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Advances in Research into Oral Vaccines for Fish

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Abstract

The oral route is an ideal approach for immunizing fish. Compared with conventional parenteral delivery, this method saves time and effort, while it is also simple to operate, avoids any operating pressure, is suitable for small and large sized fish, and is not limited by the scale of fishery farming. Different antigen delivery systems have been developed in order to prevent the antigen from being degraded prior to reaching the immune site, such as various biodegradable polymeric nanoparticles, transgenic engineering bacteria, and transgenic plants. Each antigen delivery system acts at a different level to improve the immune effect. In order to further improve the immune effect, immune adjuvants have also become important for the development of oral vaccines. However, little is known about the mechanism of action for oral vaccines and the possible causes of immune tolerance. This review considers recent progress in oral vaccines in terms of the delivery vector, immune adjuvant, and oral tolerance in order to provide new insights that may facilitate future research.

Keywords: delivery system, oral tolerance, oral vaccination

1. INTRODUCTION

The development of fishery vaccines began in the 1940s. Duff first reported the development of an inactivated *Aeromonas salmonicida* vaccine in 1942, which was effective at protecting fish and it also stimulated the body to produce appropriate antibodies [1]. After nearly 30 years of development, the first commercialized fishery vac-

cine, i.e., an inactivated *Yersinia ruckeri* oral vaccine, was approved in the United States in the 1970s [2]. However, due to the difficulty of mass producing the antigen and the imperfect immune effect of the vaccine, the market was weak and little of the commercial oral vaccine was applied [3]. Among the antiviral vaccines reported in 2014, only two of more than 17 commercially available vaccines were oral preparations[4]. Commercialized oral vaccines have low yields because they are rapidly degraded by gastric acid and other digestive fluids before reaching the immune site [3]. Nevertheless, researchers continue to study oral vaccines that can induce good immune responses. However, due to a lack of relevant knowledge about the immune mechanism for oral vaccines, the development of high-quality oral vaccines is complex and difficult.

In recent years, aquaculture has become one of the fastest growing food production industries. According to Food and Agriculture Organization of the United Nations reports, the rapid growth of aquatic products has made a lasting contribution to global economic growth [5]. The global aquaculture industry is becoming increasingly intensive and industrialized. Large-scale, high-density aquaculture and pollution of the water environment are becoming severe problems, and the damage caused by infectious viral diseases greatly hinders the development of the aquaculture industry [6]. Many factors restrict the development of the aquaculture industry, excluding typhoons, floods, droughts, and other uncontrollable natural factors, fish diseases are a major factor that hinders its development[7]. Antibiotics and parasiticides are effective solutions but long-term treatment using these types of therapeutics would lead to resistance and they could be harmful to the environment [8]. Therefore, vaccine development is an effective measure for controlling the occurrence of aquaculture diseases and ensuring the healthy development of aquaculture[9].

Fish vaccination is mainly conducted by injection (intraperitoneal or intramuscular), soaking (bathing or spraying), or oral routes [10]. Injection can effectively stimulate the body to produce antibodies, where it has the advantages of a low dosage, high titer, and long duration of immunity, but only with a large body size, and it can easily lead to a stress response, while this approach is also time-consuming and expensive to use [3; 11]. The immersion immunization method is simple and suitable for the large-scale immunization of fry, where it causes little stress. Following the first successful vaccination of fish using the soaking method [12], a *Vibrio anguillarum* vaccine was successfully applied via immersion in salmon, Japanese eel, and rainbow trout. However, the route of antigen absorption and the mechanism of action for the vaccine remain unclear in immersion immunization and it provide only a short period of protection[13]. For example, it is now known whether the antigen is absorbed via the skin, gills, side line, or other parts of the body, while it is unclear whether the vaccine induces immunity through the blood circulatory system or the mucosal system. In addition, var-

ious factors including the vaccine concentration, soaking time, aquatic animal size, adjuvant, antigen morphology, and water temperature will affect the uptake of the antigen during immersion immunization [14].

