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Pichia pastoris yeast as a vehicle for oral vaccination of larval and adult teleosts

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ABSTRACT

Oral vaccination is of major interest because it can be used for mass vaccination of fish of various size and age. Given that their administration is relatively easy and stress-free, oral vaccines have both economic and animal welfare benefits. Yet, mostly due to their limited efficacy, only very few oral vaccines are available to aquaculture industry. Here we present a method for oral vaccine delivery based on the yeast *Pichia pastoris*. We could express a model antigen, green fluorescent protein (GFP), in this yeast and subsequently show delivery of the GFP protein to the intestine of juvenile flounder or adult carp and trout. We tested this approach in several commercially-relevant fish species, from juvenile to adult stage. To test the oral delivery of antigen to larval fish, the GFP-expressing *Pichia pastoris* was first fed to planktonic crustacean *Daphnia* or rotifers that served as 'bioencapsulation vehicles' and afterwards, fed to flounder larvae. Again, we could show delivery of intact GFP protein to the intestine. In rainbow trout, the orally-administered GFP-expressing yeast elicited a rapid local innate immune response in the intestine and a subsequent systemic response in the spleen. Our results show that *Pichia pastoris* is a good vehicle for oral antigen delivery and that it can be used in non-encapsulated form for older fish or in bioencapsulated form for larval fish. We discuss the immunomodulatory properties of the yeast itself, and its potential to enhance local immune responses and act as an adjuvant.

1. Introduction

Route of administration is an important factor influencing protection and feasibility of vaccination processes. Nowadays, three main administration routes are used in aquaculture: bath/immersion, injection and oral administration [28]. In addition, anal [42] and nasal vaccination [38] have been tested in the laboratory with good results, but their practical application is doubtful because of the associated difficulty of administration. Vaccination by injection so far remains the most-effective and thus preferred route, providing the highest and longest protection and requiring the least amount of vaccine. Still, bath and oral immunizations (mucosal routes) are options at least theoretically superior to injection vaccination, given that they require a minimum of handling, induce minimal stress and reduce the possibility of side effects. In addition, mucosal vaccination allows for easy vaccination of fish at all life stages, including the larval stage. While this vaccination route holds many promises, only few successful experimental oral vaccines have been described so far (Reviewed in [9] and part of this might be caused by our restricted knowledge on mucosal immunity in fish. However, recent studies on immunoglobulin (Ig) responses at mucosal sites have described a new isotype (Igt/z) which was shown to be the main immunoglobulin type responding to infections with mucosal parasites [43–45]. In these studies, parasite-specific Igt titers were observed in all mucosal tissues, while parasite-specific Igm was restricted mainly to the serum. The discovery of Igt/z provided us with a new tool to determine the specific antibodies involved in adaptive immunity at mucosal sites. Finally, this knowledge will aid and advance the development of mucosal vaccines.

Despite that successful vaccines typically rely on the development of specific adaptive immune response, most efficacious vaccines

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simultaneously elicit a strong innate immune responses that is crucial in determining the quality and quantity of the subsequent adaptive immune response [37]. Therefore, antigens in injectable vaccines are usually administered together with an adjuvant that largely contributes to the initial steps of the immune response and assures a slow antigen release, further contributing to vaccine efficacy and a long duration of immunity. Mucosal vaccines need to induce protective immune responses comparable to those triggered by injected vaccines by activating not only specific immunity but also innate immunity. However, these innate responses need to be started at local mucosae, one of the main obstacles to overcome. Some mucosal adjuvants have been described for fish but with contrasting results [39], and none of them have yet demonstrated efficacy on the field trials. Hence, most of the mucosal vaccines available on the market confer a relative percent survival (RPS) of 60%, while many of them never reach the market because of even lower RPS values. Thus, no doubt, effective mucosal adjuvants would be a great benefit to the application of mucosal vaccines and in consequence to the aquaculture industry.

Degradation of the vaccine antigen is another important factor affecting the efficacy of mucosal vaccines. Mucosal surfaces are protected by a mucus layer that defends the fish from possible environmental threats. In this layer, a vast number of bacteria are present together with proteases and other proteins or enzymes [7,21]. Further, relevant to oral vaccination, also the low pH in the stomach can affect antigen integrity. For these reasons, the protection of antigens is of utmost importance and several strategies have been tested to protect antigens from deterioration [9,25]. In fish, nano- and microparticles of different composition, including alginate [3,8,34], chitosan [18], and PLGA [1,40] have been shown effective in encapsulating DNA, soluble antigens or formalin-killed bacteria [16]. Although some groups have used whole bacteria or yeast as vehicle for antigen delivery [9], none many have tried to combine antigen expression with adjuvant activity in a single product approach.

Here, we studied the possibility of using the yeast *Pichia pastoris* as vehicle for oral antigen delivery. *P. pastoris* is a frequently-used expression system for proteins because it has a high growth rate on relatively simple, inexpensive media. As added benefit, the main cell wall component of yeast, β-glucan, has been characterized as a dietary immune-stimulant supplement in fish [30,41]. In the present study, we observed GFP in the gut epithelium and lamina propria shortly after feeding stomach-less adult common carp and stomach-containing adult rainbow trout with *P. pastoris* expressing GFP (*Pichia*-GFP). This confirms that *P. pastoris* is capable of protecting the antigen while travelling through the gastro-intestinal tract, allowing intact antigens to reach the posterior part of the intestine and here, interact with resident immune cells. Indeed, we observed an activation of the mucosal immune system in different segments of trout intestine, suggesting the potential role of *P. pastoris* as adjuvant for oral vaccines. In parallel to the studies in adult fish, we also studied the possibility of using *P. pastoris* as vehicle for oral antigen delivery to larval fish. We also showed that by feeding flounder larvae with *Pichia*-GFP fed plankton, these microorganisms could act as bio-encapsulation vehicles and deliver intact antigen to the intestine of the larvae. In summary, our results highlight *P. pastoris* as a promising vehicle (bioencapsulated and non-encapsulated) for antigen delivery and potential adjuvant for use in oral vaccines at early and late stage of production cycle.

2. Materials and methods

2.1. Animals

European common carp (*Cyprinus carpio* L.) were reared in the Aquatic Research Facility of Carus, the animal research facility of Wageningen University, The Netherlands. Fish were kept at 23 °C in recirculating UV-treated water and fed pelleted dry food (Skretting, Nutreco) twice daily. Rainbow trout (*Oncorhynchus mykiss*) were obtained from a local fish farm (Trout factory, Peramola, Spain) and acclimatized at least two weeks at 15 °C in a recirculating UV-treated water system before experiments. Flounder larvae (*Platichthys flesus*) were provided by Venøsund Fish and Shellfish (Fishlab, Denmark). All studies were performed with approval of the local animal welfare committees and carried out in accordance with the recommendations of the European Union Ethical Guidelines for experimental animal care and other scientific purposes (2010/63/EU).

2.2. Genes and protein nomenclature

For the nomenclature of fish genes and proteins we refer to the official guidelines at http://zfin.org/.

2.3. Production of P. pastoris expressing GFP

P. pastoris (*Komagataella phaffii*) strain W42-A and plasmid pWM13 were provided by W42 GmbH (Dortmund, Germany). The plasmid has a strong yeast promoter *Paox1* in combination with a yeast-specific transcription termination region, and the *ble* gene from *Streptomyces* sp., which enables resistance against the antibiotic Zeocin (Invivogen) in *E. coli* and *P. pastoris*.

