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Oral vaccination of Atlantic salmon (*Salmo salar*) against salmonid rickettsial septicaemia

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ABSTRACT

Effective oral immunization systems may be very helpful to the salmon industry, particularly during the seawater growth stages in which vaccination through injection is not possible. During the seawater growing stage, fish become more susceptible to several types of disease, due to the natural decay of vaccine-induced immune responses. In this study, we demonstrate the immune response and efficacy of a new salmonid rickettsial septicaemia (SRS) oral vaccine, developed using MicroMatrix™ Technology. The vaccine, which is administered together with daily feed ration, induces a specific immune response at local and systemic levels. Anti-*Piscirickettsia salmonis* specific antibodies were detected as soon as 300 degree-days after vaccination. Furthermore, oral vaccination was able to protect fish against a lethal pathogen challenge when administered either as a primary vaccination or as a booster for an injected vaccine. Results show that oral vaccination is an efficacious treatment for the prevention of SRS outbreaks throughout the salmon culture period.

1. Introduction

The emergence of infectious disease poses a serious threat to the productivity of the aquaculture industry. Although treatments with antibiotics and chemical compounds have proven useful, both economical and environmental concerns have led to the development of vaccines as the main prophylactic measure for disease control [1]. To date, most commercial vaccines are based on inactivated pathogens or recombinant proteins administered via intraperitoneal (IP) injection. Although this method ensures precise antigen dosage to every fish in the culture system with minor vaccine loss, it has some major drawbacks such as the need for an established infrastructure of qualified personnel, the induction of stress in small fish, and in bigger fish, the risk to acquire additional infections by the injection point [2,3]. Furthermore, vaccination of small-sized fish carries an additional problem specific to fish physiology in that it elicits stress-associated immune response suppression, an effect that is responsible for most of the infectious outbreaks that occur during the on-growing stage [4].

One of the main pathogens that plagues salmonid culture during the on-growing phase is *Piscirickettsia salmonis*, the causal agent of salmonid rickettsial septicaemia (SRS) or piscirickettsiosis [5,6]. This gram-negative, fastidious intracellular pathogen, originally isolated from a coho salmon in southern Chile, produces a systemic infection characterized by colonization of several organs including kidney, liver, spleen, intestine, brain, ovary and gills [7,8]. Because of the ineffectiveness of antimicrobial agents against this bacteria and the resulting high rate of fish mortality, this pathogen has become a major problem for the Chilean salmon culture industry, accounting for annual losses of over US$100 million [9,10].

At present, several injectable vaccines against SRS are commercially available. Although they produce variable long-term results, all of these vaccines are somehow effective in preventing the initial SRS outbreaks that occur after the transfer of fish from fresh water to seawater for the on-growing stage. After this initial outbreak, the fish are susceptible to a second, more aggressive SRS outbreak which correlates with the weakening of the specific immune response elicited by the first immunization event. This outbreak usually affects large fish and occurs ten to twelve months after the transfer, resulting in greater economical losses. Protecting those fish by means of an injectable re-vaccination although appears an attractive solution, is much more difficult to do mainly due economical, practical and stress-related issues. Thus, an alternative immunization methodology is necessary in order to circumvent

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these logistical obstacles. Oral immunization presents an attractive alternative to injectable vaccinations. It has a proven efficacy in mammals and poultry, and it has shown to enhance the protection window in both species, which is correlated with stimulation of both systemic and mucosal immunity [11,12]. Because mucosal membranes constitute the prime barrier between the invading pathogens and internal tissues, and because injury to these surfaces usually results in the onset of infection, induction of an immune response at mucosal sites becomes a rational strategy for conferring specific immunity against bacterial and viral microorganisms. However, in order to achieve successful oral immunization, it is first necessary to solve certain problems, specifically, a delivery system that protect the antigens from the hydrolytic conditions in the stomach and to ensure that the antigens remain in the intestinal tract long enough to be taken up by immune cells and a method to control the dosage in order to ensure that every fish ingested the vaccine [13]. These issues have been addressed extensively in salmonids and other cultured fish species, with the general conclusion that oral delivery of antigens induces weak immune responses and poor protection against pathogen challenge [14].