Given that injections cannot be applied to small fish due to operational constraints and that the effectiveness of immersion immunization is poor, then there is a need to develop other modes of immunization for small fish and oral immunization with fish vaccines can address these problems. Compared with other immunization methods，the oral delivery of a vaccine has advantages in terms of time, labor, simplicity, and lower costs, where it avoids any operation pressure and it is not limited by the scale of the fishery[8]. However, soluble or crude antigens will generate poor immune responses because they are readily degraded by gastric acid and various proteolytic enzymes in the digestive tract [15]. Thus, various effective delivery systems have been explored in order to maintain the integrity of the antigen before reaching the target immune site. For example, nanotechnology has been used to control the vaccine specifications, cell targeting, and reduce the antigen dosage [16]. Methods for antigen encapsulation using existing packaging materials such as alginate, chitosan nanoparticles, poly(D,L-lactic-co-glycolic) acid (PLGA), and other biodegradable biopolymer materials have achieved good immune effects[17]. In addition, the vehicle for antigen delivery can prolong the drug release time and enhance therapeutic efficacy[7]. This review describes recent progress in the oral immunization of fish according to the different types of oral vaccines in order to provide new insights that may facilitate future research.

2. DIFFERENT SYSTEMS FOR DELIVERING ORAL VACCINES IN FISH

2.1. Nanoparticles

The antigen wrapped in biodegradable polymer nanoparticles so the antigen can maintain the correct epitope reaches the immune site, where the antigenic substance is released slowly and sustainably, and thus the immune effect is greatly improved [18]. Commonly used polymers for vaccine delivery are chitosan[19; 20] and PLGA[21; 22]. In recent years, chitosan has been used widely in targeted drug[23] and DNA vaccine delivery[24-26] systems because of its nontoxicity, biodegradability, excellent biocompatibility, and mucoadhesive and penetration-enhancing properties[27] [28-30]. Koppolu and Zaharoff showed that chitosan nanoparticles have the capacity to efficiently deliver capsulated antigens to activate macrophages and dendritic cells [31]. Moreover, Zaharoff demonstrated that chitosan nanoparticles can enhance the humoral and cellular-mediated immune responses to vaccination in the absence of adjuvants [32]. Carboxymethyl chitosan (CMCS) is one of the important derivatives of chitosan, where it is water soluble and negatively charged in a neutral environment[33]. Gao et al.

[34]found that negatively charged CMCS can form nanoparticles with positively charged chitosan via electrostatic interactions to maintain the stability of nanostructures in the gastrointestinal tract, and this method has great potential as an oral delivery system for antitumor drugs. They also encapsulated an antigen against *Vibrio anguillarum* with CMCS for release in the gastrointestinal tract of turbot, where it exhibited a good pH response and stability, thereby protecting the antigen from degradation by gastric acid before releasing the antigen in the intestine. Dubey et al. [35] encapsulated the recombinant outer membrane protein A of *Edwardsiella tarda* in chitosan nanoparticles and used it as an oral vaccine in *Labeo fimbriatus*. A higher post-challenge survival proportion (PCSP) was obtained compared with *L. fimbriatus* immunized orally using inactivated *Edwardsiella*, where the PCSP for the vaccine encapsulated in chitosan particles reached 73.3%[35]. As pointed out by Meenakshi et al.[36], outer membrane protein A are only protective in the presence of adjuvants and, hence, it is likely that the higher protection induced by the oral vaccine was due to the adjuvant effect of the chitosan nanoparticles used to deliver the outer membrane protein A[37]. The authors also found that the chitosan nanoparticles had an inherent adjuvant effect where the pulsed slow antigen release obtained a high antibody level with a high PCSP in the vaccinated fish. Rajesh[38] examined the efficacy of DNA vaccines against *Vibrio anguillarum* encapsulated with chitosan nanoparticles for oral delivery in bass. They demonstrated that the fish absorbed the antigen but the protective effect was not very good, where the relative percentage survival (RPS) rate was only 46%. Being one of the earliest immune-adjuvants for oral vaccine, PLGA has shown excellent enhancement of immune response in many cases, and has achieved certification by U.S. Food and Drug Administration (FDA)[7; 39]. PLGA has drawn attention due to its biocompatibility, biodegradability and high stability in biological fluids and storage, which has been used to control drug release and antigen encapsulated vaccine management[40; 41]. In general, encapsulating antigens in a variety of biodegradable polymer particles can improve the protection against viral or bacterial infections, but it remains to face barriers with regard to stimulating effective immunity. Many proteases and other enzymes are present within the small intestine, so the successful delivery of both the vector and antigen may be hindered by digestion or inactivation before cellular uptake[42]. Adomako et al.[43] encapsulated DNA vaccines against infectious hematopoietic necrosis virus (IHNV) using PLGA nanoparticles and then mixed them with feed pellets for rainbow trout. After feeding the rainbow trout for six weeks, the vaccine entered the intestine to induce low levels of gene expression and specific antibody production, but this was not sufficient to protect the fish from lethal attacks. Therefore, some researchers began to focus on making functional improvements to the vector by tailoring an intelligent shell for targeted delivery and accelerated internalization. Zhang et al. [44] described oral vaccines with an intelligent phase-transitional shielding layer, which can protect antigens in the gas-

tro-intestinal tract and achieve targeted vaccination in the large intestine, where the nanoparticles with a core-shell structure exhibit good dispersion. In acidic and weak alkaline conditions, the nanoparticles can resist trypsin degradation and provide complete protection to the antigen. This technique may facilitate the development of packaging materials for oral vaccines.

