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An evaluation of microalgae as a recombinant protein oral delivery platform for fish using green fluorescent protein (GFP)

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ABSTRACT

Recombinant proteins produced by biological systems such as bacteria, yeasts, mammalian and insect cell cultures are widely used for clinical or industrial purposes. Most therapeutic protein drugs require purification, cold chain, and injection, which make them prohibitively expensive and hinders their widespread use. Here, we describe a new economical oral vaccination platform using algae and evaluated its potential for the delivery of recombinant drugs using GFP expressed in the chloroplast of algal cells. The transplastomic algae expressing recombinant GFPs were freeze-dried for long-term storage at ambient temperature and for easy handling in feeding. GFPs bioencapsulated by lyophilized *Chlamydomonas reinhardtii* were found intact without degradation for several months at ambient temperature. The expression level of GFP in the lyophilized algae was estimated at 0.47 μg/mg dry weight. The GFPs bioencapsulated and orally delivered to *Danio rerio* were immunostained and observed in the intestinal tissues using a confocal microscope. Furthermore, the uptaken GFPs in the intestine were detected in the blood using ELISA and the detected level was 5.4 ng of GFP/μl of serum.

These results demonstrate that microalgae can be a viable protein production and oral delivery system to vaccinate fish. The results give greater justification to continue exploring the concept of microalgal-based oral vaccines. The potential of the technology would greatly benefit aquaculture farmers by providing them with affordable, environmentally sustainable, and user-friendly vaccines.

1. Introduction

Aquaculture is the most rapidly growing food sector in the world, estimated to be worth over 200 billion USD, growing at a rate of approximately 5% per annum, and producing more than 100 million metric tons of seafood annually [1,2]. The World Bank's Agriculture and Environmental Services Division predicts that by 2030, more than 62% of the seafood consumed on the market will be farmed seafood [3]. Despite the unprecedented growth of the industry, aquaculture farmers, especially in developing nations, are facing a crisis with managing disease, losing more than 4 million metric tons of productivity due to bacterial, viral, and parasitic infections each year [3]. Based on the OECD's estimates, the loss is large enough to feed an entire nation of over 90 million people for an entire year [4].

While farmers can use antimicrobial compounds to treat bacterial and parasitic infections, they cannot be used for the treatment of viral infections [5]. As an alternative approach, vaccination is much more economical and environmentally sustainable, and, unlike antibiotics, can be used to prevent viral infections [5,6]. Many private companies have tried to respond to the increased demand for effective animal health products by providing preventative vaccines against several economically significant diseases. Some of the leading aquaculture vaccine products were produced by companies such as Zoetis, Merck, Intervet, Novartis, Schering-Plough, and Bayer [7,8]. The aquatic animal health market is estimated to be worth approximately 500 million USD in 2018, and growing at a rate of 8% year-over-year [9].

Although intraperitoneal injection vaccines induce strong protective immunity against many significant diseases by precise dosage administration with minor vaccine loss, they have several important limitations which hinder widespread adoption. Injection vaccines require cold storage/transportation and professional labor to administer, which create socio-economic barriers for adoption in developing countries [7]. In addition, injection-based vaccination can induce stress to fish regardless of their size and pose the risk of additional infection through lesions produced by the injection procedure. Moreover, induction of stress-associated immune response suppression by injection,

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particularly for small-sized fish, accounts for the infectious outbreaks during the on-growing stage [5]. Currently, injectable vaccines are only commercially viable for high value fish species. The vast majority of vaccines sold on the market are for salmon, trout, or sea bass in Europe and North America [10]. Oral vaccines have the potential to make vaccination economically feasible for a much larger and diverse range of fish species which are currently underserved by the animal health industry. With oral administration, there are no self-injection safety hazards, no cold storage requirements, and no need to hire professional labor [6].

Despite the clear advantages of oral vaccines, there are very few available on the market because they generally display poor efficacy. Out of the 17 most widely studied viral vaccines for fish, only two of them were oral-based [11]. Still, several animal health firms have developed or licensed technologies to address the challenges of oral delivery. For instance, Merck Animal Health have a proprietary encapsulation technology that has been shown to improve antigen stability and protective immunity in rainbow trout challenged with Enteric Redmouth Disease [12,13]. Other companies, such as Centrovet, have licensed MicroMatrix technology to encapsulate their oral vaccines, which are also sold with some commercial success in Latin America [13]. Still, the market remains highly segmented and underserved, because oral vaccines generally show weak or short protection [6]. Most oral vaccines are ineffective due to palatability issues, low antigen stability in the gastrointestinal tract, poor bioavailability, oral tolerance, or failure to elicit an appropriate immunity response [6].

