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Review

Chitosan and Alginate in Aquatic Vaccine Development

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Abstract

The global aquaculture industry faces a number of challenges, including the risk of infection spreading in closed aquatic ecosystems. Since 1942, vaccination has become a mainstream approach in fish cultivation. However, the immune system of cold-blooded organisms differs significantly from that of mammals, which must be taken into account when developing vaccines for aquaculture. Modern technology employs delivery systems for antigens to protect them from degradation in the water and the digestive tract. Packaging the antigen into a biodegradable structure protects the protein or target gene from degradation and enhances antigen delivery to immune cells. The combination of chitosan and alginate is widely used for the development of various types of nano- and microcarriers. New vaccines based on these polysaccharides are more effective, increasing survival rates in some fish species by up to 100% compared to 20% in the control group. However, the correlation between the observed effects and the physicochemical characteristics of the polysaccharides/carriers, and the mechanisms of their action, remains unclear. This review summarizes and analyzes the data on the use of chitosan and alginate in aquaculture vaccines. Particular focus is given to the physicochemical properties and sources of the polysaccharides, and their potential implementation in aquaculture vaccination practices.

Keywords: chitosan; sodium alginate; fish vaccination; aquaculture

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1. Introduction

Effective vaccine development depends critically on an organism's immune system's ability to mount an appropriate response to exogenous materials, whether natural or artificial. The mammalian immune system has been extensively characterized, revealing intricate pathways and mechanisms. Comparative research has uncovered significant interspecies differences, particularly between humans and the laboratory rodents commonly used in immunological studies. A precise understanding of these subtleties is essential for developing effective human vaccines. However, the immunological landscape for aquatic organisms, such as teleosts, is considerably more complex. Fish possess immune characteristics shared across vertebrate lineages alongside unique features that distinguish them from mammals. Furthermore, substantial variation in immune responses exists among different fish species, an area that remains underexplored.

The immune systems of many commercially important fish species have not been thoroughly characterized, presenting a major obstacle for rational aquaculture vaccine

design. As the demand for sustainable aquaculture intensifies, there is a pressing need to tailor vaccination strategies to the specific immunological profiles of target species. This individualized approach is crucial, given that vaccine efficacy can vary dramatically across different fishes.

Vaccination has transformed modern public health, preventing numerous infectious diseases through successful immunization campaigns [1]. The impact of vaccination extends beyond human medicine; effective disease control is equally critical in aquaculture, where it underpins both the economic viability and environmental sustainability of fish farming. The pioneering work of D. Duff, who first successfully immunized trout orally against *Bacterium salmonicida*, established the foundation of fish vaccinology [2]. Since this breakthrough, the field has evolved substantially, with a steady increase in the number of vaccines developed to meet industry needs. A literature search yields approximately 36,900 publications related to "fish vaccination," with over half published between 2000 and 2024, highlighting the field's rapid expansion.

Currently, more than 150 aquaculture vaccines are in various stages of approval [3], representing a significant research and development investment. Nonetheless, this progress faces considerable challenges. Obstacles include weak or variable immunogenicity, the requirement for specialized equipment or skilled personnel for administration, difficulties in large-scale production, and high costs. Vaccines can be administered via intraperitoneal (i/p) or intramuscular (i/m) injection, by gavage, orally with feed (*per os*), or by immersion (imm). The latter two methods are subjects of active investigation due to their practicality: they are less labor-intensive, do not require specialized skills or anesthesia, induce minimal stress, and facilitate the administration of booster doses. However, *per os* and imm vaccines have not achieved widespread commercial success, primarily due to their relatively low efficacy and the rapid degradation of antigens in water [4].

The efficacy of these vaccines can be enhanced by encapsulating active ingredients (nucleic acids, proteins, and peptides) within various nano- and microparticulate systems. The challenges and advantages associated with such delivery systems are discussed in detail elsewhere [5–8].