For the production of enhanced green fluorescence protein (eGFP), its DNA sequence (AAG49427) was synthesized with optimized codon usage for *P. pastoris*. To facilitate display, the GFP sequence was modified to express a yeast-specific signal-peptide (N-terminal) in combination with a yeast-specific GPI-anchor (C-terminal). The construct was cloned in the pWM13 vector between the *Eco*RI and *Xba*I sites and subcloned into *E. coli*. The resulting expression plasmid pWGFP1-1 was transferred to *P. pastoris* in linearized form by standard electroporation to enable genomic integration of the plasmid in the *Paox1* gene locus of the yeast. GFP-producing clones were obtained through selection on agar plates containing increasing concentrations of Zeocin. Correct genomic integration of the *gfp* gene was confirmed by PCR. Using synthetic medium MMY (Invitrogen) and induction by methanol, the clone expressing the highest amount of recombinant protein was identified using fluorescence image analysis. For large-scale production of recombinant *Pichia*-GFP, a lab-scale fermentation with 2 L working volume was carried out in a defined synthetic medium. *P. pastoris* cells from fermentation were freeze-dried after harvest and kept at 4 °C until further use.

2.4. Culturing of plankton

Rotifer *Brachionus plicatilis* was purchased from Reefphyto in the United Kingdom as Pod-Shot– live Roti-Shot – live concentrated rotifers in 50 ml pouches. The ‘shots’ were transferred into 5 L flasks with flat bottoms in 2 L saltwater with a gravity of 1.020 and aeration. The 5 L flat bottle flasks were covered to avoid dust and contaminants. *Daphnia magna* was purchased from Frederiksen, Denmark (Frederiksen Scientific A/S) and cultured at Fishlab facilities. *Daphnia* were cultured in flasks with flat bottoms and 2 L freshwater with aeration. Both, rotifers and *Daphnia* were fed with baker's yeast (final concentration in culture was 1,25 mg yeast/L *Daphnia* culture and 2,5 mg yeast/L rotifers culture) mixed with sterile water and different kinds of live phytoplankton, which were kept at 7 °C and dark environment. Saltwater phytoplankton was purchased from Reefphyto (UK), and mainly contained a mixture of 5 phytoplankton species (*Nannochloropsis oculata*, *Tetraselmis*, *Isochrysis*, *Pavlova*, *Thalassiosira weissflogii*). *Pseudokirchneriella* sp. a freshwater algae, was cultured at Fishlab and were kept at room temperature (15-20°C) before its administration to rotifers and *Daphnia*.

Planktonic rotifers and *Daphnia* were left to starve for 1 day prior to provision with *P. pastoris*. Planktonic microorganisms, $n = 20–50$ rotifers or $n = 5$ *Daphnia*, were transferred to 1.5 ml vials containing a high concentration of *P. pastoris*-GFP (3×10^6 cells/ml water) and were left for 24 h. Flounder larvae were fed with *Pichia*-GFP-fed plankton (100–200 copepods/rotifers per 5 flounder larvae) and larvae were taken 1 h after feeding for fluorescence microscopy inspection.

2.5. Oral administration of Pichia-GFP to carp and trout

Freeze-dried *Pichia*-GFP was washed twice with PBS for 5 min at 500 × g, and resuspended in PBS at a concentration of 20 mg/ml. Carp of six months old (± 10 g, $n = 3$/group) were anesthetized using 0.3 g/L Tricaine Methane Sulfonate (TMS, Crescent Research Chemicals). One group received 100 μl of *Pichia*-GFP suspension (2 mg/carp) by oral gavage, whereas the control group received 100 μl PBS. The two groups were then placed in two separate thanks and euthanized 24 h post-treatment using 0.6 g/L TMS and by bleeding through the caudal vein. The intestine was removed, cleaned from outside fat and the lumen was rinsed once with PBS to remove non-internalized Pichia. The complete intestine was mounted in KP CryoCompound mounting medium (Klinipath) and was snap-frozen in liquid nitrogen. Five μm cryosections were air-dried, fixed in 4% paraformaldehyde in PBS for 30 min, rinsed twice with PBS and embedded in Vectashield with DAPI (Vectorlabs). Uptake of *Pichia*-GFP was visualized using a M205 FA fluorescence stereomicroscope (Leica).

Freeze-dried Pichia-GFP was washed twice and resuspended in PBS at a concentration of 100 mg/ml. Rainbow trout (100–125 gr) were placed in two different tanks in the same recirculating system, corresponding to Pichia-fed and control group. Twenty-seven fish received 100 μl of Pichia-GFP solution (10 μg/trout) by oral gavage, while 12 fish received 100 μl of PBS as control group. At 6 h, 24 h, 3 days and 7 days after treatment, 3 fish from the Pichia-GFP group and 2 from the control group were euthanized with an overdose of MS-222 (Sigma). Spleen and intestine were harvested and were frozen immediately in liquid nitrogen for RNA extraction. Samples from the control group isolated at different time points were pooled for processing. At 6 h or 24 h after administration, also intestine from 3 Pichia-fed and 2 PBS-fed trout were dissected, washed with PBS to remove non-internalized Pichia, and the 3 sections of the intestine (pyloric caeca, midgut and hindgut) were placed separately in DMEM media (Sigma). Isolation of gut cells was performed as described previously [45]. Green fluorescence (GFP^+) in gut cells was analyzed using a FacsCanto (BD) and Diva software. Data analysis was performed using FlowJo software.

2.6. Complement and lysozyme activity

Complement hemolytic activity and lysozyme activities were measured as previously described [23].

2.7. Blood and gut mucus isolation

Blood was harvested from the caudal vein and serum was obtained by centrifugation for 10 min at 2000 rpm at 4 °C. Trout gut mucus was collected as previously described [45].

2.8. ELISA

Total amount of trout Igt and Igm in serum and gut mucus was measured by ELISA as previously described [45]. Briefly, 50 μl of primary antibody (mouse anti-trout Igm clone 4C10 at 1/400 dilution or mouse anti-trout Igt clone 38.5 at 5 μg/ml in PBS) was absorbed overnight at 4 °C onto Maxisorp microplates (Nunc). Nonspecific binding was blocked with 8% skim milk in PBS for 1 h at room temperature (RT). After two washes with PBS, gut mucus and serum samples from control and *Pichia*-fed fish were diluted (1/2000 for serum and 1/100 for mucus) in PBS + 10 mM EDTA, added to the plates and incubated 2 h at RT. Plates were then washed with PBS containing 0.15% Tween-20 (v/v). For analysis of the binding of Igt or Igm, biotin-labeled mouse anti–trout Igm 1.14 or biotin-labeled rabbit anti–trout Igt (2 μg/ml each) was added to each well. After 2 h of incubation at RT, plates were washed and bound antibodies were detected by incubation with Pierce High Sensitivity Streptavidin-HRP (horseradish peroxidase; 0.25 μg/ml; Thermo Fisher Scientific), followed by color development with Ultra-TMB (3,3′,5,5′-tetrametilbenzidine; Thermo Fisher Scientific) as a substrate. After 5–15 min of incubation at RT, the color reaction was stopped with 2 M sulfuric acid solution and absorbance measured at 450 nm in a Victor 2 microplate reader.

2.9. RNA isolation and cDNA synthesis

Total RNA was isolated from three different gut segments (pyloric caeca, midgut and hindgut) and from spleen of trout using TRI reagent (Sigma) according to manufacturer's instructions. Total RNA was dissolved in sterile miliQ-water and immediately stored at −80 °C until use. RNA quality was assessed by gel electrophoresis and RNA concentration was determined by NanoDropND-2000 spectrophotometer (Thermo Fisher Scientific). Total RNA (1 μg) was used as template to synthesize complementary DNA (cDNA) using the high capacity cDNA reverse transcription kit (Applied Biosystems) according to manufacturer's instructions and immediately stored at −20 °C until use.

2.10. Gene expression analysis

Real-time PCR was used to analyze the expression pattern of genes associated with immune response in spleen and in three different segments of gut of trout intubated with *P. pastoris*. Specific primers used for gene expression analysis are listed in Table 1. Real-time PCR was carried out with iTaq universal SYBR Green Supermix (Bio-Rad Laboratories) using a 1:10 cDNA dilution for target genes, or 1:50 cDNA dilution for reference genes (*ef1α* and *β-actin*). The thermal conditions used were 3 min at 95 °C of pre-incubation, followed by 40 cycles at 95 °C for 30 s and 60 °C for 30 s. An additional temperature ramping step was utilized to produce melting curves of the reaction from 65 °C to 95 °C to verify amplification of a single product. All reactions were performed using Bio-Rad CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories, USA) and data output was obtained from Bio-Rad CFX Manager software (version 3.1). Quantification was done according to the Livak method [20] using the reference gene *β-actin* (it was chosen as it showed lower variability than *elf1α*) and normalized against the control group.

