to the expected live weight and growth rate of fish. To do this the weight of fish were obtained at 0, 30 and 60 post-start of the experiment.

Fish were observed at least three times daily during the study, recording
15 any possible clinical signs and mortalities. The experiment lasted 60 days. Results during the course of the trial showed no mortalities or abnormalities attributable to the product administered at the different doses. Additionally, no macro or microscopic pathological alterations in the liver or intestine in any treated group, compared to the control group, were found. In conclusion, administration of
20 *Quillaja* extracts were safe at tested doses.

EXAMPLE 2: Use of *Quillaja* extracts for the prevention and/or treatment of *in vitro* bacterial infection in fish.

Antibacterial activity of *Quillaja* extracts against *P. salmonis*.

25 The antibacterial activity of the extracts was measured through an infection assay in CHSE-214 cell monolayers derived from chinook salmon (*Oncorhynchus tshawytscha*, ATCC Number CRL-1681, American Type Culture Collection). 1.2×10^6 cells/well were seeded in 6-well plates and incubated at a confluence of 70%.

To determine antibacterial activity, culture medium was removed and the cell monolayer was infected with a bacterial suspension of a Chilean *P. Salmonis* isolated with an approximate of 10^5 genome copies/mL in culture medium (MEM, Hyclone) supplemented with HEPES buffer 10 mM (Hyclone), non-essential aminoacids (1x) (Hyclone) and 10% Fetal Bovine Serum (Hyclone). In addition, when was necessary the culture medium was supplemented with different *Quillaja* extracts, particularly Ultra Dry® 100-Q (UD100Q®, Desert King) (65% w/w of saponins) or Quillaja Dry® 100 (QD100®, Desert King) (25% w/w of saponins). Then, the capacity of *P. salmonis* infection was compared with the inoculum without *Quillaja* extracts.

The efficiency of the infection was quantified by qPCR through amplification of the 16S ribosomal gene. All conditions were done in triplicate. As a result, it was obtained that *Quillaja* extracts inhibited bacterial replication, where the product Ultra Dry ® 100-Q managed to reduce replication by 97.98% when compared to the untreated control. Quillaja Dry® 100 product achieved a 64.40% inhibition of bacterial replication, as shown in Table 3 below.

Table 3. Results of infection inhibition of *Quillaja* extracts in CHSE-214 cells against *Piscirickettsia salmonis*.

Treatment	Ct (dRn)	Copies/mL	Inhibitory concentration of total saponins (mg/mL)	Inhibition %
Control without bacteria	15,29	7,94E+7	0	0
UD100Q® (65 % of saponin purification)	20,07	1,60E+6	0,00016	97,98
QD100® (25% of saponin purification)	14,73	1,94E+7	0,00053	64,40

EXAMPLE 3: Preparation of a fish feed impregnated with *Quillaja* extracts.

Quillaja extracts were mixed with fish oil to achieve the desired concentrations and then this mixture was impregnated at a rate of 4.9:1 (oil:dry pellet) in fish feed. This was done using an industrial mixer and incorporating oil in the food in movement for at least 5 minutes and additional mixing for at least 10 minutes.

To obtain a food composition impregnated with *Quillaja* extracts at the effective dose, important parameters to be considered are saponin doses/live weight of fish to be administered in the range from 0.9 to 12 mg of saponins per kilogram of live weight, fish weight and the amount of food they consume. The percentage of saponins in each particular *Quillaja* extract product should also be noted beforehand. Thus, any skilled person can obtain the ratio of saponins to be incorporated in the food.

As an example, to prepare a food composition considering the QL Perm ® product, it must be used in a proportion of 6% w/w (60 g of product per kilogram of fish food).

EXAMPLE 4: Use of *Quillaja* extracts for the prevention and/or treatment of fish bacterial infections *in vivo*.

In vivo efficacy of *Quillaja* products against *Piscirickettsia salmonis*

One hundred and eighty Atlantic salmon (*Salmo salar*) weighting 30 g (fry) were used. Selected fishes had no history of ISA and *P. salmonis* infection, which was checked by sampling and subsequent analysis of molecular diagnosis by RT-PCR in real time. Additionally, fishes were checked for bacterial and viral diseases. Before transferring to the experimental station, 60 fish were sampled for checking health status, which considered necropsy, gill inspection, intestine and skin sampling, Gram staining in internal organs (spleen, kidney and brain), staining with acridine orange in gills, IFAT analysis for BKD and SRS, and RT-PCR for IPNV. The general clinical appearance of the fish was acceptable and was within

what is considered normal for the salmon industry and therefore representative of the Chilean fish industry.