Naturally derived polysaccharides represent a distinct category among these delivery systems [9]. Chitosan and alginate are the most widely used biopolymers, favored for their low cost, chemical versatility, biocompatibility, and biodegradability. Their opposing charges enable the formation of diverse vaccine delivery systems via electrostatic interactions, including nanoparticles, microcapsules, gels, and films [10]. Furthermore, they exhibit intrinsic adjuvant and immunomodulatory properties [11,12]. While several excellent reviews have discussed the general use of chitin, chitosan, and their oligosaccharides as immunostimulants [7,9,10] or food additives [11,12], a focused and critical analysis specifically on the combination of chitosan and alginate for aquatic vaccines is lacking. Previous publications often treat these polysaccharides as a broad category without delving into the critical impact of their inherent variability on vaccine performance. This review seeks to fill this gap by providing a comprehensive synthesis that not only compiles recent advances but also critically examines the correlation between the physicochemical characteristics of chitosan and alginate and their functional efficacy as adjuvants and carriers. Furthermore, we place a particular emphasis on the pressing issue of standardization and the current understanding of their mechanisms of action within the unique framework of fish mucosal and systemic immunity. By connecting polymer chemistry with immunological outcomes and translational barriers, this review aims to provide a roadmap for the rational design of next-generation, polysaccharide-based aquaculture vaccines.

2. Chitosan and Sodium Alginate as Adjuvants and Carriers for Fish Vaccines

2.1. Structural Properties and Sources of Chitosan and Alginate

In aquaculture, the sea-to-sea concept is increasingly adopted, utilizing marine polysaccharides both as vaccine carriers and as feed additives to promote growth and enhance disease resistance in aquatic species [13–15]. Polysaccharides derived from algal sources, such as fucoidan, carrageenan, ulvan, kelp, and sodium alginate, and from crustacean shells, such as chitin and its derivatives, find widespread application [11,16–22]. Among these, chitosan and sodium alginate (hereafter alginate) are the most prevalent. The polycationic nature of chitosan, with its multiple amino groups, facilitates the immobilization of biological molecules and the formation of nanoparticles. Conversely, the carboxyl groups of alginate enable antigen loading and complexation with chitosan to form core–shell structures [23]. The combination of chitosan and alginate has been extensively employed in developing various nano- and microcarriers for delivering recombinant proteins, nucleic acids, and inactivated pathogens [24].

Chitosan, a partially or fully deacetylated derivative of chitin (Figure 1), is widely used in medicine, pharmaceuticals, cosmeceuticals, the food industry, and agriculture due to its polycationic nature and solubility in acidic aqueous media [25]. It possesses a unique set of physicochemical and biological properties. The presence of reactive hydroxyl and amino groups allows for the preparation of derivatives with tailored properties, the loading of bioactive substances, and the synthesis of polymers with enhanced mucoadhesive characteristics [8].

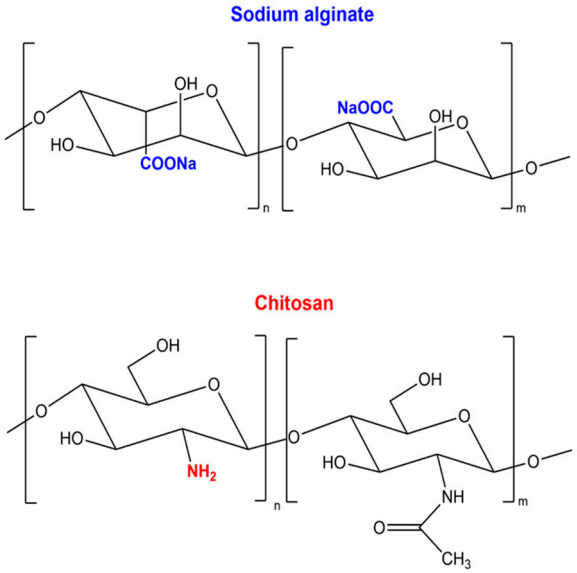

Figure 1. Chemical structures of sodium alginate and chitosan.

Alginate is a natural anionic polysaccharide, a copolymer of β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues linked by 1,4-glycosidic bonds (Figure 1). A fundamental property of alginate is its ability to form gels in the presence of divalent cations such as Ca^{2+}, Sr^{2+}, and Ba^{2+} [26]. This ionotropic gelation involves the formation of polyelectrolyte complexes through electrostatic interactions between the negatively charged carboxyl groups on the polysaccharide chain and the positively charged metal ions.

2.2. A Difficult Choice: Problems of Chitosan and Alginate Characterization and Standardization for Aquatic Vaccine Development

Chitosan and alginate represent families of natural polysaccharides. The terms 'chitosan' and 'alginate' are not universal and require precise definition, including source, molecular weight (MW), viscosity, degree of deacetylation (DD) for chitosan, or the blockwise arrangement of M and G residues for alginate [27]. The MW and DD of chitosan are critical parameters governing its physicochemical and biological properties. The polydisperse nature of chitosan and its tendency to aggregate in aqueous solutions can yield conflicting MW results, even when using a single analytical method. This variability complicates the comparison of studies from different research groups and hinders the standardization of chitosan-based products for pharmaceutical applications. Notably, only approximately 20% of published studies on aquaculture vaccine development report the key characteristics of the biopolymers used and/or their manufacturers [28,29].