2.11. Statistical analysis

One-way ANOVA was used to explore differences in the different experiments registered between each time-point and control group. GraphPad software v5.0 was used to calculate the mean and standard error (SEM), and to perform statistical tests. In the analyses, a p-value < 0.05 was considered statistically significant.

3. Results

3.1. P. pastoris can deliver intact antigens to the intestine of adult stomachless and stomach-containing fish

Using the recombinant yeast *P. pastoris* expressing GFP, we followed the uptake of intact protein to the intestine of carp and trout after oral delivery by gavage. This allowed us to evaluate the stability of the yeast and its suitability for antigen delivery to the gut of both stomachless teleost fish (common carp) and a teleost with stomach and pyloric caeca (rainbow trout).

As shown in Fig. 1, we detected GFP protein in the epithelium and lamina propria of both the first and second segment of the intestine of carp, 24 h after administration of *Pichia*-GFP. No major differences in yeast uptake and localization of GFP were observed between the two intestinal segments. These results indicate that the yeast protects the

Table 1
Primers used for real-time quantitative PCR analysis of gene expression.

Gene	Primer sequence (5′-3′)	Accession number	Amplicon size
EF1a	Fw: 5′-ATGCCCCCAAGTTCCTGAAG-3′ Rv: 5′-AACAGCAACAGTCTGCCTCA-3′	NM_001124339.1	140
β-actin	Fw: 5′-GGACTTTGAGCAGGAGATGG-3′ Rv: 5′-ATGATGGAGTTGTAGGTGGTCT-3′	NM_001124235.1	186
mIgM	Fw: 5′-AAAGCCTACAAGAGGGAGACCGAT-3′ Rv: 5′-AGAGTTATGAGGAAGAGTATGATGAAGGTG-3′	U04616.1	128
mIgT	Fw: 5′-TTTTCACATGCGCCGTCAAG-3′ Rv: 5′-AGCGAAGCCCGCCTCAG-3′	AY870265.1	116
MPO	Fw: 5′-ATCCACACGGGCATCACCTG-3′ Rv: 5′-GCAGAGTCACCAATGACACCA-3′	GBTD01119227	68
MCSFR-α	Fw: 5′-ATCTCCACTCATGGCGACACA-3′ Rv: 5′-CATCGCACTGGGTTTCTGGTA-3′	NM_001124739.1	177
IL-1β	Fw: 5′-TGAGAACAAGTGCTGGGTCC-3′ Rv: 5′-GGCTACAGGTCTGGCTTCAG-3′	NM_001124347.2	148
IL-8	Fw: 5′-CACAGACAGAGAAGGAAGGAAAG-3′ Rv: 5′-TGCTCATCTTGGGGTTACAGA-3′	NM_001124362.1	162
IL-10	Fw: 5′-CCGCCATGAACAACAGAACA-3′ Rv: 5′-TCCTGCATTGGACGATCTCT-3′	NM_001245099.1	105
TGF-β1	Fw: 5′-GCCAAGGAGGTCCACAAGTT-3′ Rv: 5′-GTGGTTTTGATGAGCAGGCG-3′	NM_001281366.1	146
C3	Fw: 5′-GAGATGGCCTCCAAGAAGATAGAA-3′ Rv: 5′-ACCGCATGTACGCATCATCA-3′	L24433.1 91	
Lysozyme	Fw: 5′-TGCCTGTCAAAATGGGAGTC-3′ Rv: 5′-CAGCGGATACCACAGACGTT-3′	NM_001124716.1	152
TLR3	Fw: 5′-AGCCCTTTGCTGCCTTACA-3′ Rv: 5′-GTCTTCAGGTCATTTTTGGACACG-3′	NM_001124578.1	61
IFNγ	Fw: 5′-CAAACTGAAAGTCCACTATAAGATCTCCA-3′ Rv: 5′-TCCTGAATTTTCCCCTTGACATATTT-3′	NM_001160503.1	210
Mx1	Fw: 5′-GGTTGTGCCATGCAACGTT-3′ Rv: 5′-GGCTTGGTCAGGATGCCTAAT-3′	NM_001171901.1	102

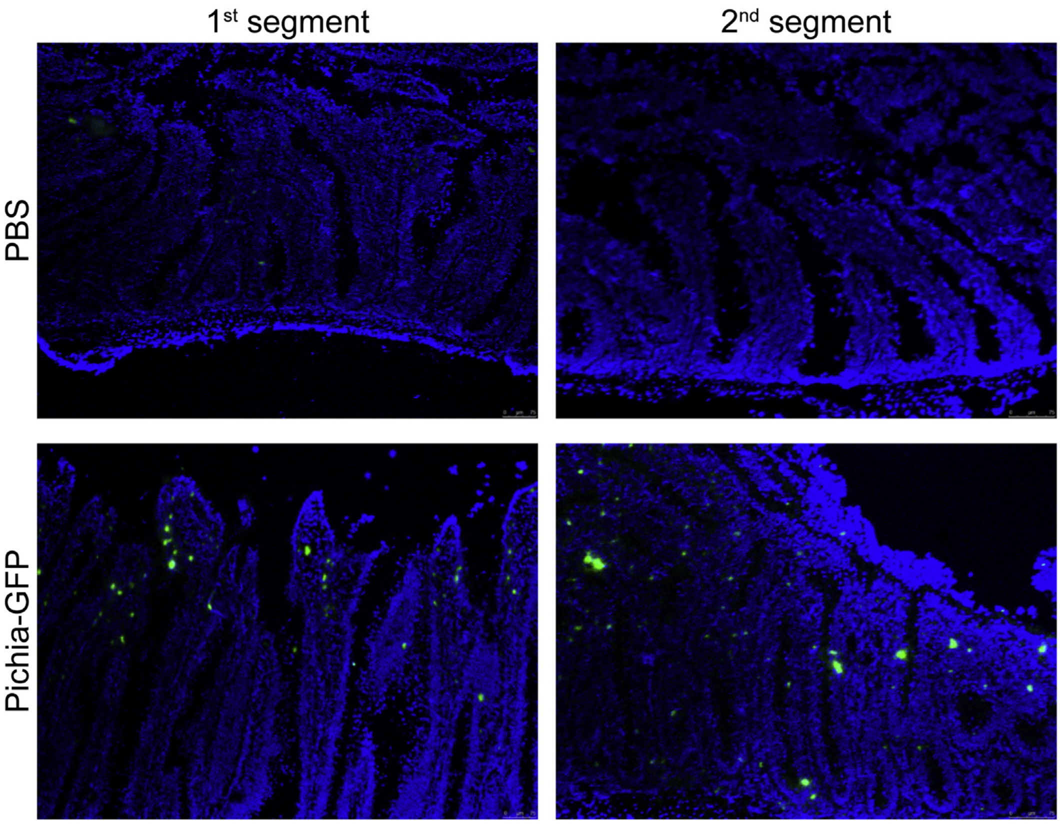

Fig. 1. Uptake of *Pichia*-GFP in the first and second intestinal segment of common carp. Cryosections of 5 μm were prepared 24 h after fish received 2 mg of Pichia-GFP by oral gavage. Control fish received 100 μl PBS. Vectashield-DAPI was used to visualize cell nuclei. Uptake of Pichia is visible by green fluorescence (GFP), as was analyzed by microscopy. Shown are representative images of one fish out of 3 analysed.