2.2. Engineering microbes as vaccine vehicles

2.2.1. *Bacillus subtilis* spores

B. subtilis is a Gram-positive bacterium that under the condition of nutrient deficiency or other stress condition, it can form drought-resistant dormant-spores, whose spores can tolerate the digestive tract environment and thereby protecting the antigen[45; 46]. This spore is one of the most resistant living structures where it exhibits high stability, as well as resistance to oxidation, high temperature, anti-chemical drugs, and radiation, which allow it to survive in harsh environments for tens of years [47; 48]. Thus, a heterologous protein can be stably exposed on its surface and avoid degradation, which can also be used as a non-specific immune factor that can pass through the cell wall to stimulate immune cells to activate macrophages and immune responses[49; 50]. The spores can also enter the mesenteric lymph nodes to elicit humoral immunity, improve immunoglobulin (Ig) secretion in the blood and the nitrogen negative balance, and enhance the immune recognition capacity [51]. Previous studies have shown that *B. subtilis* spores can be engineered to express vaccine antigens to generate systemic and mucosal antibodies, where *B. subtilis* appears to play an important role in inducing a balanced Th1/Th2 response [45; 46; 52]. This shows the *B. subtilis* antigen delivery system has advantages compared with other traditional carriers and *B. subtilis* spores are being used increasingly as delivery vectors for oral vaccines. Jiang et al.[53] assessed the immune response of grass carp after the oral administration of *B. subtilis* spores expressing *Clonorchis sinensis* enolase. The results showed that *C. sinensis* enolase induced specific antibodies and immune-related genes, and protection from *C. sinensis* infection to a certain extent. This was the first study to focus on applying an intermediate host in freshwater fish to protect against *C. sinensis*. Tang et al. [54] used *B. subtilis* WB600 spores as a vehicle to deliver another antigenic protein cysteine protease from *C. sinensis* fused to CotC, a coat protein, into the gastrointestinal tract. The antigens were expressed on the surface of *B. subtilis* spores, which were mixed with commercial fish feed particles for grass carp, thereby eliciting a high level of mucosal and humoral immunity. The *B. subtilis* spores did not appear to produce signs of toxicity or damage in grass carp[54]. In addition, Valdez et al. [55] combined the Vs26 and Vp28 genes separately with the capsid protein CotC gene from *B. subtilis* to present the Vp26 and Vp28 proteins on the surfaces of spores as vaccines. At the end of

shrimp growth, the Vp26 and Vp28 proteins were used for immunization and they obtained 100% and 90% survival, respectively. Zhou [52] and Wang [56] combined the 22.3-KDa membrane protein and enolase from *C. sinensis* with the *B. subtilis* capsid protein CotC gene, which is expressed on the surface of the spore, and vaccination of rats induced an immune response in the intestinal mucosa and a systemic immune response. Thus, the use of *B. subtilis* spores as a carrier for delivering oral vaccines provides the basis for the development of new oral vaccines.

2.2.2. Yeasts

Yeasts are an immune adjuvant and a carrier for oral vaccines because they improve various shortcomings in terms of oral palatability, as well as prolonging the duration of immunity in organisms[57]. Yeasts comprise a eukaryotic expression system for exogenous proteins, with a complex transcription, translation, and modification system, where the protein can be processed, cut, glycosylated, and modified with ethyl phthalocyanine, and the peptide is secreted with normal folding to maintain the natural activity of the protein molecule[58-60]. Yeasts reproduce as rapidly as prokaryotes and they are readily amenable to genetic manipulation. In addition, yeasts are rich in vitamins, other nutrients, enzymes, and some important synergistic factors. Yeast cells can maintain their metabolic activity in an anaerobic environment, as well as tolerating dry, hot, and acidic stressful environments [61]. Yeasts and their expression products can be applied directly in the pharmaceutical industry with good application prospects and potential commercial value[62]. Yeast-based vaccines have several advantages compared with other vaccines, including their safety, ease of use, limited stress effects, and efficiency in terms of cost, time, and labor[63]. More importantly, the yeast cells are immunostimulatory when administered orally and they can act as immune adjuvants[64]. Studies have shown that β-glucan-containing yeast extract may activate some innate immune responses in sea bass, and particularly under conditions of immunodepression related to environmental stress[65].