In vaccine development, the presence of impurities such as proteins, heavy metals, and endotoxins must be considered, as these can significantly alter adjuvant properties and induce toxicity [30,31]. Davydova et al. [32] demonstrated that purifying chitosan leveled the proinflammatory cytokine response (IL-1β, TNF-α, IL-6) in J774A.1 macrophages, whereas variations in MW and DD had a less pronounced effect on the cytokine profile. Conversely, bacterial endotoxins can potentially stimulate innate immunity [33].

Even with nearly identical MW and DD values, the polysaccharide source can significantly influence polymer properties [34]. Shrimp and crabs are the most common raw materials for chitosan, though other sources like lobsters, crayfish, and oysters are also utilized [35]. Chitosan derived from squid gladius has been shown to be purer than that from crab and shrimp shells, as it lacks carotenoid impurities. Such polysaccharides also exhibited a higher DD and greater viscosity compared to crustacean chitosans [36].

The primary sources for alginate production are brown algae and bacteria. Recent years have seen progress in the large-scale bacterial synthesis of alginates with predefined composition and M/G sequences [37]. However, the classical extraction method from brown algae, established in 1881, remains in use. The specific extraction process is crucial, as it determines the MW and M/G ratio of the final product [38].

Table 1 summarizes data from recent publications on chitosan and alginate in aquaculture vaccines. Most studies utilize commercial low-MW chitosan from crustacean sources with a high DD, likely due to its greater solubility and lower solution viscosity, or based on precedent from prior studies. Consequently, chitosan with an MW range of 60–140 kDa is often recommended for nucleic acid delivery, including DNA and mRNA vaccines. While this recommendation may be valid, the issue remains a subject of debate [39].

However, a clear gradation for commercial chitosan by MW is currently lacking. For instance, Thu Lan et al. [40] and Sukkarun et al. [41] classified Sigma-Aldrich Chemie GmbH (St. Louis, MO, USA) chitosan with an MW of 50–190 kDa as low MW, while Sangchai et al. [42] employed Marine Bio Resources Co., Ltd. (Bangkok, Thailand) 160 kDa chitosan as a high MW polymer. Many of the analyzed studies fail to report chitosan or alginate characteristics altogether, thereby significantly complicating the standardization of such vaccines and their translation into practical application.

Therefore, accelerating the implementation of chitosan and alginate in aquaculture necessitates urgent measures for their standardization and unification. Progress has been made in this area for chitosan. Heppe, a leading global manufacturer, recommends using at least 13 different parameters to characterize chitosan produced under Good Manufacturing Practice standards [43]. The company also provides a characterization service employing various analytical techniques. Both the European Pharmacopeia and the United States Pharmacopeia define a wide range of parameters for chitosan hydrochloride and

chitosan [44]. However, such comprehensive characterization remains challenging for most research laboratories.

Consequently, we recommend specifying at least three primary characteristics for chitosan: the degree of deacetylation (DD), molecular weight (MW)—including the method and conditions of determination—and ash content. The DD, for which ^{1}H-NMR is the standard method, is typically provided by manufacturers and generally requires no further verification. Determining chitosan's MW Mw presents a greater challenge, as various methods, including size exclusion chromatography with multi-angle laser light scattering—considered the gold standard—can yield results differing by several-fold [43,45]. We have previously proposed agarose gel electrophoresis as an accessible method for Mw estimation [46], though its validation requires well-characterized commercial or in-house standard samples.

The heavy metal and endotoxin content of a vaccine formulation are also desirable parameters to control [47,48]. For endotoxin analysis, specifying the detection method is particularly important, as several techniques are prone to false-positive results [43].

When applying these polymers for vaccine development, the process of forming particulate carriers from these biopolymers requires careful optimization, as the choice of method presents distinct advantages and limitations. The widely used ionotropic gelation technique for creating sodium tripolyphosphate (TPP)-chitosan nanoparticles, while operationally simple, is highly sensitive to solution pH, which directly impacts chitosan charge density and consequently affects colloidal stability, as demonstrated in [41,42]. This variability can lead to challenges in batch-to-batch reproducibility [49,50]. In contrast, the complex coacervation between chitosan and alginate typically yields more robust core–shell structures with superior stability across a wider physiological pH range, beneficial for oral vaccine delivery. However, this method often involves more complex multi-step procedures. For all systems, rigorous post-formation purification—such as removing unbound polymer via low-speed centrifugation and eliminating small molecules like TPP using size-exclusion filters—is critical to obtaining a defined and consistent nanoparticle population. Ultimately, the selection of a specific polyelectrolyte and cross-linking system must balance methodological simplicity against the required stability and scalability for industrial application.