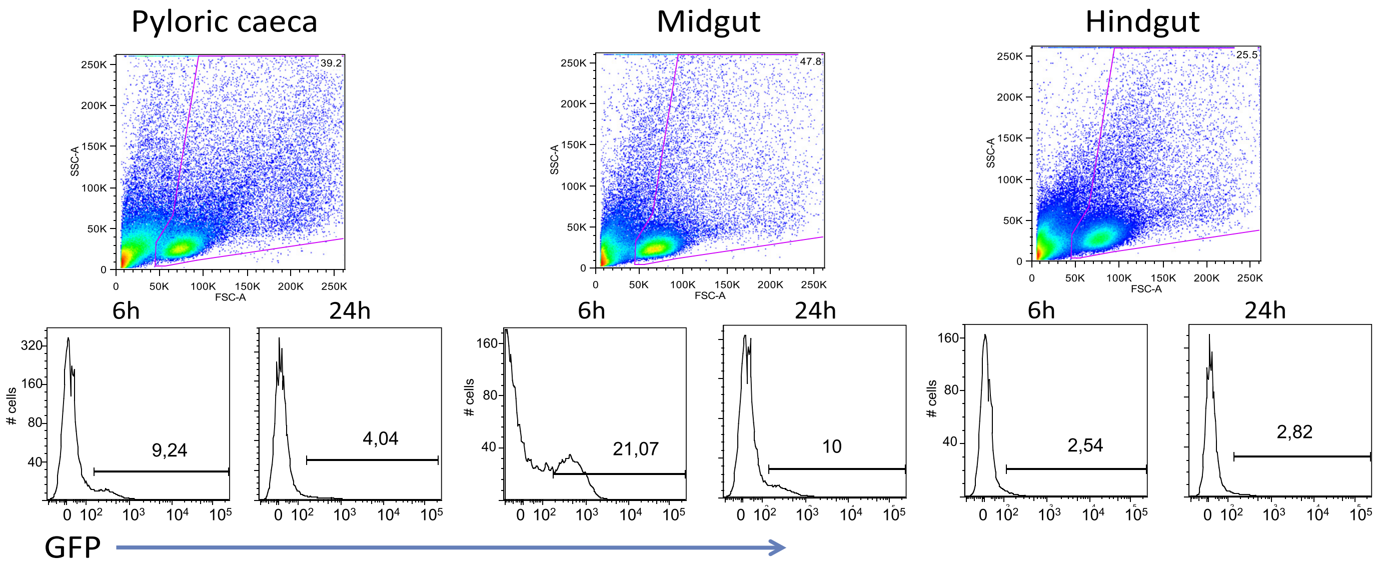

Fig. 2. Internalization of *Pichia*-GFP in trout intestine. Flow cytometry data of intestine of adult rainbow trout after administration of Pichia-GFP by oral gavage. Pyloric caeca (left), midgut (middle) and hindgut (right) were harvested at different times after treatment (6 h and 24 h) and analyzed for green fluorescence (GFP$^+$) with a FACSCanto. Data show a representative graph from three independent experiments in which a total of $n = 7$ fish were analysed. The gating for the GFP signal in the histogram plots was set using cells isolated from fish fed with PBS.

antigen from digestion and reaches the lamina propria still intact, which is a crucial fact for mucosal vaccination. In parallel to the carp experiment, the presence of GFP$^+$ cells in *Pichia*-fed trout intestine was confirmed by flow cytometry. This analysis shows a slight difference in yeast uptake and localization of GFP between the segments of the trout intestine, with the hindgut displaying lower GFP fluorescence at both time points analyzed (6 and 24 h). No GFP was detected in control fish not receiving the treatment (not shown). Moderate GFP levels were found in the pyloric caeca and the highest GFP signal was observed in the midgut (Fig. 2). When comparing the two time points we found higher GFP signals in both pyloric caeca and midgut at 6 h post-treatment than at 24 h post-treatment, indicating that the GFP present at 6 h post-treatment is already degraded or processed at 24 h post-treatment. Thus, with these experiments we were able to detect antigen delivery to the intestine by two different methods in two different teleosts. It is worth to point out the important difference between the digestive tracts of carp (stomachless fish) versus rainbow trout (stomach-containing fish) and also point out that, independent of this difference, *P. pastoris* was able to facilitate delivery of intact antigen to the intestine.

3.2. Bio-encapsulation of Pichia-GFP in planktonic microorganisms allows the delivery of intact antigen to the intestine of fish larvae

There are several fish species which can be fed with pelleted dry food shortly after yolk sac absorption, but larvae of other fish species, including flounder, are fed with live planktonic microorganisms. Some vaccines are coated to the pellet in order to deliver the antigen orally. However, the use of plankton for vaccine delivery to larvae has been scarcely studied. In order to develop this idea, we fed planktonic organisms with *Pichia*-GFP. We noticed green fluorescence in the digestive tract of rotifers and *Daphnia* 24 h after being fed with *Pichia*-GFP (Fig. 3a and b), effectively transforming these microorganisms into live vehicles for vaccine delivery. Notably, feeding flounder larvae with these *Pichia*-GFP-fed plankton resulted in the detection of intact fluorescent yeast in the fish intestine (Fig. 3c). This indicates that bio-encapsulation of *P. pastoris* can serve as a very promising way of delivering antigens to fish larvae.

3.3. Mucosal responses induced by oral delivery of Pichia-GFP

Mucosa and their secretions are among the first barriers that protect animals from environmental threats and the local activation of the mucosal immune system may lead to the production and secretion of several immune components to the mucus layer. Among these immune components described in the intestine are the complement system and lysozyme. Here, we have observed indications for an increase, although not significant, of complement hemolytic activity in trout gut mucus 3 days after administration of *Pichia*-GFP (Fig. 4a), but no change in lysozyme activity (Fig. 4b). Other important contributors to mucosal and systemic immunity are the immunoglobulins (Ig) found in the mucosal layer. While no significant differences were detected in the levels of Igm and Igt in trout serum (Fig. 5a), levels of Igt in trout intestinal mucosa increased immediately after feeding with *Pichia*-GFP, with a peak at 24 h (Fig. 5b). In contrast, trout mucosal Igm levels decreased significantly over time (Fig. 5b), corroborating the role of Igt as main mucosal Ig.

3.4. Gene expression analysis in intestine and spleen after oral delivery of Pichia-GFP

Trout intestine can be divided in three main segments: an anterior segment with pyloric caeca; a medial segment or midgut; and a posterior segment or hindgut, each one presenting different functions and immune reactivity [2,4]. Indeed, we showed that levels of expression of immune genes were different in each of the three intestinal segments after oral delivery of *Pichia*-GFP. We also compared induced gene expression in the intestine (mucosal response) with induced gene expression in the spleen (systemic response). Immune gene responses which could be classified as innate and/or anti-viral, such as *ifnγ*, or *tlr3*, were induced locally in the intestine but not systemically in spleen (Fig. 6). Local gene expression of *ifnγ* increased rapidly in all three segments of the intestine, although it was only significantly upregulated in the hindgut at day 7 post-treatment. Local gene expression of *tlr3* was only significantly upregulated in the hindgut at day 7 post-treatment.

The expression of the B cell receptor *igm* was upregulated at 24 h post-treatment in the midgut and later, at 7 days post-treatment, in the spleen. *igt*, on the other hand, was upregulated at 7 days post-treatment