During the test, fishes were fed with a diet of 15 micro EWOS 15CP® at a daily rate of 0.75% of body weight (bw/day) impregnated with a *Quillaja saponaria* extract (Ultra Dry® 100-Q o Quillaja Dry® 100, Desert King) in a dose of 3.75 mg product/Kg of body weight.

The experiment was designed with the following experimental groups: positive control (fish challenged with the bacteria and fed without *Quillaja* extracts products), negative control (fish not challenged with bacteria and fed without *Quillaja* extracts products), and treatment groups (fish challenged with the bacteria and fed with *Quillaja* extracts products).

The challenge was performed using an experimental infection with *Piscirickettsia salmonis* by intraperitoneal injection in the ventral line at a rate of 0.1 mL of inoculum per fish, which had a title of 3×10^6 bacterial genome copies/mL. For the negative control group, fish were inoculated with 0.1 mL of culture medium. All experimental groups followed the following schedule, as indicated in Table 4 below.

Table 4. Time schedule for the *in vivo* efficacy study of *Quillaja* extracts for the prevention and / or control of piscirickettsiosis.

Day	-15	0	1	7	45
Acclimation	Start	Finish			
Treatment with orally given quillay extracts Ultra Dry® 100-Q or Quillaja Dry® 100			Start		Finish
Infection with <i>Piscirickettsia salmonis</i>				Start / Finish	
Post-infection follow up				Start	Finish
Post-treatment follow up			Start		Finish
Assay time	Start				Finish

20

Results showed that Ultra Dry ® 100-Q reduced mortality associated with infection of *Piscirickettsia salmonis* (SRS) by 37% when compared with the group challenged with the bacteria and fed with normal diet (without *Quillaja* extract). *Quillaja* Dry® 100 achieved a reduction of 18% compared to the same control, as shown in Table 5.

Table 5. Results of the *in vivo* study with functional diets containing *Quillaja* extracts.

Experimental groups	Mean mortality (%)	Mean survival (%)	Level of protection compared to controls (%)
Normal diet	0	100	-
Normal diet + infection with <i>Piscirickettsia salmonis</i>	45,8	54,2	-
Diet with UD100Q® (66% saponin purity) + infection with <i>P. salmonis</i>	29	71	36,68%
Diet with QD100® (26% saponin purity) + infection with <i>P. salmonis</i>	37,5	62,5	18,12%

Thus, results regarding the use of *Quillaja saponaria* extracts for the prevention and/or control of bacterial diseases in fish showed that these are highly effective, reflecting a protective effect against *in vitro* *Piscirickettsia salmonis* with efficacy over 60%; while *in vivo* a decrease in mortality associated to the bacterial infection was achieved between 18 to 36% at a concentration of 3.75 mg of product/Kg live weight of fish using *Quillaja* extracts at different saponin purities.

CLAIMS

1. Use of a *Quillaja saponaria* extract as sole active ingredient and a veterinary acceptable formulating excipient for the manufacture of a medicinal composition, useful for controlling a bacterial disease caused by *Piscirickettsia salmonis* in salmonid fish, wherein said *Quillaja saponaria* extract contains saponins, and wherein the medicinal composition is prepared to provide an oral dose range between 0.9 to 12 mg of saponins/Kg of live weight of fish.
2. The use according to claim 1, wherein the medicinal composition is prepared in combination with fish food.
3. The use according to claim 1, wherein the salmonid fish is selected from the group consisting of *Salmo salar*, *Salmo trutta*, *Salmo gairdnerii*, *Oncorhynchus mykiss* and *Oncorhynchus kisutch* species.
4. A medicinal composition for controlling a bacterial disease caused by *Piscirickettsia salmonis* in salmonid fish, wherein the medicinal composition comprises a *Quillaja saponaria* extract as sole active ingredient and a veterinary acceptable formulating excipient, wherein said *Quillaja saponaria* extract contains saponins, wherein the saponins are in the medicinal composition in a proportion between 2% and 90% w/w of the total weight of the composition.
5. A food composition for controlling a bacterial disease caused by *Piscirickettsia salmonis* in salmonid fish, wherein the food composition comprises a *Quillaja saponaria* extract as sole active ingredient and a veterinary acceptable additive, wherein said *Quillaja saponaria* extract contains saponins, wherein the saponins are in the food composition in a proportion less than 6% w/w of the total weight of the composition.