Table 1. Characteristics of aquaculture vaccines utilizing chitosan or alginate-based delivery systems.

Chitosan Based Vaccines									
Fish Species	**Antigen**	**Administration Route**	**Source**	**Manufacturer**	**MW/Viscosity**	**DD**	**Endotoxins /Residual Proteins**	**Form**	**Reference**
Nile tilapia (*Oreochromis niloticus*)	*Streptococcus agalactiae*	i/p, imm + i/p, i/m + *per os* or *per os*	n/d	Qingdao Hehai Biotechnology Company (China)	MW ≤ 1500 Da	n/d	n/d	A formalin-inactivated vaccine mixed with chito-oligosaccharides.	[51]
Asian seabass (*Lates calcarifer*)	*Vibrio harveyi*	imm	n/d	Sigma-Aldrich (USA)	MW 50,000–190,000 Da/ 20–300 cps 1 wt.% in 1% acetic acid (25 °C, Brookfield)	>75%	n/d	An inactivated vaccine mixed with chitosan solution in acetic acid	[40]
Nile tilapia (*Oreochromis niloticus*)	*Edwardsiella tarda*	imm	n/d	n/d	Water soluble, MW < 100,000 Da	n/d	n/d	A formalin-inactivated vaccine mixed with 0.5% chitosan solution	[52]
Hybrid red tilapia (*Oreochromis* spp.)	*Flavobacterium oreochromis*	*per os*	n/d	n/d	n/d	n/d	n/d	An inactivated bacterium, incapsulated in a chitosan/alginate /bentonite microparticles	[53]
Nile tilapia (*Oreochromis niloticus*)	*Edwardsiella tarda*	*per os*	n/d	Sigma Aldrich (USA)	Low MW	n/d	n/d	Bacterin incorporated in chitosan/alginate scaffolds	[54]
Pacific white shrimp	Spot syndrome virus (pva-pre-miRNA 11881)	imm	Shrimp	Marine Bio Resources (Thailand)	MW 160,000 Da	90%	n/d	TPP-chitosan nanoparticles	[42]

Table 1. *Cont.*

Chitosan Based Vaccines									
Fish Species	**Antigen**	**Administration Route**	**Source**	**Manufacturer**	**MW/Viscosity**	**DD**	**Endotoxins /Residual Proteins**	**Form**	**Reference**
Striped catfish (*Pangasianodon hypophthalmus*)	*Edwardsiella ictaluri*	imm	n/d	Merck (Thailand)	Low MW	n/d	n/d	Cationic lipid-based nanoparticles coated with chitosan	[55]
Red tilapia (*Oreochromis* sp.)	*Aeromonas veronii*	imm	n/d	Sigma Aldrich (USA)	MW 50,000–200,000 Da	n/d	n/d	TPP-chitosan nanoparticles	[41]
Zebrafish (*Danio rerio*)	*Haemorrhagic septicaemia virus (miRNA-155)*	i/p	n/d	Sigma Aldrich (USA)	MW 50,000–200,000 Da	n/d	n/d	TPP-chitosan nanoparticles	[56]
Pacific white shrimp	*Spot syndrome virus*	*per os*	Shrimp or squid	Marine Bioresources (Thailand).	MW 35,000 Da.	higher than 90%	n/d	TPP-chitosan nanoparticles	[57]
Alginate Characteristics									
Fish Species	**Antigen**	**Administration Route**	**Source**	**Manufacturer**	**MW**	**M/G Ratio**	**Endotoxins**	**Form**	**Reference**
Asian seabass (*Lates calcarifer*)	*Vibrio harveyi*	*per os*	n/d	n/d	n/d	n/d	n/d	Calcium alginate Microparticles with sodium bentonite	[58]
Hybrid red tilapia (*Oreochromis* spp.)	*Flavobacterium oreochromis*	*per os*	n/d	n/d	n/d	n/d	n/d	An inactivated bacterium, incapsulated in chitosan/ alginate microparticles	[53]
Nile tilapia (*Oreochromis niloticus*)	*Edwardsiella tarda*	*per os*	n/d	n/d	n/d	n/d	n/d	Bacterin incorporated in chitosan/alginate scaffolds	[54]

Abbreviations: DD—degree of deacetylation, MW—molecular weight, *per os*—oral vaccination, i/m—intramuscular, i/p—intraperitoneal, imm—immersion, TPP—sodium tripolyphosphate.