This study presents results using an oral vaccine formulation capable of inducing a lasting and specific immune response against *P. salmonis* at both the mucosal and systemic levels in Atlantic salmon. Fish were effectively protected against a lethal *P. salmonis* challenge when immunized with the oral vaccine either as a first vaccination or as a booster for an injected vaccine. This oral vaccine technology was developed jointly by Centrovet (Santiago, Chile) and Advanced BioNutrition (Columbia, MD, USA), using the MicroMatrix™ proprietary technology.

2. Materials and methods

2.1. Fish maintenance

Disease-free 30 g Atlantic salmon (*Salmo salar*) fish were obtained from local aquaculture facilities and maintained at Centrovet animal facilities in 0.1 m^3 tanks at a density of 15 kg/m^3. The average water temperature and flow were $12.5 \pm 0.3\,^{\circ}C$ and 150 l/h, respectively, with a water flow per tank of 150 l/h. Fish were fed *ad libitum* with oil-coated feed (Transfer 15R, 2.2 mm, Ewos, Chile) until vaccination.

2.2. Vaccination protocols

For vaccination, fish were fed with vaccine formulated feed made by mixing the vaccine with feed in a final concentration equivalent to 6 mg vaccine/fish/day, administered in the first 90% daily ratio, in order to ensure mass vaccination.

Solution containing injectable vaccine antigen (*P. salmonis* PS2C field strain grown in cell culture, Centrovet, Chile) was incorporated in an oral delivery vehicle containing a bioadhesive cationic polysaccharide formulation (MicroMatrix™ [15]) made at Advanced BioNutrition. A commercial feed was top-coated with oil containing the MicroMatrix™ vaccine preparation at a final concentration of 1EXP10 cells/g feed. To assess antibody production and intestinal histology a total of 180 fish (*S. salar)* were separated into three experimental groups of sixty fish each. The groups received injected vaccine, oral vaccine, or no vaccine. Fish in the oral vaccine group were vaccinated by feeding every three days at 2% of the total biomass for 30 days with the vaccine top-coated feed, a vaccination program which could ensure a constant vaccine delivery during a month. The injected vaccine group was immunized according to the procedure recommend by the manufacturer. Control and IP-vaccinated fish were fed with oil only top-coated feed. To ensure that all fish in the tank were fed with equal amounts of feed fish were monitored until vaccine was totally consumed. Fish weight was monitored during the entire feeding period.

To evaluate the efficacy of the oral vaccination as a primary immunization, a total of 200 fish (*S. salar)* were randomly divided into four groups of fifty fish each. Each group received either injected vaccine or fed with oral free (non MicroMatrix™ formulated) antigen, oral MicroMatrix™ vaccine preparation, or no vaccine containing feeds (oil-only coated). For the challenge, control and vaccinated fish were IP injected with 0.2 ml of pathogenic *P. salmonis*, at 300 or 600 degree-days after vaccination and mortality was monitored on a daily basis. In order to assess the efficacy of the oral vaccination as a booster for injected vaccines, twenty fish (*S. salar)* receiving the injected SRS vaccine were separated into two experimental groups of ten fish each. At 1500 degree days after the IP injection, one group was fed with oral MicroMatrix™ SRS vaccine preparation, while the control group was fed oil only top-coated feed. For the challenge, control and orally boosted fish were injected intraperitoneally with 0.2 ml of pathogenic *P. salmonis*, at either 300 or 600 degree-days after the booster feeding, and mortality was monitored on a daily basis.

2.3. Fish sampling

At different time points after vaccination, five fish from each group were euthanized with Kalmagin 20%® (Benzocaine, Centrovet Ltd.). Blood samples were taken from the caudal vein with 1 ml syringe, stored at $4\,^{\circ}C$ for 24 h and then centrifuged for 10 min at $6000 \times g$. Serum samples were stored at $-20\,^{\circ}C$ until use. The second segment of the intestine was aseptically extracted with a scalpel and processed immediately for histology.

2.4. Antibody ELISA

Nunc Maxisorp plates were activated with 100 μg/ml heat-inactivated *P. salmonis* (strain PS2C, isolated from Atlantic salmon) in bicarbonate buffer, pH 8.5. The plate was blocked with PBS containing 1% BSA, and serial dilutions of either blood serum or supernatants from intestine samples obtained according to Rombout et al. [16] were incubated in the plates overnight. The next day, the plates were washed, and incubated with monoclonal mouse anti-salmon IgM, isotype IgG1 (BiosChile, IGSA, Chile) for 1 h at $30\,^{\circ}C$. The plates were then washed again and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (KPL, USA). Serum antibody titers were determined using 3,3′,5,5′-tetramethylbenzidine as a chromogenic substrate and H_2SO_4 to stop the reaction. Values were obtained by measuring the absorbance at 450 nm.