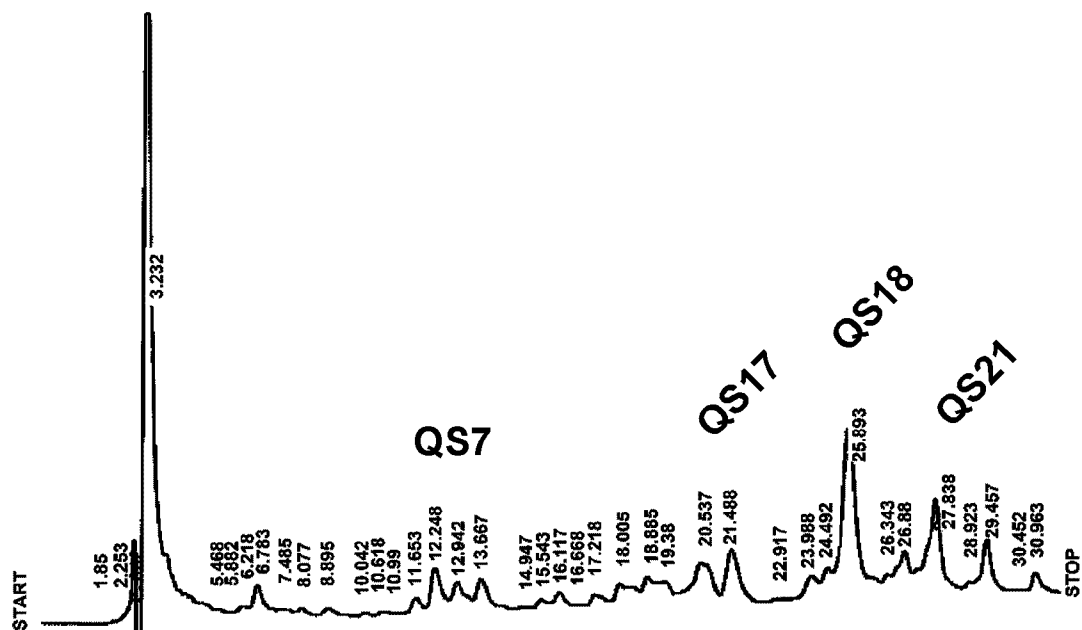


FIGURE 1

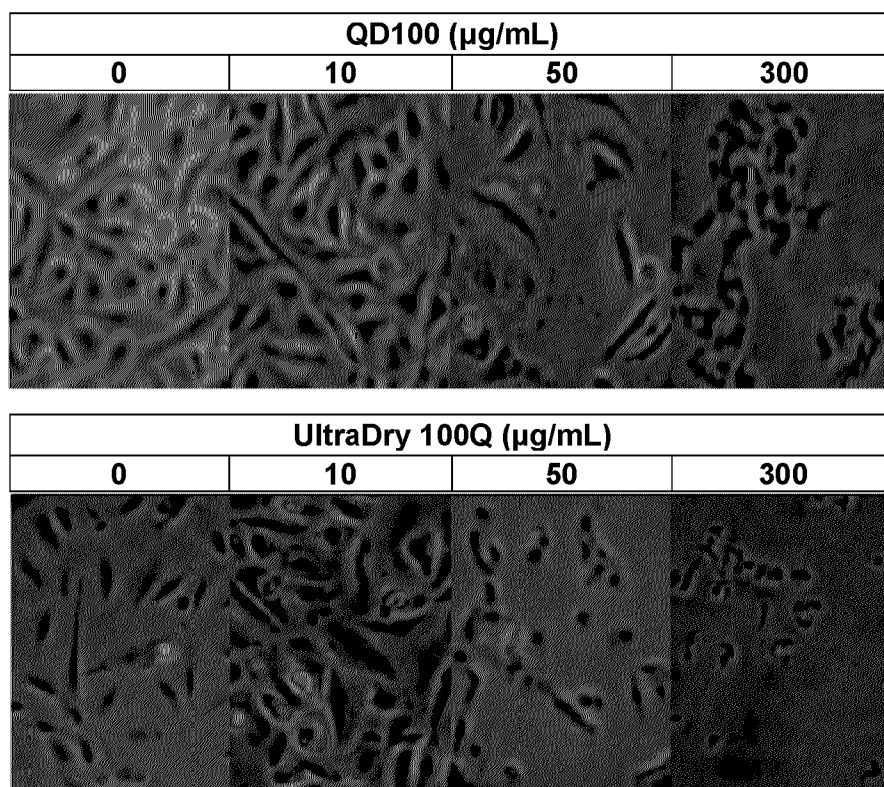


FIGURE 2