Microalgae as a protein production and drug delivery platform have the potential to make the aquaculture animal health industry more sustainable and profitable by solving many of the existing technical and commercial challenges with oral vaccines [14]. Algae are generally recognized as safe (GRAS) by the U,S. Food and Drug Administration (FDA) [15], which means algae can be safely used as a fish feed additive. As recognized in plant production systems, recombinant proteins made using algae can be produced with less capital input, and no risk of contamination with animal pathogenic agents [16]. In addition, the commercial-scale production of microalgae is already feasible [14,16–18]. Moreover, microalgae are a natural part of the fish diet and an essential nutrient source for many commercially harvested fish species [19]. They are a rich source of natural antimicrobial compounds, immunostimulants, and other essential nutrients. They are routinely used in aquaculture farms to improve fish health [20–22]. Consequently, the fish have no palatability issues consuming microalgae, since they can easily be digested by commensal bacteria upon arrival to the gut [23,24]. In addition, many commercially valuable vaccine antigens [25–38] and high-value molecules [39–52] can be expressed and bio-encapsulated by the rigid cell wall of the microalgae [16,17], which provide protection to proteins expressed in the microalgal cells and allows them to remain intact after they are consumed [30]. However, the cell wall can still be broken down in the hind gut by commensal bacteria [53–59], and consequently, the recombinant proteins are released. Once freeze-dried, the bio-encapsulated recombinant proteins can eliminate the need for cold storage and transportation, which is required for injectable vaccines [30]. These robust advantages make microalgae an excellent oral delivery platform for recombinant proteins. Oral delivery of vaccine antigens is much more suitable for large-scale vaccination than the parenteral method since it can stimulate both mucosal and systemic immunity at the entry point against pathogens [60]. The controlled production of protein therapeutics by culturing transgenic algae in closed bioreactors can also eliminate any possible transgene flow into environment [14,16,61,62]. The advantages of algal expression systems can be further improved using chloroplast transformation of transgenes, which can avoid several disadvantages caused by nuclear transformation such as transgene silencing, lower yields, and potential risk of gene escape [14,16]. Despite the obvious advantages of using microalgae as an oral delivery platform for fish, the concept remains largely unrealized and poorly understood. In this study, we orally delivered whole-cell, lyophilized transplastomic microalgae (*Chlamydomonas reinhardtii*) expressing GFP to larval and adult zebrafish to assess whether recombinant proteins could be orally delivered and uptaken in the tissues and circulatory system of the model fish, *Danio rerio*.

2. Materials and methods

2.1. Creation, cultivation, and lyophilization of Chlamydomonas reinhardtii expressing GFP

Codon-optimized GFP was synthesized as described in a previous report [63], and the synthetic coding region was cloned into a transformation vector downstream of the *psb*D promoter/5'UTR and upstream of the *rbc*L 3′ UTR. The GFP expression cassette was flanked by homologous DNA sequences adjacent to the psbH locus called the 3HB site which would facilitate recombination of the expression cassette into the chloroplast genome [40]. The kanamycin-resistance gene *aphA6*, under the control of the *atp*A promoter/5′ UTR and *rbc*L 3′ UTR was subcloned into the 3HB vector [64]. Cultured CC-2937 cells as described previously [65] was used for chloroplast transformation using particle bombardment [41].

The two *C. reinhardtii* strains (wild-type and chloroplast transformant *psb*D-GFP) were cultivated in Tris-acetate-phosphate (TAP) medium with revised trace recipe [66] in illuminated incubators at 100 μmol/m^2/s, 120 rpm shaking and 25 °C. At pilot-scale, the strains were grown in an illuminated, temperature controlled room with a single-use hanging bag system provided by Supreme Health (Nelson, New Zealand). For each strain, two cultivation bags of the system were filled with 25 L of autoclaved TAP medium each, sparged with air from the bottom and illuminated with 100 μE $m^{-2}s^{-1}$ using ten Osram Lumilux Cool daylight fluorescence tubes. The cultures were cultivated for three days at 25 °C and harvested at the end of the logarithmic phase by centrifugation, with growth monitored by optical density at 750 nm. The wet cell pellets were frozen at −20 °C and then lyophilized using an Edwards Modulyo freeze dryer.