Historically, *Saccharomyces cerevisiae* has been used as an additive in animal feeds because it may prevent disease and it has a strong immune adjuvant capability [66; 67]. In addition, *S. cerevisiae* exhibits high survival in the digestive environment [68]. *S. cerevisiae* has many advantages in oral vaccine production, including its generally accepted safety, ease of cultivation, inexpensive production, and adjuvant function [69]. Thus, *S. cerevisiae* can serve as a potential delivery vector for oral vaccination of DNA vaccines. In fact, protein antigen delivery by *S. cerevisiae* has been repeatedly demonstrated to produce adaptive immune responses in mice [70; 71] and humans [72; 73]. Yan et al. [74]also demonstrated that orally delivered *S. cerevisiae* can be transported as a carrier of protein antigens and DNA vectors to mouse intestinal dendritic cells (DCs)

and trigger an immune response. Studies have shown that the use of recombinant *S. cerevisiae* expressing VP2 protein from infectious pancreatic necrosis virus via oral administration in rainbow trout can induce a protective immune response [75]. The protein expressed on the surface of yeast is easily detected by enzymatic or immunofluorescence methods, but it can only induce a weak immune response because of its low expression level. In order to address this problem, Zhao et al. [76] used an improved yeast surface display technique (arming technology in yeast) (Fig. 1) to increase the expression of IHNV glycoprotein and improve the immune effect. In immunized rainbow trout, the vaccine prepared using the improved yeast surface display technique increased the RPS in rainbow trout from 25% to 45.85% compared with the traditional yeast surface display technique. In recent years, *Pichia pastoris* has also been employed in the field of aquaculture, where the main focus has been on the viral capsid protein, envelope protein, and some specific functional protein expression studies. Fu et al. [77] cloned glycoprotein G gene from spring viremia of carp virus (SVCV) into *P. pastoris* to achieve expression of the viral glycoprotein. Liu et al. [78] successfully expressed the VP37 protein, which plays a major role in the treatment of shrimp white spot syndrome virus (WSSV), in *P. pastoris* with pGAPZa-A as the carrier. Yeast oral vaccines are safe and they elicit immune protective effects, while they are also easy to manipulate, so they have a promising future in the field of oral vaccines.

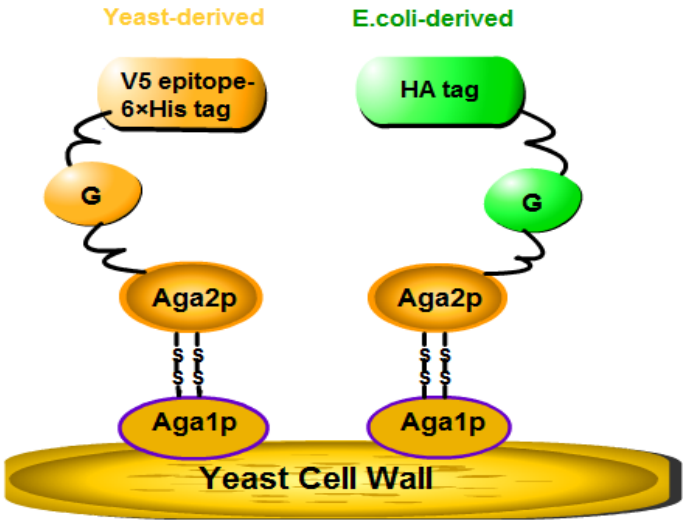

Fig. 1 [76]. Schematic diagram of a yeast-based anti-IHNV oral vaccine. Using the expression plasmid, yeast-derived G protein (yellow) is displayed on the yeast cell surface with conventional methods. *Escherichia coli*derived G protein (green) is anchored to the same yeast surface by disulfide bonds between Aga1p and Aga2p under artificial oxidation conditions.