3. Vaccines Based on Chitosan and/or Alginate Delivery Systems

3.1. Inactivated Vaccines with Biopolymer Adjuvant

Adjuvants are commonly employed to enhance vaccine immunogenicity. For aquaculture applications, any selected adjuvant must be affordable, safe, and effective. Freund's complete and incomplete adjuvants and aluminum hydroxide are among the most frequently used. However, their administration via intraperitoneal (i/p) or intramuscular (i/m) routes can induce side effects in fish, including melanosis, granuloma formation, kidney damage [59] and toxicity in splenic melanomacrophage centers [60]. Consequently, the development of new biocompatible adjuvants for fish vaccination is a high priority. Chitosan, a natural polysaccharide characterized by low cost and high bioavailability, presents an attractive option. Multiple studies have demonstrated that chitosan enhances the efficacy of inactivated vaccines delivered via i/p or *per os* routes [59,61,62].

Zhu et al. [63] evaluated the efficacy of three adjuvant types—astragalus polysaccharides, chitosan, and poly (I:C)—for an inactivated oral vaccine against *Edwardsiella ictaluri*. Vaccination with astragalus polysaccharides and chitosan significantly enhanced the protective effect in yellowhead catfish (*Tachysurus fulvidraco*), yielding relative percent survival (RPS) rates of 55% and 60%, respectively. Histological analysis of intestinal tissues supported these findings, revealing only minor changes in adjuvant-treated groups compared to pronounced lesions in infected fish.

In another study, Sukkarun et al. [64] demonstrated that *A. veronii* formalin-killed cells (bacterin) encapsulated in chitosan provided a remarkable protective effect in red tilapia (*Oreochromis* sp.) compared to bacterin alone or empty nanoparticles. A single immersion administration of the nanovaccine resulted in a 25% mortality rate upon challenge, versus 100% in the control and 70% in the bacterin-only group. Notably, the source of chitosan was not specified.

The development of multi-component adjuvants represents another promising research direction. Recently, the effect of *V. anguillarum* inactivated bacteria (FKC) mixed with chitosan or chitosan/alum nanoparticles was studied in turbot (*Scophthalmus maximus*) following *per os* vaccination [61]. The vaccination induced both systemic and local immune responses in the gut, and the survival rate post-challenge increased from 0% in the control group to 26%, 32%, and 47% for FKC, FKC-chitosan, and FKC-chitosan-alum, respectively. Similarly, encapsulation of two salmonid bacterial pathogens, *L. garvieae* and *S. iniae*, in chitosan/alginate microparticles significantly increased specific IgM titers following *per os* administration [65]. Kitiyodom et al. [66] developed a biomimetic mucoadhesive vaccine for red tilapia against *F. columnare* (columnaris disease) using chitosan, which significantly enhanced both mucosal immune response in the gills and systemic response in the blood.

Examples also exist of combined vaccines comprising multiple antigens packaged within polymeric carriers. Kole et al. [67] developed chitosan and poly(lactic-co-glycolic acid) (PLGA) microparticles containing inactivated hepacivirus (HCV), *S. parauberis*, and *M. avidus* antigens. Immunization by immersion increased survival rates in olive flounder (*P. olivaceus*) challenged with the respective pathogens. A chitosan-based combined *per os* vaccine against infectious salmon anemia (ISAV), comprising alphavirus replicons and inactivated ISAV components, was protective in up to 77% of cases, despite the absence of a detectable humoral response [68]. A recent study by Thu Lan et al. [40] demonstrated the efficacy of a combined approach for immersion vaccination of Asian sea bass (*Lates calcarifer*) against *Vibrio harveyi*. Their strategy involved oxygen nanobubbles in the immersion bath, coupled with chitosan adjuvant, followed by an oral booster. This regimen increased antigen uptake, enhanced specific IgM levels in serum and mucus, and achieved a post-infection survival rate of 63%.