2.2. Evaluation of GFP level in lyophilized transplastomic C. reinhardtii

To quantitate GFP protein expression, immunoblot and densitometric assays were performed using anti-GFP antibody with known amounts of standard GFP proteins. For total protein extraction, powdered lyophilized *C. reinhardtii* cells expressing GFP were suspended in extraction buffer (100 mM NaCl, 10 mM EDTA, 200 mM Tris-Cl pH 8.0, 0.05% (v/v) Tween-20, 0.1% SDS, 14 mM β-ME, 400 mM sucrose, and proteinase inhibitor cocktail) in a ratio of 10 mg per 300 μl and were sonicated (pulse on for 5 s and pulse off for 5 s, Q125, QSonica, USA) twice after vortexing (~30 s). Total homogenate proteins were quantified using a Bradford assay and were loaded after the samples were mixed with Laemmli buffer and heated for 15 min at 70 °C, then separated on SDS-polyacrylamide with known amounts of GFP (Vector laboratories MB-0752-100). To detect the GFP, nitrocellulose membrane was immunoprobed with mouse monoclonal anti-GFP antibody (1:5000 in 1X PBST: 0.1% Tween-20, EMD MILLIPORE MAB3580, USA) and then goat anti-rabbit IgG-HRP secondary antibody (1:4000 in 1X PBST, Southern Biotechnology, 4030-05, USA). Signals developed on X-ray films were quantitated using Image J software (IJ 1.46r; NIH).

2.3. Immunostaining and confocal imaging of transplastomic Chlamydomonas reinhardtii expressing GFP

To observe GFP expressed in lyophilized, transplastomic *Chlamydomonas reinhardtii*, 20 mg of the powder were fixed using 2% formaldehyde solution. The samples were incubated at 4 °C overnight. Afterwards, the solution was aspirated and then washed with PBS three times. Chick anti-GFP, diluted 1:200 with blocking solution (PTB): 1X

PBS, 0.3% triton X-100, 4% bovine serum albumin and 0.1% NaN_3, was then added and vortexed, then placed on a rocker in 4 °C for 24 h. The samples were then washed 3 times with 1X PT buffer (PBS, 0.3% triton X-100) and were incubated with donkey anti-chick (diluted with blocking solution of 1:200, Jackson ImmunoResearch Laboratories, Inc.) and DAPI (diluted 1:200 with PTB) on a rocker overnight at 4 °C. The antibody-treated samples were washed with 1X PT buffer 3 times and soaked in 80% glycerol for 1h, then mounted on a slide. A drop of 100% glycerol was placed on top of a microscope slide and surrounded by a thin strip of petroleum jelly, then a sample of algae was placed on top and covered with a slide cover. The fixed and immunolabeled algae was then imaged using Zeiss LSM 710 confocal microscope.

2.4. Spawning, maintenance, and imaging of wild type Danio rerio larvae

Adult zebrafish bred in-house were raised in recirculated tank water kept at 26 °C with a 12 h light cycle. temperature, salinity, pH, and oxygen levels were checked daily and kept within range. Male and female wild type zebrafish grown in holding tanks were taken and placed in crossing tanks before end of day. Next morning, laid and viable eggs from wild type zebrafish *(Danio rerio)* were collected and washed of debris, then stored in a 27 °C incubator in a petri dish with egg water (6.0 g sea salt, 1.5 g $CaSO_4$, 20 l distilled water). Fry were allowed to hatch and develop, and after 36 h, PTU (0.003% 1-phenyl-2-thiourea in 10% Hanks Saline) was added to stop production of melanin in scales. At 1–2 days post fertilization, fish eggs were separated into four groups with equal numbers of fish in each group, then allowed to hatch.

At 7 days post fertilization, they were fed based on the following regimens: Group I was fed a diet of GFP-expressing *C. reinhardtii*, Group II was fed wild type *C. reinhardtii*, Group III was fed commercial fry food (BioMarine Artemac fry food, USA), and Group IV was not fed anything. The feeding rate for each feed was approximately 2% body weight of the fish. The algae were fed to fish without formulation with fish feed. After approximately 2 h, the fish were anesthetized using 0.4 mg/ml tricaine (Sigma, USA) solution. Embryos were placed in 1.5 ml Eppendorf tubes. Excess water was aspirated out and then embryos were fixed by adding 1 ml of 2% formaldehyde solution (940 μl PEM, 60 μl 37% formaldehyde) and placed on a rocker at 4 °C overnight for 24 h. The fish were then removed from solution and washed in PBS. The outer ventral skin was removed to expose the gut and other organs for immunostaining. Embryos were soaked in blocking solution for 24 h at 4 °C on a rocker, then soaked in primary antibody (1 in 200 with blocking solution) for 24 h at 4 °C. Fish were then washed three times with 1X PT (0.3% Triton) 30 min each. Secondary antibody (1 in 200 with blocking solution) and DAPI (1 in 200 with blocking solution) was added to the specimens and incubated at 4 °C overnight on a rocker. Fish were then washed with 1X PT 3 times, then placed in 80% glycerol solution. Mounting was done as described above. Embryos were placed in 100% glycerol ventral side up and a slide cover was placed on top. Embryos were then imaged using a Zeiss LSM 720 confocal microscope.