2.2.3 *Lactobacillus*

Lactobacillus is an important probiotic that has many benefits in both human and animal health. It has a wide range of applications in food fermentation and biopharmaceuticals and is recognized as a safe microorganism[79; 80]. It can not only survive in the intestine[81], but also can induce nonspecific immunity[82]. These characteristics of *Lactobacillus* make it an attractive candidate for presentation of vaccine antigens[83-87]. Their potential as vaccine vectors has been reaffirmed in several publications, which document *Lactobacillus casei* prototype vaccines against transmissible gastroenteritis virus[88],and *Lactobacillus plantarum (L. plantarum)* prototype vaccines against *Eimeria tenella*[89]. Previous studies have found that lactic acid bacteria have intrinsic adjuvanticity, but there are significant differences in the Lactobacillus-induced cytokine profiles of different strains. Thus not all *Lactobacilli* strains have intrinsic adjuvanticity and can be used as vaccine adjuvants[90]. Cui[91] engineered, for the first time, the recombinant *L. plantarum* coexpressing glycoprotein of SVCV and ORF81 protein of koi herpesvirus, and used it as oral vaccine for cyprinid fish to induce protective immunity against SVCV and koi herpesvirus infection. Compared with the control group, the level of immunoglobulin M in the carp fed with recombinant *Lactobacillus* was significantly increased, and the survival rate of the immunized carp was 71% after sixty-fifth days of inoculation. And it's worth noting that the survival rate in group of fish bait-fed with *L. plantarum* was higher (approximate 10%) than that in group of fish received daily fish feed only, which indicated that the *L. plantarum* strain showed beneficial effects on the animals, or maybe activated certain non-specific innate immune response for responding to virus infection[91].

2.3. Oral vaccines developed using transgenic plants

Transgenic plant vaccine production usually involves the use of certain antigenic proteins from one or more pathogens to construct a plant expression vector, which is expressed in edible plants and prepared as food for immunization when ingested by aquatic animals. In the past decade, plants have been employed widely as antigen production systems because they readily allow the expansion of antigen production, with decreased antigen production costs and good safety margins [92-94]. The use of transgenic plant vaccines can avoid the digestion of antigens in the foregut without the need for purified proteins and they retain their protein activity [95]. Plants are less expensive to produce and maintain compared with conventional recombinant protein expression systems, and they do not produce additional components other than expressing the heterologous antigens, whereas the proteins expressed in yeasts may be excessively glycosylated[96]. The use of plant expression vectors for the production of fish vaccines facilitates the low cost and large-scale manufacture of vaccines, but

compared with the traditional attenuated vaccines, they are non-toxic in fish and, more importantly, the plant expression vector can effectively transcribe the modified antigen to ensure its immunogenicity [92].

Plants have been used as vehicles for immunization in many animals, including fish[97]. Companjen et al. [98] achieved the expression of *Escherichia coli* heat-labile enterotoxin B subunit (LTB) and a viral polypeptide or green fluorescent protein in potato stems, and after immunization with the transgenic plants, carp exhibited increased absorption of LTB with specific immune responses. Siripornadulsil et al. [99] used the microalga *Chlamydomonas reinhardtii* as an expression system for the P57 protein of *Renibacterium salmoninarum* to produce a vaccine that could be used for immunization by soaking and oral routes to trigger the production of specific antibodies. Plant expression systems generally select plants with suitable characteristics, including leafy crops, cereal and legume seeds, oilseeds, fruits, vegetables, higher plant tissue and cell cultures, hydroponic systems, algae, and halobios[100; 101] However, it should be noted that plant systems lack the inherent benefits of cell culture, where it is relatively more difficult to control the growth conditions and batch inconsistencies occur [102]. The current plant expression system has not been employed for the commercial production of oral vaccines but it is still an important direction for future research in aquaculture [92].

3. **ORAL VACCINE IMMUNOADJUVANTS**

An immunoadjuvant is a substance that is used in conjunction with an antigen to enhance nonspecific immunity and the immunogenicity of the corresponding antigen, but it does not possess intrinsic antigenic properties. The ideal adjuvant should be able to induce humoral immunity and cell-mediated immune responses, as well as altering the intensity of the immune response [103]. In addition, an adjuvant should eliminate immune tolerance in the organism and reduce the side effects of biological immunity, thereby decreasing the amount of antigen required for immunization. However, the commonly used vaccine adjuvants comprising aluminum salt adjuvants, and oil emulsions with microbiological compositions, can only promote immune responses[104-106], while other issues such as stickiness and the injection of oil adjuvants causing local reactions mean that few adjuvants are commercially available for fish. Still, there are some oral vaccine adjuvants have been developed for fish. Merino-Contreras conducted oral immunization with LTB as an adjuvant in spotted sand bass and found that the antibody titer was highest in the group with LTB as the adjuvant, where the immune protection rate was up to 70%, and it prevented *Aeromonas veronii* causing tissue damage [107]. Dong et al. [108] investigated the possibility of using chitosan micro-nanoparticles as a new adjuvant in the development of fish vaccines and prepared