2.5. Statistical analysis

Statistical calculations and analyses were performed using the statistical software GraphPad Prism 5 (GraphPad Software, Inc.). Differences in serum antibody titers were analysed by applying Student's *t*-Test, differences in weight gain were tested for significant differences between treatments using ANOVA. Differences were considered significant when $p < 0.05$.

3. Results

3.1. The oral SRS vaccine induces immunity against P. salmonis in vaccinated fish

Previous studies have shown that a specific and detectable immune response in fish can be elicited by oral vaccination, which is characterized primarily by a sudden increase in the specific antibody in the blood, followed by a decline in antibody titer three

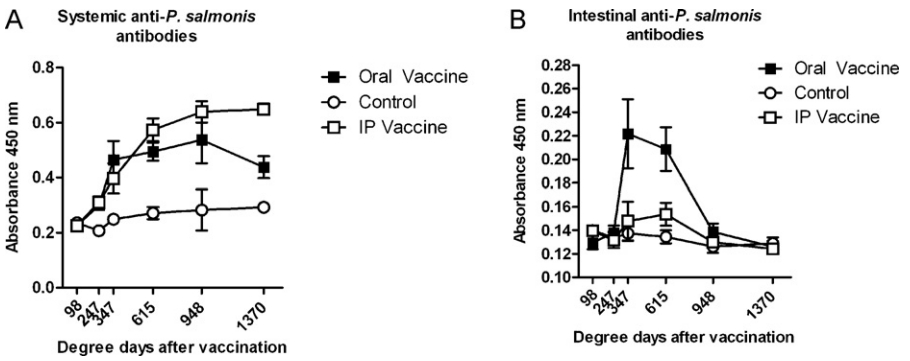

Fig. 1. The effect of IP or oral vaccination on the specific anti-*P. salmonis* response at the local and systemic level. Atlantic salmon were immunized with injectable vaccine, oral vaccine or unvaccinated. Samples of blood (A) and the second segment of the intestine (B) were obtained at different day-degrees post vaccination. Specific IgM titers were assayed by ELISA against inactivated *P. salmonis*. Data are the mean ± SE from 5 fish ($n = 50$) per group at each time point. *T* test analyses showed significant differences between the vaccinated groups and the control group ($p < 0.05$).

weeks post-vaccination [16,17]. To establish that SRS oral vaccine induces the production of specific antibodies, the presence of anti-*P. salmonis* IgM in both serum and the second segment of intestinal mucosa was determined by ELISA. Fig. 1 shows that oral MicroMatrix™ vaccine preparation induced a specific anti-*P. salmonis* response in both serum and the intestinal mucosa. Systemic anti-*P. salmonis* antibodies were detected in the sera as soon as 300 degree-days post vaccination, with a magnitude slightly higher than what was observed in the sera obtained from IP-vaccinated fish (Fig. 1A). High antibody titers were detected up to 900 degree-days post-vaccination, followed by decay in the specific IgM response. Although this period of acquired immunity is shorter than that of IP-vaccinated fish, it is significantly higher than what has been reported in previous studies of oral vaccination [16,17]. Correspondingly, treatment with the SRS oral vaccine induced a considerable local response ($p = 0.0065$, Student's *t* test), as indicated by the detection of intestinal anti-*P. salmonis* antibodies in the intestinal mucosa of the oral vaccine-treated group at 350 degree-days post-vaccination (Fig. 1B). These results suggest that oral vaccination of fish results in efficient significant systemic antibody response as well as in the production of specific intestinal antibodies.

3.2. Oral vaccination does not affect fish weight gain

Previous studies have shown that immersion or oral vaccination strategies may locally activate inflammatory cells at the antigen contact site, resulting in antibody secretion from various tissue surfaces such as the skin, liver, and intestinal mucosa [16]. Because oral vaccination elicited a local specific IgM response, we wanted to determine the effect of oral vaccination in fish nutrition and performance. This is because a successful oral vaccination must not affect the entire gut function in order to maintain the assimilation rate in the intestine of vaccinated fish. As a way to determine if oral vaccine could interfere with nutrient absorption, we measured potential effects on weight gain in orally vaccinated and control fish. Fig. 2 shows that vaccinated groups had similar weight to that of unvaccinated and vaccinated fish at all stages of growth (Fig. 2). This result suggests that administration of the SRS oral vaccine induces no effect in nutrient assimilation in orally vaccinated fish.