2.5. Immunostaining and confocal imaging of Danio rerio intestinal tissues

Albino adult wild type zebrafish 1 g in size were pulled out of stock tanks and placed in tanks inside the lab at room temperature, allowing the fish to acclimate to room temperature for 24 h and then were fed GFP algae or untransformed wild type algae at a feeding rate of approximately 2% body weight. Two hours later, animals were anesthetized using 3–5 ml of tricaine solution and were placed in 1.5 ml Eppendorf tubes filled with 2% formaldehyde, then incubated at 4 °C overnight on a rocker. Fish were removed from fixing solution and then washed in PBS. Using precision forceps under a dissecting microscope, the ventral skin was removed, exposing the gut cavity. Gut tissues, including the stomach, intestine, pancreas, gallbladder, and liver were removed. Gut was placed back into Eppendorf tubes containing blocking solution for 24 h at 4 °C on a rocker. Blocking solution was removed and chick anti-GFP primary antibody (diluted 1:200 in blocking solution) was added and incubated at 4 °C on a rocker overnight. The next day, the gut was washed 3 times with 1X PT over 2 h and both donkey anti-chick secondary antibody and DAPI were added (diluted 1: 200 in blocking solution) then incubated overnight at 4 °C on a rocker. Intestinal tissue was washed with 1X PT 3 times over 2 h then placed into 80% glycerol solution. The tissue was prepared as described above and observed under a Zeiss LSM 720 confocal microscope.

2.6. Evaluation of uptake of orally delivered GFP bioencapsulated in Chlamydomonas reinhardtii using GFP ELISA kit

Two groups of apparently healthy adult wild type *Danio rerio* were obtained from the in-house breeding program at Sanford Burnham Prebys for the study and separated into two groups. Group A contained 20 adult *Danio rerio* which were fed lyophilized *C. reinhardtii* expressing GFP each day for 5 days. Group B contained 14 adult fish which were fed a commercial fish diet for 5 days. The feeding rate of the lyophilized algae-GFP powder was approximately 2% body weight (average body weight was ~1g). Both the treatment and control groups were anesthetized, then sacrificed and bled via the heart cavity 2 h after the last feeding using non-heparinized capillary tubes (0.55 μm, ThermoFisher, USA). Approximately 100 μl of blood from 20 zebrafish fed with algae-GFP, and 50 μl of blood from 14 unfed zebrafish were harvested. The collected blood samples were kept in clot activation gel tubes (BD Vacutainer, Thermofisher, USA) and were allowed to clot for 30 min at room temperature, then subjected to centrifugation for 5min at 10,000 rpm. The harvested volumes of sera were 14 μl and 6 μl for algae-GFP fed and non-fed, respectively. Each serum was combined with a dilution solution provided by ELISA kit manufacturer (Cell Biolabs, USA) to the final volume of 50 μl each. Therefore, the serum sample from zebrafish fed with algae-GFP was diluted 3.57 fold and the sample from unfed was diluted 8.33 fold.

3. Results

3.1. Evaluation of transplastomic algae expressing GFP

For a bioavailability study of recombinant proteins bioencapsulated in *C. reinhardtii* and orally delivered to fish, lyophilized transplastomic *C. reinhardtii* expressing codon-optimized GFP [63,67] was used. The expression of GFP was driven by *psb*D promoter and 5'UTR, and *rbc*L 3′ UTR. To check the intactness of the GFP, the fluorescence signals were observed under the confocal microscope. As shown in Fig. 1A, strong GFP signals were detected from the transplastomic algae expressing GFP, while the untransformed algae showed only background noise due to the autofluorescence by chlorophylls. To further confirm the intactness of GFP expressed in the chloroplast, proteins extracted from the lyophilized materials were run on SDS-PAGE and immunoprobed with anti-GFP antibody. The GFP was detected at the expected size, 26 kDa, as seen in Fig. 1B, while there was no band detected from untransformed wild type *C. reinhardtii*. In addition, there were no cleaved bands detected below the expected molecular weight of GFP, which indicates that the expressed GFP in the chloroplast of *C. reinhardtii* exists intact. The expression level of GFP was calculated approximately at 0.47 μg/mg dry weight using a densitometry assay (Fig. 1B). The lyophilized transplastomic *C. reinhardtii* expressing GFP was used to study whether orally delivered GFP bioencapsulated by *C. reinhardtii* to zebrafish can reach the gut.