chitosan nanoparticles loaded with antigen EATE_1227. The protective effect in immunized zebrafish showed that the chitosan particles with the EATE _1227 antigen and an oily adjuvant obtained similar immune gains in the antigen presentation process. Thus, chitosan could be used as a new adjuvant for future fish vaccines. In addition, liposomes[109], sodium alginate[110], cholera toxin[111], and cytokines [112-114]can effectively promote immune responses in animals. Novel vaccine adjuvants include saponins and their derivative QS-21, intrinsic immune agonists such as TLR natural and synthetic ligands, bacterial/fungal-derived β-glucans, novel cytokine adjuvants, novel Th1/Th2 adjuvants, and mucosal adjuvants [115]. Research into fish oral vaccine adjuvants is developing rapidly. However, previous studies of adjuvants for oral administration in fish have mainly focused on determining adjuvant effects rather than understanding the mechanisms involved. In the future, it will be necessary to explore how immune adjuvants interact with immune cells and immune molecules in the body and the pathways involved.

5. ORAL TOLERANCE

Oral tolerance is a major factor that hinders the development of oral vaccines. Oral tolerance is determined by oral antigens, where there is no or a low immune response to the antigen, whereas other antigens can still produce a normal immune response [116]. Oral tolerance is a known phenomenon in fish(rainbow trout [117]; salmon [118; 119]; common carp [120]) during different growth stages, which is considered to be caused by the inhibition of antibodies and it is easily induced, but the specific pathways for inducing oral tolerance are not clear. In higher vertebrates, studies have shown that the causes of antigen tolerance are: low band tolerance and high band tolerance, B cell tolerance and T cell tolerance, the antigen type and configuration, antigen immune pathway (oral delivery more readily leads to systemic tolerance, followed by intravenous injection, intraperitoneal injection, and muscular and subcutaneous injection), antigen persistence, antigen epitope characteristics, age and developmental stages, and genetic background [121-124]. The tolerance mechanism in the mammalian intestine involves the induction of Treg, which is associated with Foxp3 upregulation and the production of TGF-β. In humans, cells such as M cells, DCs, Th1, Th3, Th17, Foxp3+ Treg, and LAP+ cells, as well as cytokines including TGF-β, IL-10, IFN-, pathway-like Cox2, Retinoic acid, and Foxp3 are involved in the induction of oral tolerance [125]. In fish, these mechanisms are not understood in any detail and most of have been shown to reduce the antibody response after repeated antigen immunization [116; 122; 126]. Recently, the inhibition of antibody production was shown to be accompanied by the induction of Foxp3, TGF-β, and IL-10 [126]. The immune tolerance mechanism in higher vertebrates may provide insights into the immune tolerance mechanism in fish.

6. CONCLUSIONS

In recent years, the number of fish oral vaccines has increased but the uptake of fish oral vaccines and the immune mechanisms induced remain poorly understood. Oral vaccines are readily degraded by gastric acid and proteases before reaching the immune site, but the use of poly-biodegradable nanoparticles, transgenic engineered bacteria, and plant systems to encapsulate antigens can address this problem. In order to further improve the immune effect, the combination of an oral vaccine with an adjuvant can improve the specific immunity to achieve the desired immune effect. Studies of the structure and function of the digestive tract in fish indicate that the foregut is the main site of digestion, whereas the posterior intestine is the primary location for antigen uptake and immune responses[115]. Studies have shown that some immune-related cells occur in the intestine of the teleost, thereby providing a basis for the immune response[115]. However, the type of cells involved in antigen uptake as well as the molecular mechanisms and immune organs involved are not clearly understood. In addition, a key issue in oral immunization is whether delivery of the antigen via the mucosal surface (oral, skin, gill, nose) might cause local and systemic reactions. The antigen must directly reach the target site and not cause immune tolerance to produce a suitable commercially available oral vaccine. The immune response elicited by an oral vaccine after entering the body mainly occurs in the intestinal mucosa, but this system is immune to the large amounts of protein found in food and the immune response to individual antigens is weak. Further research is needed to elucidate the mechanism of oral vaccination-induced immunity, and the susceptibility of oral vaccines to degradation by gastric acid and other digestive juices is expected to be resolved. In general, fish oral vaccines have good development prospects for aquaculture practices and they have many advantages for disease control.

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