3.3. The SRS oral vaccine effectively protects against P. salmonis challenge when administered either as a primary vaccination or as a booster to an IP injection

Our results show that the SRS-oral vaccine enhances specific immunity against SRS by producing a strong antibody response both locally and systemically, suggesting that oral- and IP-vaccinated fish could be efficiently protected against pathogen challenge in a similar way. To compare the efficacy of the oral vaccine versus an IP-injected vaccine, we challenged both oral- and IP-vaccinated fish at 300 and 600 degree-days after vaccination with a lethal IP-injection of *P. salmonis*, and monitored fish survival on a daily basis. Fig. 3 shows that both routes of vaccination conferred a significant protection, at each time point. Notably, orally vaccinated fish showed higher survival rates compared to bacterin-injected fish in response to the lethal IP dose of *P. salmonis* at 300 degree-days after vaccination (85% versus 45%, respectively; Fig. 3A). These results corroborated with the enhanced systemic and local anti-*P. salmonis* IgM response observed in orally immunized fish at this time point. At 600 degree days after immunization, both immunization strategies continued to provide efficient protection against challenge with *P. salmonis*, achieving a 90% survival rate in both groups (Fig. 3B). These results also correlate with the specific IgM response described above, as both immunization routes elicited the highest antibody titers at around 600 degree days after vaccination.

Considering that oral immunization as the primary vaccination efficiently protected against a lethal challenge and because antigenic re-exposure generally elicits faster and stronger secondary immune responses, we assessed the application of the SRS oral vaccine as a means to boost and prolong the immunity conferred by the primary immunization with an injected vaccine. To test this, IP-vaccinated fish were fed with oral vaccine 1500 degree days after the first vaccination event (as a booster), and the efficacy of the different vaccination strategies was evaluated by challenging the fish with an IP injection of lethal *P. salmonis* at 300 or

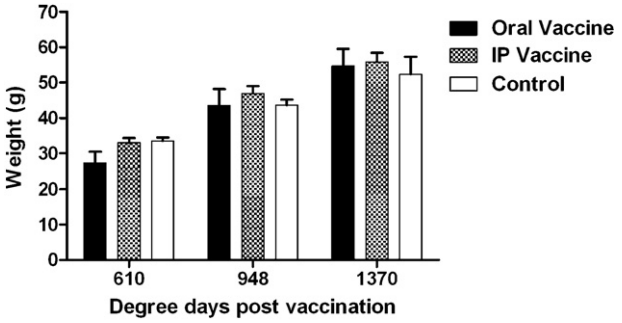

Fig. 2. The effect of IP or oral vaccination on fish growth. The weight of fish immunized with oral or injected vaccine was measured at different time points after vaccination. Unvaccinated fish were used as a control for growth comparison. Data are the mean ± SE from 5 fish ($n = 50$) per group at each time point. ANOVA analyses showed no significant differences (95% confidence).

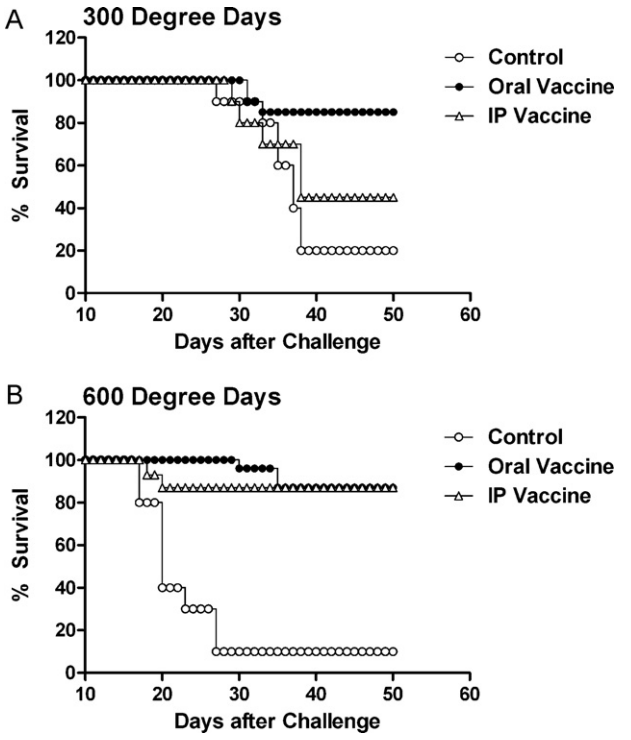

Fig. 3. The effect of IP or oral vaccination on survival of fish infected with lethal *P. salmonis* Atlantic salmon, *Salmo salar* were vaccinated orally or by IP injection. At 300 (A) and 600 (B) degree-days post vaccination, the fish were challenged with a lethal intraperitoneal injection of *P. salmonis*. Survival was monitored on a daily basis.