3.2. Immunostaining and confocal imaging of Danio rerio larvae fed Chlamydomonas reinhardtii expressing GFP

To observe the uptake of GFP into the gut and the surrounding tissue, zebrafish larvae were immunostained using anti-GFP antibody and the detected locations of GFP were imaged using a confocal

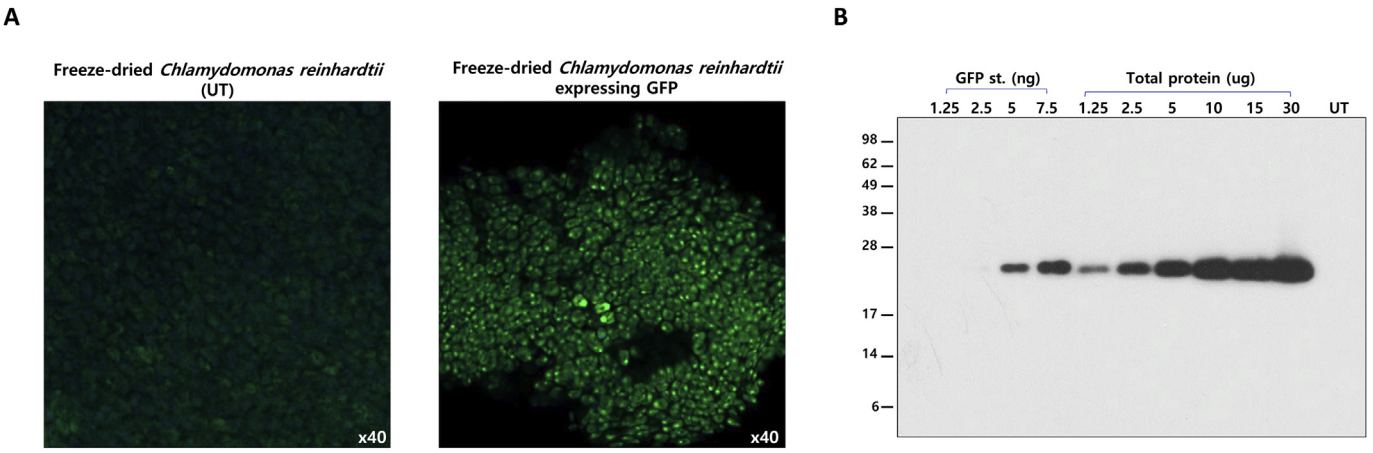

Fig. 1. Evaluation of GFP intactness and level of the expression in lyophilized transplastomic *Chlamydomonas reinhardtii*. (A) Confocal images for observation of GFP signals. Transplastomic *C. reinhardtii* expressing GFP was observed under the confocal microscope and the images were captured at the 40x magnification. UT, untransformed wild type Chlamydomonas. (B) Densitometry assay using western blot for the evaluation of level of GFP expressed in chloroplast of the lyophilized *C. reinhardtii*. Extracted total proteins were loaded as indicated and probed with anti-GFP antibody (1 in 5000). Known amounts of GFP protein were loaded for the standard curve to quantitate the expressed GFP (26 kDa).