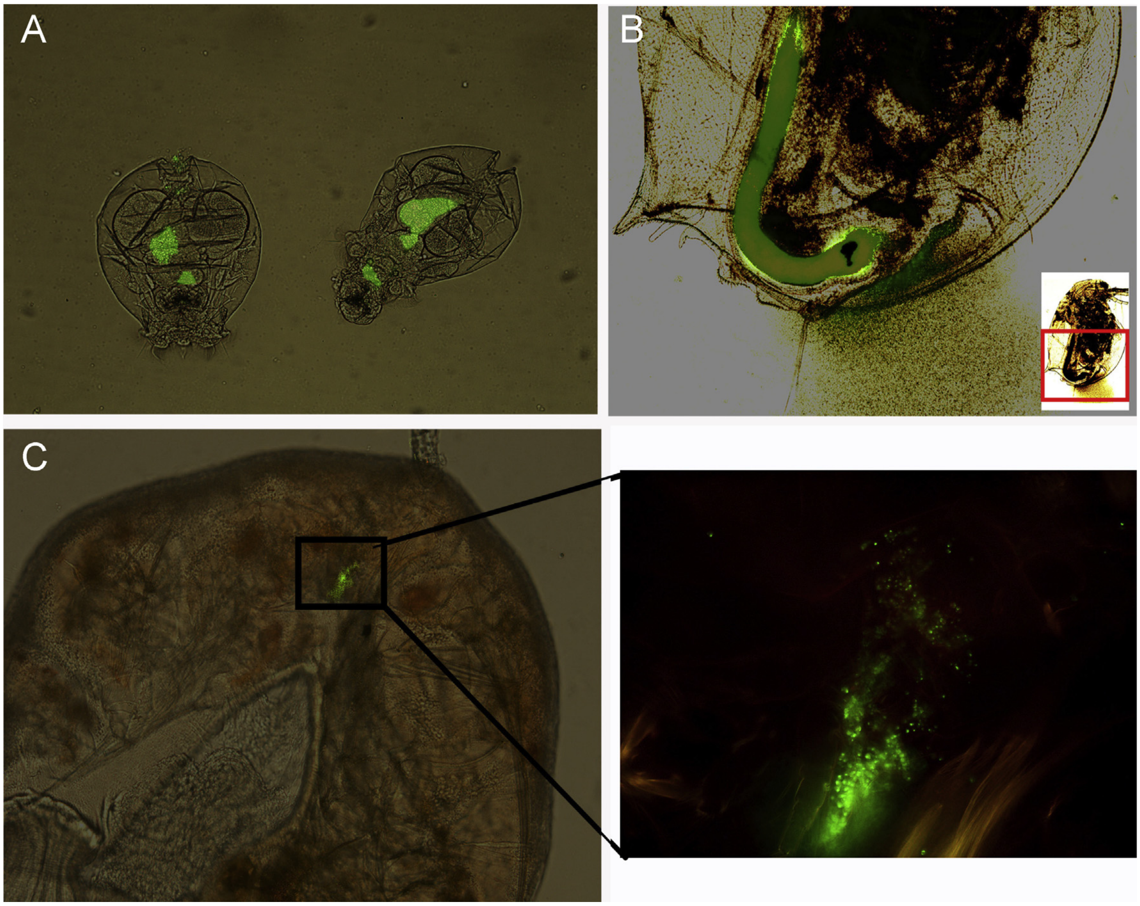

Fig. 3. Visualization of yeast location in microorganisms fed with Pichia-GFP and in flounder larvae fed with Pichia-GFP-fed microorganisms. Rotifers (A) and *Daphnia* (B) were fed with Pichia-GFP. *Daphnia* that were fed on Pichia-GFP were used for feeding of flounder larvae and GFP expression was studied in the intestine (C and lower right corner). Positive cells for GFP were found in the intestine of the larvae (C; magnification in lower right corner). The presence of GFP was analyzed by microscopy.

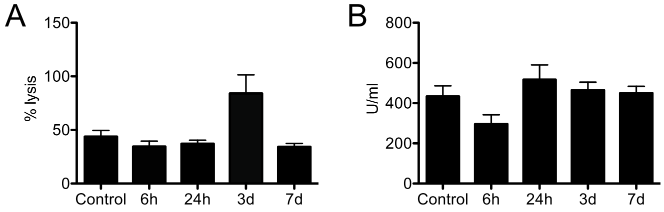

Fig. 4. Complement and lysozyme activity in gut mucus of rainbow trout after administration of *Pichia*-GFP. Gut mucus was collected at different time points post-treatment and complement hemolytic activity (A) and lysozyme activity (B) were analyzed. Graphs show data from two different experiments ($n = 7$). One-way ANOVA with a post-hoc Dunnett's test was used for statistical analysis.

in both hindgut and spleen.

The expression analysis of other immune-related genes (*c3*, *lysozyme*, *mx*, *mpo*, *mcsfR-α*, *il-1β*, *il-8*, *il-10*, *tgf-β1*) did not reflect any significant regulation in pyloric caeca (Supplementary Fig. 1), midgut (Supplementary Fig. 2), hindgut (Supplementary Fig. 3), or spleen (Supplementary Fig. 4).

4. Discussion

Vaccination has become essential for aquaculture industry in order to overcome disease outbreaks. Although several different vaccines are offered in the market, only a part of them can be applied through a mucosal delivery route. Even though mucosal vaccination has clear practical and welfare-related benefits, injectable vaccines are still the preferred option by the aquaculture industry owing to their high efficacy. Here, we present the yeast *Pichia pastoris* as vehicle for oral administration of (recombinant) antigens. Given that this microorganism

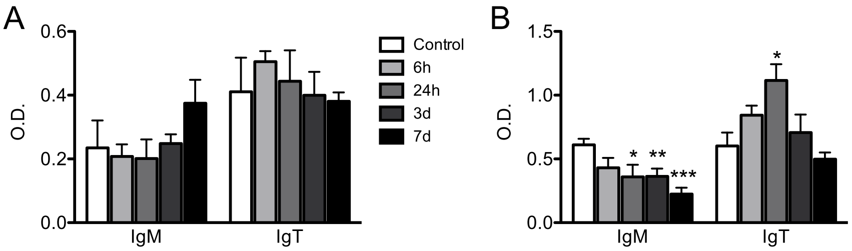

Fig. 5. Immunoglobulin levels in serum and gut mucus of rainbow trout after administration of *Pichia*-GFP. Total amount of Igm and Igt was measured by ELISA in serum (A) and gut mucus (B) at various time points after treatment. Graphs show data from three independent experiments (n = 10 samples/time point). One-way ANOVA with a post-hoc Dunnett's test was used for statistical analysis. *P* values < 0.05 were considered significant and are depicted with asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