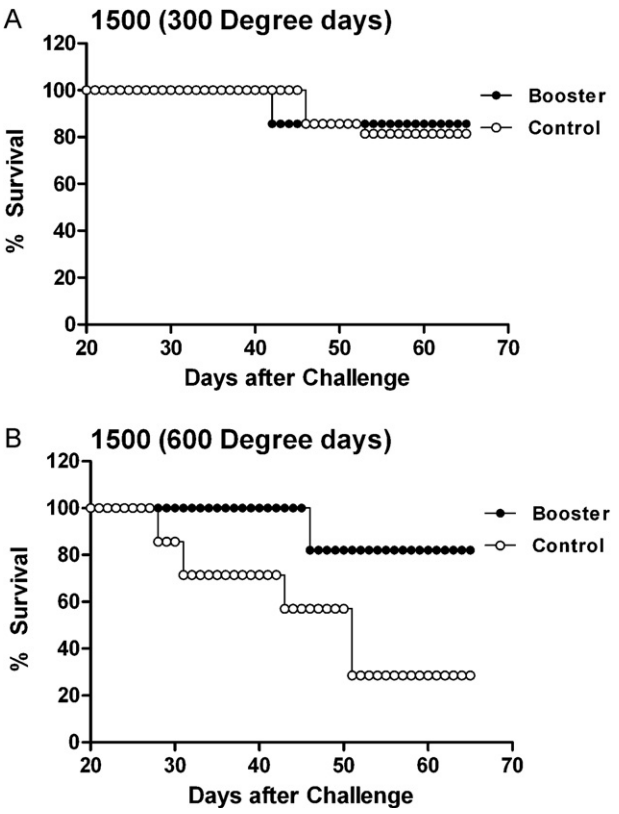

Fig. 4. The effect of IP vaccination only or IP vaccination + oral boostering on survival of fish infected with lethal *P. salmonis*. Atlantic salmon, *Salmo salar* previously vaccinated with an injected bacterin were re-vaccinated with the SRS oral vaccine. At 1800 (A) and 2100 degree-days (B) after IP vaccination (or after booster in parentheses), fish were challenged with a lethal intraperitoneal injection of *P. salmonis*. Survival was monitored on a daily basis.

600 degree-days after feeding (Fig. 4A). When challenged at 300 degree-days after oral vaccination, the survival of both boosted and control groups were very similar, indicating that injected vaccine is able to efficiently protect against a lethal dose of *P. salmonis* for up to 1800 degree days (Fig. 4B). However, the protection conferred by bacterin injection was drastically reduced at 2100 degree-days after IP-vaccination, as was indicated by the massive mortality in control group after 28 days following the lethal challenge (only 30% survival by the end of the trial, Fig. 4B). Notably, oral vaccination as a booster effectively prevented high mortalities compared to the control group, conferring 100% protection until 46 days after challenge and a final survival of 80% at the end of the trial (Fig. 4B). This protection effect has been obtained by applying vaccine on feed during 10 days every three days (this study), or when administered during 10 consecutive days (SRS-oral vaccine registry, Centrovet, Chile, and manuscript in preparation). Taken together, these results demonstrate that oral vaccination efficiently protects fish against lethal infection with *P. salmonis* when used either as a primary vaccination or as a booster vaccination to support primary immunization with an injected vaccine.

4. Discussion

Vaccination is one of the most important and effective method of intervention used in the prevention of infectious diseases in aquaculture. Vaccines confer specific and long lasting protection and significantly reduce the need for antibiotic treatments.