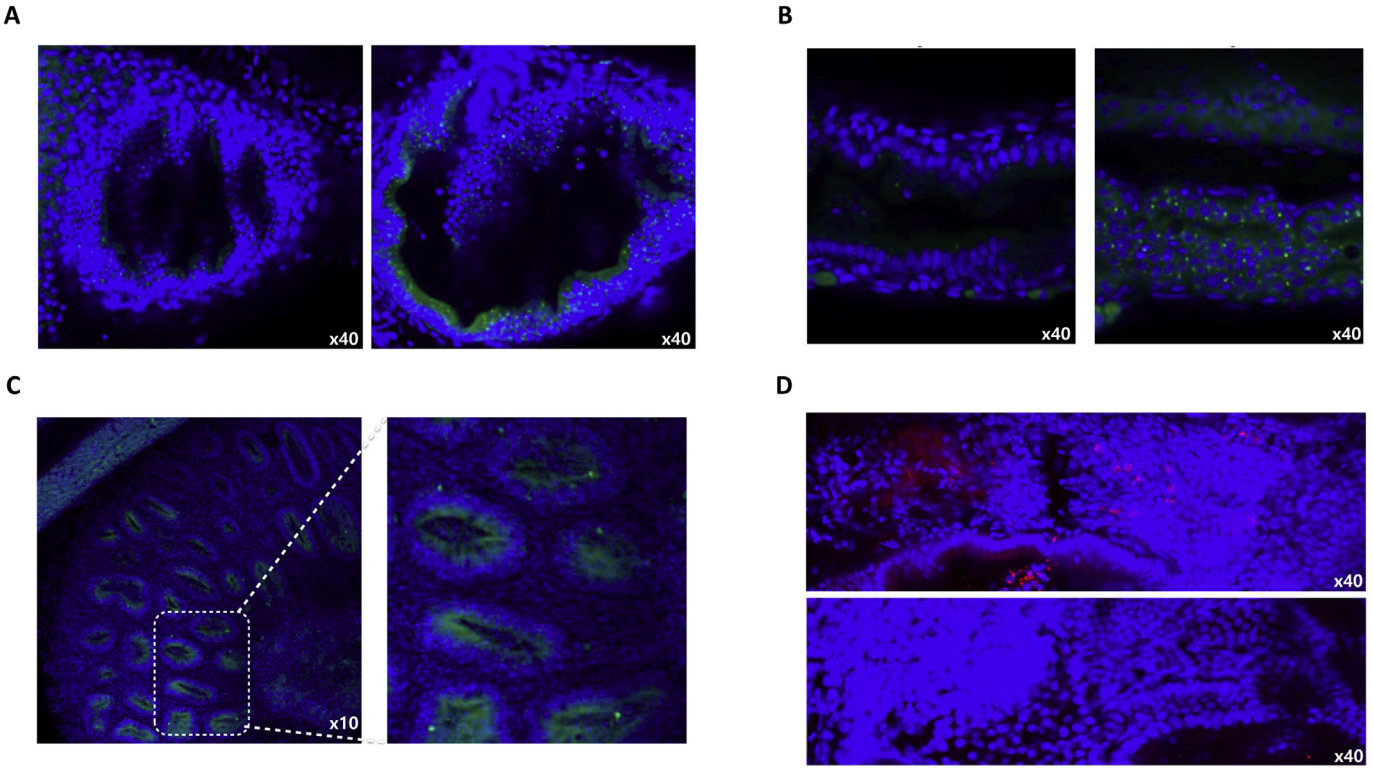

Fig. 2. Immunostaining and confocal imaging of GFP orally delivered detected GFP signals within adult and larval *Danio rerio* intestinal tissues post-feeding. (A) Transverse cross-section of intestinal tissues from *Danio rerio* fed untransformed *C. reinhardtii* (left), or *C. reinhardtii* expressing GFP (right) at 40x magnification. (B) *Danio rerio* fed untransformed *C. reinhardtii* (left), or *C. reinhardtii* expressing GFP (right) at 40x magnification. (C) Transverse cross-section of adult *Danio rerio* intestine after oral feeding of *C. reinhardtii* expressing GFP (left). Image enlarged for detail (right). (D) Lateral cross-section of larval *Danio rerio* intestine after oral feeding of *C. reinhardtii* expressing GFP (top) and untransformed *C. reinhardtii* expressing (bottom).

microscope. Images were taken in z-stack and analyzed. For this, three independent experiments were performed and the results showed similar image patterns. During the first and second round of imaging, fluorescence was seen in the form of small particles in intestinal tissue past the mucosal lining, found in between the epithelial cells surrounding the outside of the intestine (Fig. 2A and B, right image each). Larvae fed wild type algae showed background autofluorescence in the mucosal lining of the intestine as well as in various random areas outside the gut (Fig. 2A and B, left image each). GFP protein infiltrating the surrounding tissue was also observed in multiple stages of the intestine, and not just in one specific section (Fig. 2C). To counteract green autofluorescence, the third round of staining was done using a secondary antibody in the 647 spectrum, which fluoresces red. When imaged, GFP protein could be seen past the mucosal lining in the surrounding epithelial cells as well as in the surrounding mesodermal tissue of the lateral muscle of the fish (Fig. 2D).