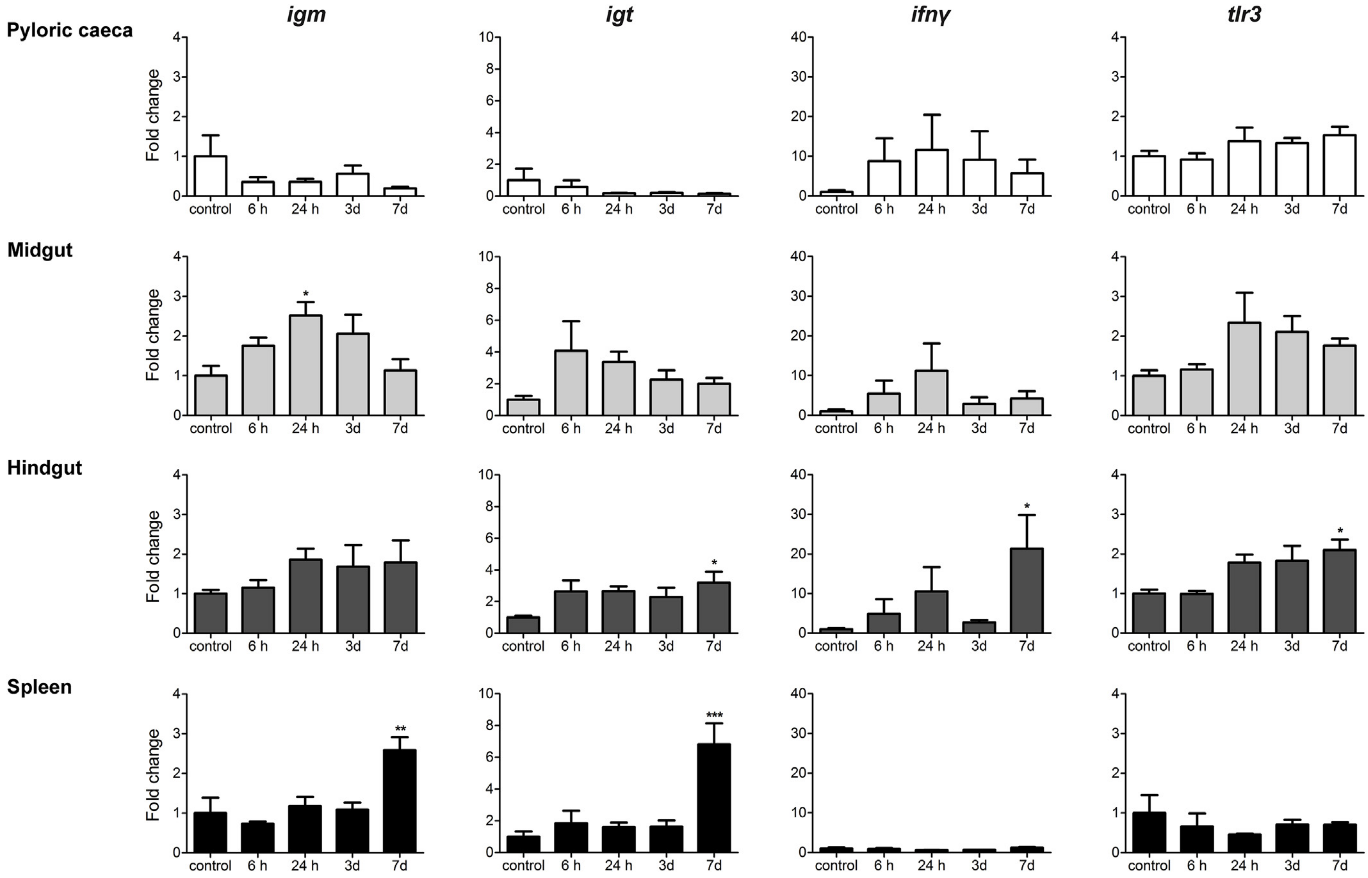

Fig. 6. Expression of immune-related genes in rainbow trout after administration of *Picha*-GFP. Pyloric caeca, midgut, hindgut, and spleen were sampled from trout at multiple time points after administration of *Pichia*-GFP. Total RNA was extracted and the expression of genes associated to humoral (*igm, igt*) and anti-viral (*ifnγ* and *tlr3*) responses was evaluated by real-time quantitative PCR. Graphs represent mean values ± SEM of two independent experiments, each using $n = 4$ fish per time point. One-way ANOVA with a post-hoc Dunnett's test was used for statistical analysis. *P* values < 0.05 were considered significant and are depicted with asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

can be genetically modified, it can express almost all desired antigens and can hereby serve as a widely-applicable vaccine vehicle. Here, although surface expression of GFP on *P. pastoris* could not be confirmed, we delivered the model antigen GFP to the intestine of several fish species. Formulations were given to the fish by either oral intubation of adult fish, or by feeding fish larvae with microorganisms themselves fed with modified yeast (*Pichia*-GFP). Interestingly, we observed that yeast alone could trigger a moderate but rapid and local immune response and, at a later stage, also a systemic response in the spleen. Early local immune responses have been demonstrated to be crucial for vaccine success [37], indicating that *P. pastoris* could be a promising vehicle for oral vaccines for fish.

Some pathogens are able to infect fish larvae at very early stages, even before the animal is capable of mounting an adaptive response, complicating vaccine administration and success of any long term vaccination regime [27]. Although some authors have proposed that vaccination of fish larvae could imply an increase in the susceptibility to infection [24], specific treatments against those pathogens that infect larvae are needed to avoid disease outbreaks. Given the small size of fish larvae, injection vaccination is not feasible and oral or immersion vaccination become the only possible routes. In this study we could show delivery of a fluorescent antigen to the intestine of flounder larvae using rotiphers and *Daphnia* fed with the *Pichia*-GFP. Although results were not fully conclusive in adults, *P. pastoris* could possibly act as an adjuvant inducing innate and protective immune responses in larvae before specific antibodies become present. Whether this type of oral vaccination method could indeed protect fish larvae from an infection early in life is an important question that needs to be addressed in further studies. In most aquaculture fish species, intraperitoneal vaccination is administrated prior to a phase of intensive culture. After vaccination, fish is kept in culture for several months, or even years, depending of the species reaching market size. Hence, a long duration of immunity is one of the main goals when formulating fish vaccines. The possibility of increasing protection in adult fish by administrating an oral boost could help to avoid important losses due to infections and would improve fish welfare. Here, we described a promising method that could be applied also at late stages during the production cycle, using *P. pastoris* as vehicle and oral vaccination as route of administration.

Many reports discuss the use of yeast as an immunostimulant and its potential use as an adjuvant in vaccine formulations [9,25,30,41]. The microbiota of the fish intestine includes a large number of yeast species [26], of which some can be used as probiotics [6]. Here, we studied innate immune responses triggered by *P. pastoris* in the intestine and in systemic organs. The complement pathway and lysozyme activity are two main innate mechanisms that protect fish in the initial phase of the immune response [13,22]. Previous studies in common carp and tilapia showed the induction of complement and lysozyme activity at a systemic level after administration of commercial yeast-based products [15,32], as well as an increase in complement activity after administration of yeast or β-glucan [30,31]. We observed a (minor) increase in hemolytic activity in trout gut mucus three days after oral delivery of *Pichia*-GFP, which was not reflected in the gene expression level of complement component 3 (C3), one of the main components of the complement pathway. However, since the role of C3 is determined by its cleavage, gene expression levels do not directly reflect complement activity. Furthermore, other (antimicrobial) complement factors, such as factor B, can be induced after a pathogen challenge [14,19] are therefore potential candidate genes for future investigations. Contrary to some studies that described an increase in lysozyme activity after yeast or β-glucan stimulation [30], we did not observe differences activity or gene expression of lysozyme after delivery of *Pichia*-GFP to trout. Differences in the source of β-glucans, fish species and time points could explain these differences.

Besides investigating the innate response induced by the administration of *Pichia*-GFP, we also analyzed Igm and Igt. While both immunoglobulins are present at mucosal and systemic sites [29,36], Igt is the main responder during infections with mucosal parasites and Igm responses are more pronounced in systemic organs [45]. The combined results after administration of *Pichia*-GFP in trout suggest an early response at the mucosal site and a later response in both intestine and spleen, which correlates with the notion that the immunization route will determine the site of the initial immune response [28]. While we did not observe differences in Ig levels in serum, a moderate increase in Igt was observed in gut mucus at 24 h. The significant decrease in total Igm levels in gut mucus could indicate an overall downregulation of the local production or increased loss (in the absence of replenishment) due to binding to Pichia. With respect to gene expression, both *igm* and *igt* were downregulated in the pyloric caeca at all tested time-points. This observation is in contrasts to those published before, where the recruitment of Igm$^+$ and Igt$^+$ B cells in the pyloric caeca was demonstrated after oral vaccination [2]. However, this increase was observed at a later time-point (10 days post-vaccination) and using another antigen vehicle (alginate particles) and antigen (DNA). Surprisingly, there was a notable increase of both *igm* and *igt* gene expression in the spleen 7 d post-treatment, hinting at a rapid reaction of both Igm$^+$ and Igt$^+$ B cells to the *Pichia*-GFP. The increased expression observed in systemic organs coincides with a slight increase, although not significant, observed in serum Igm levels. Conversely, no significant increase in total Ig levels was observed in gut mucus at 7d. Whether the increment observed in systemic organs will lead to an increase in the intestine or other mucosal sites needs to be evaluated in future studies. In the current study, total Igm and Igt levels were not increased in the mucus 7d after administration.

One interesting feature of yeast, and more specifically mainly through the expression of β-glucan on their cell surface, is the ability to induce an anti-viral-like immune responses [5,17,30]. A significant upregulation of immune genes involved in anti-viral responses (*tlr3* and *ifnγ*) was observed also in the midgut at 7 days post-treatment, which is in accordance with a previous report where *tlr3* was upregulated in the midgut of common carp that were fed a β-glucan-supplemented diet [11]. Since in our study the increase of *ifnγ* and *mx* was only significantly upregulated in the hindgut, we suggest that at the given dose, yeast acted as a mild adjuvant. Importantly, the moderate anti-viral response suggested in our study is promoted in the intestine but not in spleen. Compartmentalization of the response might be the reason of these differences, although it could also be due to a difference in the onset of the response in both tissues. Furthermore, given the moderate response at local tissues also suggests that a systemic response in distant tissues might be even less prominent.