There are several routes for the administration of immunological agents, with IP injection and immersion being the most commonly used. The first method has the advantage of eliciting a strong and prolonged immune response, albeit with a delay of approximately 600 degree-days between vaccination and the onset of immune competence. This is the main reason why in salmonids the IP vaccination against seawater-related pathogens, such as *P. salmonis*, is restricted to freshwater stage, where it is applied about 600 degree-days after salmon sea transfer in order to get SRS-protected fish in saltwater stage. In addition, other drawbacks of IP vaccination are the stress imposed on the fish due to the associated handling and the limited applicability to the freshwater growth stage where fish is still small. Vaccination by immersion, on the other hand, significantly reduces the amount of stress placed on the fish and can be applied to small fish. However, it is not an effective means of immunization during the seawater phase. Additionally, vaccination by immersion induces shorter periods of protection than those generated by IP vaccination. For these reasons, it is important to develop a method of immunization that is easily delivered, applicable to fish at all sizes and stages of development, and most importantly, capable of eliciting a specific and long lasting immune response. The current study has demonstrated that the oral delivery of antigens can fulfill these objectives.

One of the challenges in the development of effective oral delivery technology is to protect the antigen from degradation in the acid environment in the stomach and to ensure that it is exposed to gut-associated lymphoid tissues, thereby enhancing the uptake, processing and presentation of the antigen to the fish innate immune system at the mucosal level.

The findings presented here are promising in this respect and were obtained using an oral vaccine against *P. salmonis*. When administered correctly, the oral vaccination induces the produc-

tion of specific antibodies against *P. salmonis*, in both the systemic and intestinal mucus levels. The ability of this vaccine to elicit a local immune response is significant, because it has been reported that antibodies in the intestines of Atlantic salmon have a short life span [18]. The antibody levels measured in the present study correlated with the presence of inflammatory cells and the infiltration of lymphocytes into the subepithelial region of the small intestine (data not shown). This inflammatory cell infiltration could be either T or B cells, probably IgM-cells, which have been reported to have the ability to capture and degrade bacterial antigens [19]. Future work must be done in order to obtain antibodies against T or B cells which could clear this asseveration. Taking the knowledge in Salmon immune response we have to date, our data suggests that, in addition to a humoral response, indicated by the detection of specific IgM associated antibodies, there was also an active cellular immune response to *P. salmonis*.

The immune response elicited by administration of the oral vaccine, either as a primary or booster vaccination, correlated with the induction of a significant level of protection in fish challenged by intraperitoneal injection of pathogenic *P. salmonis*. As a primary vaccination, the earliest levels of protection were observed at 300 degree-days post-vaccination, a time point that has been previously reported for other oral vaccines [16,17]. These vaccines exhibited an early response peak upon vaccination, but the period of protection did not exceed three to four weeks post-immunization. The finding that the MicroMatrix™ preserves oral-SRS vaccine maintained significant levels of protection until 600 degree-days post-vaccination and conferred a level of protection comparable to that of the injected vaccine it has not been described for a fish oral vaccine.

Re-vaccination is a well-known method for enhancing the magnitude and specificity of the immune response. Generally, the antigen is presented to the already primed immune cells, mainly T lymphocytes, which induce the multiplication of reactive lymphocytes. Re-vaccination is necessary in cases where the primary immune response is weak and a high probability exists that the fish will be re-infected, or in situations where the levels of immunity have decreased and the fish are challenged by a new environment, such as during the seawater phase of salmon culture. The oral-SRS vaccine is a promising solution to this problem. Its route of administration eliminates handling-associated stress and its mechanism of action enhances the immunity established by previous vaccination. Future work must be done regarding the duration and magnitude of the specific immune response once fish receives a second, third or even a fourth oral booster vaccination. We expect that memory cells which could be generated upon first and second immunization would act as “immunological magnifiers” in charge of amplify the immune response in every consecutive re-vaccination. This phenomenon, although it is needed to be confirmed, will acquire remarkable importance when fish species to be vaccinated presents a long life cycle before harvest, such as salmonids.

The MicroMatrix™ technology utilized to produce the oral-SRS vaccine is potentially applicable to other antigens for oral administration. This technology eliminates problems commonly associated with other oral vaccines. For example, MicroMatrix™ preserves the antigen in the stomach, allows for a prolonged intact with the intestinal mucosa and recruits immunological elements, such as macrophages and lymphocytes, at the same time it delivers the encapsulated antigen into the gut mucosa. Currently, this technology is evaluated for oral vaccination against other fish pathologies, such as infectious salmon anemia (ISA). This technology represents a major advance for the salmon industry, allowing for the optimization of the immunization process.

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