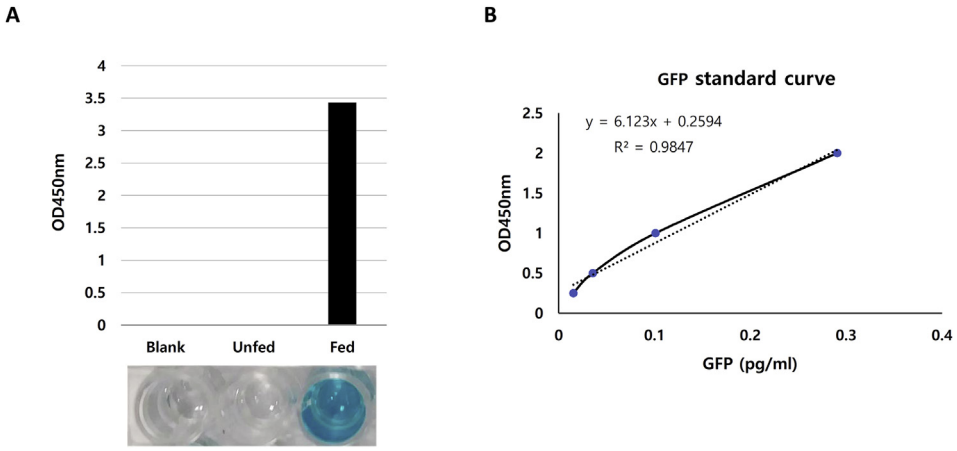

Fig. 3. Evaluation of GFP level in blood of zebrafish using ELISA. (A) ELISA assay for the evaluation of GFP levels in pooled serum from zebrafish orally fed with lyophilized *C. reinhardtii* expressing GFP. Detection of GFP was performed using GFP ELISA kit. Please see materials and methods section for detail. Unfed, pooled serum collected from zebrafish untreated; Fed, pooled serum collected from zebrafish orally-fed with lyophilized *C. reinhardtii* expressing GFP. (B) Plot of optical density values at 450 nm for quantification of GFP based on standard curve. Solid line shows data points. Broken line represents trend line.

3.3. GFP uptake in zebrafish after oral delivery of lyophilized algal cells

To evaluate the systemic GFP level, 1 g zebrafish housed in tanks were fed lyophilized *C. reinhardtii* cells expressing GFP, which were sprayed using a spatula once per day for 5 days. The blood from both orally-fed zebrafish with *C. reinhardtii* expressing GFP and zebrafish with no treatment was collected 2 h after the last oral feeding and pooled due to the low volume-to-weight ratio in zebrafish. Then the serum samples were separated from the blood for the GFP detection. Due to the low volume of blood available, all the obtained serum samples were used for ELISA in singlet. As seen in Fig. 3A, the serum obtained from zebrafish orally-fed with *C. reinhardtii* expressing GFP showed a strong OD 450 value, up to 3.5, and the quantification result based on the standard curve (Fig. 3B) showed that the amount of GFP in zebrafishserum was 5.4 ng/ul.

4. Discussion

In this study, we confirmed the potential use of algae for the delivery of protein drugs via oral administration to fish. The orally delivered, bioencapsulated GFP was detected in both intestinal and mesoderm tissues using immunostaining and confocal imaging (Fig. 2). The presence of systemically circulating GFP was also confirmed in the blood serum (Fig. 3). We have demonstrated that microalgae can function as an effective oral delivery platform and could be used as an alternative to injection delivery of biologically active proteins to aquatic organisms.

The major hurdle in the oral delivery of protein drugs is their degradation by acid and proteases in the harsh environments of the stomach and intestines, respectively. However, the recombinant proteins bioencapsulated by the microalgal cell wall can be protected from the acidic stomach and can then be released through cell wall degradation [30] by cellulolytic bacteria in the gut [53–59]. In addition, algae are routinely used as both a nutritional additive and an immunostimulant [20–22], so using algae as an oral delivery system can provide additional benefits for fish. As seen in Fig. 2, the GFP signals were detected in the intestines and inside of intestinal cells, which indicates that the orally delivered proteins were protected until they were released in the gut. Furthermore, the strong GFP signal was detected in the blood of adult zebrafish which were orally fed with lyophilized *C. reinhardtii* expressing GFP (Fig. 3), which means that the released GFPs were uptaken by intestinal cells and reached systemic circulation. Here, we showed the potential of algae as an oral delivery platform for recombinant proteins using stomachless zebrafish. However, for the universal application of this delivery platform, further studies are required for both herbivorous and carnivorous fish with or without a stomach.

In this study, there was no control experiment with naked GFP protein alone. There was a technical difficulty in preparing an appropriate feeding formulation for the GFP protein only as a control. We fed the lyophilized algae powders expressing GFP to fish without being formulated with fish food. In previous experiments with stomachless carp done by both Companjen et al. and Rombout et al. [68,69], they used oral or anal intubation methods for the delivery of naked GFP proteins, which can potentially be used for larger fish without injury, but such methods would not be feasible for small fish like zebrafish, which are less than 1 g in weight and 4 cm in length. We already confirmed that there was no palatability issue for zebrafish to eat the lyophilized algae. In our preliminary study, zebrafish could eat up to 2% body weight of lyophilized algae. We fed the zebrafish lyophilized algae powder without formulation with fish feed to mimic natural feeding conditions.