As expected, given the highly tolerogenic environment of the intestine, expression levels of pro-inflammatory cytokines such as *il-1β* were slightly downregulated in the trout intestine after administration of *Pichia*-GFP. Accordingly, the expression of inflammatory-related genes in midgut was not modulated in carp fed with β-glucan supplemented diet after 14 days of feeding [10]. Control of the inflammatory response at mucosal sites is crucial to mantain homeostasis [12]. Similarly, we could not detect any upregulation of *mpo* nor *mcsfRα*, as neutrophil or macrophage-specific markers, respectively. This could be an indicative of a controlled infiltration of monocytes in the site of yeast uptake in the intestine, avoiding an excessive inflammatory response.

In conclusion, aquaculture being (one of) the fastest growing food industries in the world highlights the importance of controlling pathogen outbreaks. Vaccination probably is the best tool to control pathogen load without affecting product quality, such as linked with chemical or pharmaceutical treatments. However, most of the vaccines that are available for the aquaculture industry are administered by injection which can cause negative side-effects in the animal and increase the possibility of opportunistic infections due to the stress induced during vaccination. Vaccination without stress or side-effects will enhance animal welfare and will improve the final product quality while reducing costs. To obtain this, we need to improve the efficacy of reported mucosal vaccines, or develop new mucosal vaccination strategies. Here, we presented a novel method for oral delivery using *Pichia pastoris* in adult fish and, maybe even more important, in larval fish. Modified yeast can deliver antigen to the mucosal epithelium in both larval and adult stages, boosting innate immunity without inducing stress and most probably without side-effects. Taken together, the use of yeast in oral or immersion vaccine formulations may have a great impact on fish welfare and also on product quality. Future studies should aim at investigating the potential using yeast for expression and delivery of true vaccine antigens, and should investigate efficacy and long term protection. Furthermore, besides oral gavage, which allows for the administration of exact amounts of vaccine/antigen, mixing the yeast with fish feed should be included in future studies.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2018.07.033.

References

[1] M. Adomako, S. St-Hilaire, Y. Zheng, J. Eley, R.D. Marcum, W. Sealey, B.C. Donahower, S. LaPatra, P.P. Sheridan, Oral DNA vaccination of rainbow trout, Oncorhynchus mykiss (Walbaum), against infectious haematopoietic necrosis virus using PLGA [Poly(D,L-Lactic-Co-Glycolic Acid)] nanoparticles, J. Fish Dis. 35 (2012) 203–214, https://doi.org/10.1111/j.1365-2761.2011.01338.x.

[2] N.A. Ballesteros, R. Castro, B. Abos, S.S. Rodríguez Saint-Jean, S.I. Pérez-Prieto, C. Tafalla, The pyloric caeca area is a major site for IgM+ and IgT+ B cell recruitment in response to oral vaccination in rainbow trout, PLoS One 8 (2013) e66118, https://doi.org/10.1371/journal.pone.0066118.

[3] N.A. Ballesteros, S. Rodriguez Saint-Jean, S.I. Perez-Prieto, Food pellets as an effective delivery method for a DNA vaccine against infectious pancreatic necrosis virus in rainbow trout (Oncorhynchus mykiss, Walbaum), Fish Shellfish Immunol. 37 (2014) 220–228, https://doi.org/10.1016/j.fsi.2014.02.003.

[4] N.A. Ballesteros, S. Rodríguez Saint-Jean, S.I. Pérez-Prieto, C. Aquilino, C. Tafalla, Modulation of genes related to the recruitment of immune cells in the digestive tract of trout experimentally infected with infectious pancreatic necrosis virus (IPNV) or orally vaccinated, Dev. Comp. Immunol. 44 (2014) 195–205, https://doi.org/10.1016/j.dci.2013.12.009.

[5] J. Beaulaurier, N. Bickford, J.L. Gregg, C.A. Grady, A.L. Gannam, J.R. Winton, P.K. Hershberger, Susceptibility of pacific herring to viral hemorrhagic septicemia is influenced by diet, J. Aquat. Anim. Health 24 (2012) 43–48, https://doi.org/10.1080/08997659.2012.668511.

[6] M. Caruffo, N. Navarrete, O. Salgado, A. Díaz, P. López, K. García, C.G. Feijóo, P. Navarrete, Potential probiotic yeasts isolated from the fish gut protect zebrafish (Danio rerio) from a Vibrio anguillarum challenge, Front. Microbiol. 6 (2015) 1–9, https://doi.org/10.3389/fmicb.2015.01093.

[7] H. Cordero, M.F. Brinchmann, A. Cuesta, J. Meseguer, M.A. Esteban, Skin mucus proteome map of European sea bass (Dicentrarchus labrax), Proteomics J (2015) 1–29, https://doi.org/10.1002/pmic.201400614.This.

[8] A.I. de las Heras, S. Rodríguez Saint-Jean, S.I. Pérez-Prieto, Immunogenic and protective effects of an oral DNA vaccine against infectious pancreatic necrosis virus in fish, Fish Shellfish Immunol. 28 (2010) 562–570, https://doi.org/10.1016/j.fsi.2009.12.006.

[9] C.W.E. Embregts, M. Forlenza, Oral vaccination of fish: lessons from humans and veterinary species, Dev. Comp. Immunol. 64 (2016) 118–137, https://doi.org/10.1016/j.dci.2016.03.024.

[10] A. Falco, P. Frost, J. Miest, N. Pionnier, I. Irnazarow, D. Hoole, Reduced inflammatory response to Aeromonas salmonicida infection in common carp (Cyprinus carpio L.) fed with β-glucan supplements, Fish Shellfish Immunol. 32 (2012) 1051–1057, https://doi.org/10.1016/j.fsi.2012.02.028.

[11] A. Falco, J.J. Miest, N. Pionnier, D. Pietretti, M. Forlenza, G.F. Wiegertjes, D. Hoole, β-Glucan-supplemented diets increase poly(I: C)-induced gene expression of Mx, possibly via Tlr3-mediated recognition mechanism in common carp (Cyprinus carpio), Fish Shellfish Immunol. 36 (2014) 494–502, https://doi.org/10.1016/j.fsi.2013.12.005.

[12] W.S. Garrett, J.I. Gordon, L.H. Glimcher, Homeostasis and inflammation in the intestine, Cell 140 (2010) 859–870, https://doi.org/10.1016/j.cell.2010.01.023.

[13] D. Gomez, J.O. Sunyer, I. Salinas, The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens, Fish Shellfish Immunol. 35 (2013) 1729–1739, https://doi.org/10.1016/j.fsi.2013.09.032.

[14] S.F. Gonzalez, K. Buchmann, M.E. Nielsen, Complement expression in common carp (Cyprinus carpio L.) during infection with Ichthyophthirius multifiliis, Dev. Comp. Immunol. 31 (2007) 576–586, https://doi.org/10.1016/j.dci.2006.08.010.

[15] S. He, Z. Zhou, K. Meng, H. Zhao, B. Yao, E. Ringø, I. Yoon, Effects of dietary antibiotic growth promoter and Saccharomyces cerevisiae fermentation product on production, intestinal bacterial community, and nonspecific immunity of hybrid tilapia (Oreochromis niloticus female × Oreochromis aureus male), J. Anim. Sci. 89 (2011) 84–92, https://doi.org/10.2527/jas.2010-3032.

[16] J. Ji, D. Torrealba, À. Ruyra, N. Roher, Nanodelivery Systems as New Tools for Immunostimulant or Vaccine Administration: Targeting the Fish Immune System, Biology, Basel, 2015, p. 4, https://doi.org/10.3390/biology4040664.

[17] Y. sin Kim, F. Ke, Q.Y. Zhang, Effect of β-glucan on activity of antioxidant enzymes and Mx gene expression in virus infected grass carp, Fish Shellfish Immunol. 27 (2009) 336–340, https://doi.org/10.1016/j.fsi.2009.06.006.