If the naked GFPs are orally or anally delivered to zebrafish, it is assumed that considerable amounts of the naked GFPs would be able to survive in the zebrafish intestine and be uptaken by epithelial cells because of the lack of an acidic stomach in zebrafish. There is an anterior intestine called the intestinal bulb which has a larger lumen space than the posterior intestine, so it can function as a reservoir comparable to a stomach. But the bulb has no gastric glands and therefore does not have acidic pH [70]. According to Nalbant et al.'s study, the pH of zebrafish intestines never goes below 7.5 under homeostatic conditions [71], but when any proteins reach the intestines, they are subject to degradation due to digestive proteases [72]. Therefore, it is assumed that GFP would survive for some time. As shown in the Companjen et al.'s study [68], carp, which also has no stomach like zebrafish, was anally intubated with GFP protein alone and the intubated GFP proteins were still detected after more than 6 h in the intestines.

In Rombout et al.'s study [69], the first and second gut section of stomachless carp can absorb most of the intact macromolecules. Enterocytes in the second gut, which are considered to have a similar function to mammalian M cells, help facilitate the transfer of antigens to immune cells. In the study, HRP was taken up via a selective route. The HRPs orally or anally intubated to carp were captured by receptors in coated pits or by saccular invagination on the enterocyte surface. The proteins were then internalized and the pinched-off, coated vesicles were transported through the lamellar folding to the intercellular space, and ultimately into the blood. Ferritin appears to be taken up non-selectively via pinocytosis and then accumulated in vesicles or vacuoles, which are then fused with lysosome-like bodies in the second segment of gut. The absorbed ferritin reached the large supranuclear vacuole and was phagocytized by intra-epithelial macrophages, some of which seem to migrate to the spleen.

Consequently, we assume that the orally delivered GFP in our study, regardless of whether it is bioencapsulated or not, can reach the immune cells via a similar pathway to ferritin. Even if massive oral

vaccination of stomachless fish could be done using naked antigen proteins due to having no stability issues with the orally delivered antigens, a substantially large amount of purified vaccine proteins should be secured using either biological fermenter systems or chemical synthesis. The protein antigens would then need to be extracted or isolated, and purified. In addition, those purified proteins require cold storage until use. Such expensive production and storage systems are impractical and unaffordable for large-scale vaccination. However, if we use algae as a production and delivery platform, we can provide very cheap vaccine products to the aquaculture industry. The scale-up of algae is very easy and fast, and there is no need for the purification of recombinant proteins because algae are a natural part of the fish diet, so they can be directly fed to fish [16]. The recombinant proteins encapsulated by the algal cell walls are protected from the stomach environment and can be released in the intestines with the help of cellulolytic commensal bacteria [53–59]. In addition, lyophilized algae can be stored at room temperature for several years [27,30], which further decreases the product price via elimination of cold storage and transportation. Furthermore, the lyophilization can also increase potency by concentrating the recombinant therapeutic proteins through the removal of water, allowing for more flexible vaccination regimens [14].

Our algal-based delivery platform is aimed for oral vaccination of commercial fish such as sea bass or salmon, which have an acidic stomach. In Dreesen et al.'s report [30], a recombinant fusion protein consisting of the cholera toxin B subunit (CTB) fused with D2 fibronectin-binding domain of *Staphylococcus aureus* (CTB-D2), was expressed in *C. reinhardtii* and the lyophilized algae was shown in the study to protect the fusion protein from a stomach-mimicking environment (37 °C, pH 1.7, 0.5 mg/ml pepsin, 20 min). We also expect a similar protective effect when we apply our algal-based vaccine to commercial fish. .

For this study, lyophilized algal materials which were produced using small-scale photobioreactor. The cost for cultivation and lyophilization for small scale biomass increase of algae is very expensive; e.g., a 300 l production of 300 g of lyophilized algae materials costs several thousand euros. However, if massive biomass increase is conducted, the cost can be dramatically reduced. According to a previous study on economic incentives for vaccination of either 1 million Atlantic salmon or sea bass, the combined cost for vaccination, such as prices for vaccine, vaccination labour and equipment, is €0.132/fish and €0.051/fish, respectively [73]. For dried algae production, the cost to produce 36 tons per hectare per year is €12.4/kg [74]. Assuming that 1 g of microalgae dry weight can vaccinate a 10 g juvenile sea bass, the estimated cost for vaccination of a sea bass is approximately €0.0124. We expect that the cost of oral vaccination using lyophilized algae is competitive and can provide economic benefits to the aquaculture industry.

From this study, we have demonstrated that microalgae can be an alternative protein production and delivery system for the aquaculture industry. The technology could overcome many of the core commercial challenges associated with oral vaccines (bioavailability, palatability, and production cost), and give greater justification to continue exploring the concept of microalgal-based oral delivery of vaccines for the aquaculture industry.

Declarations of interest

None.

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