[18] L. Li, S.-L. Lin, L. Deng, Z.-G. Liu, Potential use of chitosan nanoparticles for oral delivery of DNA vaccine in black seabream Acanthopagrus schlegelii Bleeker to protect from Vibrio parahaemolyticus, J. Fish. Dis. 36 (2013) 987–995, https://doi.org/10.1111/jfd.12032.

[19] X. peng Li, L. Sun, A teleost complement factor Ba possesses antimicrobial activity and inhibits bacterial infection in fish, Dev. Comp. Immunol. 71 (2017) 49–58, https://doi.org/10.1016/j.dci.2017.01.021.

[20] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method, Methods 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.

[21] L. Lowrey, D.C. Woodhams, L. Tacchi, I. Salinas, Topographical mapping of the rainbow trout (Oncorhynchus mykiss) microbiome reveals a diverse bacterial community with antifungal properties in the skin, Appl. Environ. Microbiol. 81 (2015) 6915–6925, https://doi.org/10.1128/AEM.01826-15.

[22] B. Magnadóttir, Innate immunity of fish (overview), Fish Shellfish Immunol. 20 (2006) 137–151, https://doi.org/10.1016/j.fsi.2004.09.006.

[23] I. Mauri, A. Romero, L. Acerete, S. MacKenzie, N. Roher, A. Callol, I. Cano, M.C. Alvarez, L. Tort, Changes in complement responses in Gilthead seabream (Sparus aurata) and European seabass (Dicentrarchus labrax) under crowding stress, plus viral and bacterial challenges, Fish Shellfish Immunol. 30 (2011) 182–188, https://doi.org/10.1016/j.fsi.2010.10.006.

[24] I. Mulero, M.P. Sepulcre, I. Fuentes, A. García-Alcázar, J. Meseguer, A. García-Ayala, V. Mulero, Vaccination of larvae of the bony fish gilthead seabream reveals a lack of correlation between lymphocyte development and adaptive immunocompetence, Mol. Immunol. 45 (2008) 2981–2989, https://doi.org/10.1016/j.molimm.2008.01.017.

[25] S. Mutoloki, H.M. Munang´andu, O. Evensen, H.M. Munang'andu, Ø. Evensen, Oral vaccination of fish- antigen preparations, uptake and immune induction, Front. Immunol. 6 (2015) 519, https://doi.org/10.3389/fimmu.2015.00519.

[26] P. Navarette, D. Tovar-Ramirez, Use of Yeasts as Probiotics in Fish Aquaculture, in: Sustainable Aquaculture Techniques, (2014), pp. 135–172.

[27] A. Nematollahi, A. Decostere, F. Pasmans, F. Haesebrouck, Flavobacterium psychrophilum infections in salmonid fish, J. Fish. Dis. 26 (2003) 563–574, https://doi.org/10.1046/j.1365-2761.2003.00488.x.

[28] D. Parra, F.E. Reyes-Lopez, L. Tort, Mucosal Immunity and B Cells in teleosts: effect of vaccination and stress. Front, Immunology 6 (2015) 1–12, https://doi.org/10.3389/fimmu.2015.00354.

[29] D. Parra, F. Takizawa, J.O. Sunyer, Evolution of B cell immunity, Annu. Rev. Anim. Biosci 1 (2013) 65–97, https://doi.org/10.1002/ana.22528.Toll-like.

[30] J. Petit, G.F. Wiegertjes, Long-lived effects of administering β-glucans: indications for trained immunity in fish, Dev. Comp. Immunol. 64 (2016) 93–102, https://doi.org/10.1016/j.dci.2016.03.003.

[31] N. Pionnier, A. Falco, J. Miest, P. Frost, I. Irnazarow, A. Shrive, D. Hoole, Dietary β-glucan stimulate complement and C-reactive protein acute phase responses in common carp (Cyprinus carpio) during an Aeromonas salmonicida infection, Fish Shellfish Immunol. 34 (2013) 819–831, https://doi.org/10.1016/j.fsi.2012.12.017.

[32] N. Pionnier, A. Falco, J.J. Miest, A.K. Shrive, D. Hoole, Feeding common carp cyprinus carpio with β-glucan supplemented diet stimulates C-reactive protein and complement immune acute phase responses following PAMPs injection, Fish Shellfish Immunol. 39 (2014) 285–295, https://doi.org/10.1016/j.fsi.2014.05.008.

[34] J.L. Romalde, A. Luzardo-Alvárez, C. Ravelo, A.E. Toranzo, J. Blanco-Méndez, Oral immunization using alginate microparticles as a useful strategy for booster vaccination against fish lactoccocosis, Aquaculture 236 (2004) 119–129, https://doi.org/10.1016/j.aquaculture.2004.02.028.

[36] I. Salinas, Y.-A. Zhang, J.O. Sunyer, Mucosal immunoglobulins and B cells of teleost fish, Dev. Comp. Immunol. 35 (2011) 1346–1365, https://doi.org/10.1016/j.dci.2011.11.009.

[37] F. Sallusto, A. Lanzavecchia, K. Araki, R. Ahmed, From vaccines to memory and back, Immunity 33 (2010) 451–463 https://doi.org/10.1016/j.immuni.2010.10.008.

[38] L. Tacchi, R. Musharrafieh, E.T. Larragoite, K. Crossey, E.B. Erhardt, S. a. M. Martin, S.E. LaPatra, I. Salinas, Nasal immunity is an ancient arm of the mucosal immune system of vertebrates, Nat. Commun. 5 (2014) 5205, https://doi.org/10.1038/ncomms6205.

[39] C. Tafalla, J. Bøgwald, R. a Dalmo, Adjuvants and immunostimulants in fish vaccines: current knowledge and future perspectives, Fish Shellfish Immunol. 35 (2013) 1740–1750, https://doi.org/10.1016/j.fsi.2013.02.029.

[40] J. Tian, X. Sun, X. Chen, J. Yu, L. Qu, L. Wang, The formulation and immunisation of oral poly(DL-lactide-co-glycolide) microcapsules containing a plasmid vaccine against lymphocystis disease virus in Japanese flounder (Paralichthys olivaceus). Int, Immunopharmacology 8 (2008) 900–908, https://doi.org/10.1016/j.intimp.2008.02.006.

[41] E. Vallejos-Vidal, F.E. Reyes-López, M. Teles, S. MacKenzie, The response of fish to immunostimulant diets, Fish Shellfish Immunol. 56 (2016) 34–69.

[42] K.R. Villumsen, L. Neumann, M. Ohtani, H.K. Strøm, M.K. Raida, Oral and anal vaccination confers full protection against Enteric Redmouth Disease (ERM) in rainbow trout, PLoS One 9 (2014) e93845, https://doi.org/10.1371/journal.pone.0093845.

[43] Z. Xu, D. Parra, D. Gómez, I. Salinas, Y.-A. Zhang, L. von Gersdorff Jørgensen, R.D. Heinecke, K. Buchmann, S. LaPatra, J.O. Sunyer, Teleost skin, an ancient mucosal surface that elicits gut-like immune responses, Proc. Natl. Acad. Sci. U. S. A 110 (2013) 13097–13102, https://doi.org/10.1073/pnas.1304319110.

[44] Z. Xu, F. Takizawa, D. Parra, D. Gómez, L. von Gersdorff Jørgensen, S.E. LaPatra, J.O. Sunyer, Mucosal immunoglobulins at respiratory surfaces mark an ancient association that predates the emergence of tetrapods, Nat. Commun. 7 (2016) 10728, https://doi.org/10.1038/ncomms10728.

[45] Y.-A. Zhang, I. Salinas, J. Li, D. Parra, S. Bjork, Z. Xu, S.E. LaPatra, J. Bartholomew, J.O. Sunyer, IgT, a primitive immunoglobulin class specialized in mucosal immunity, Nat. Immunol. 11 (2010) 827–835, https://doi.org/10.1038/ni.1913.