3.2. Subunit Vaccines

The development of subunit vaccines marks considerable progress in vaccinology. These vaccines utilize purified antigenic components, such as individual proteins or coding DNA/RNA from a pathogen, rather than the whole organism [1]. Most modern commercial subunit vaccines employ genetically engineered surface antigens. Currently, they constitute over 80% of vaccines in national human immunization programs in many countries [1]. Their application is also valuable in veterinary medicine, particularly for pathogens that are difficult to culture [69,70]. A challenge for subunit vaccines is the frequent need for adjuvants and multiple booster doses, which increases cost. Nano- and microparticle-based delivery systems based on biopolymers offer a potential solution to these limitations [5,9].

Chitosan and its derivatives, known for their intrinsic adjuvant and immunomodulatory properties, are widely investigated for protein antigen delivery in mammalian vaccines [71], though their application in aquaculture remains less extensive. Behera & Swain [72] developed a vaccine against *A. hydrophila* for rohu (*L. rohita*) by encapsulating pathogen outer membrane proteins in PLGA, chitosan, and alginate microparticles, also comparing them to Freund's adjuvant. Intramuscular immunization with the composite microparticle vaccine generated high levels of specific IgM antibodies, exceeding the response elicited by PLGA alone or Freund's adjuvant. This *A. hydrophila* vaccine demonstrated high efficacy, with 100% mortality in the control group compared to only 10% in the microparticle-vaccinated group [73]. The potential of chitosan nanoparticles as a delivery system was also confirmed for a bacterial cell envelope protein of *E. tarda* [74]. The use of polyelectrolyte microspheres based on chitosan and alginate offered significant advantages in developing an *S. iniae* vaccine for channel catfish (*Ictalurus punctatus*), achieving a survival rate of up to 60% post-challenge [75]. Similarly, chitosan-alginate microparticles containing a fragment of the *M. salmoides* G2 protein for intragastric immunization of largemouth bass (*M. salmoides*) increased survival from 26% in controls to 55% [20].

3.3. DNA/RNA Vaccines Based on Chitosan and Alginate

DNA and RNA vaccines represent a promising immunologic strategy with significant potential for disease prevention. DNA vaccines are pharmaceutical preparations comprising purified recombinant plasmid vectors produced using DNA technology. These vectors contain one or more DNA sequences designed to elicit a protective immune response against a pathogen. DNA-based systems, along with RNA interference technologies utilizing small interfering RNA and microRNA, are under active investigation [76].

Chitosan, being a polycation, has been widely used to form complexes with negatively charged nucleic acids and serves as a basis for non-viral transfection agents [77]. The transfection efficacy of chitosan-DNA complexes depends on multiple factors, including polymer MW and charge, the presence of hydrophobic groups, complex formation conditions, and plasmid size [78,79]. Our previous work demonstrated that among various chitosan derivatives complexed with a 3065 bp pPFP pmKate2 plasmid, only 20 kDa hexanoyl-chitosan with a specific degree of substitution achieved transfection efficiency in HEK293 cells comparable to the liposomal reagent Metafectene Pro [80]. Given the high cost of commercial transfection agents, polycationic biopolymers like chitosan present a viable alternative. Multiple studies report the successful delivery of small plasmid DNA to HEK293T cells using chitosan and its derivatives [81–84].

Many fish cell lines are notoriously difficult to transfect with commercial lipid-based agents (Table 2). To address this, strategies such as combining chemical methods with electroporation, pre-treatment with colchicine or thymidine, and protocol optimization have been employed. For instance, transfection efficiency in EPC cells was improved from 13% to 55% through such adaptations [85]. Dominko et al. [86] developed effective chemical trans-

fection protocols for zebrafish Pac-2, ZF4, and ZF cell lines. Sandbichler et al. [87] compared four commercial agents, achieving a maximum efficiency of 25–30% with Extreme Gene HP in the Z3 line. Similar results were obtained for the EPC line using Fugene (37% of cells transfected with pCMVβ plasmid) [88]. A 40% transfection efficiency was demonstrated for ZBE3 cells derived from *D. rerio* blastomeres using Lipofectamine 3000 [89].

Lipid-based agents are not the only method for transfecting fish cell lines. Falco et al. [90] isolated a 15 kDa polyethyleneimine (PEI) fraction exhibiting low toxicity and high transfection activity, achieving rates of 30% in EPC and 6% in RTG-2 cells. In contrast, pcDNA3.1-VP4C or pcDNA3.1-VP56C plasmid-loaded chitosan/alginate microcapsules showed low transfection efficiency (~2%) in FHM cells [91]. Similar low efficacy was observed for PEGFP-N1 plasmid-loaded chitosan nanoparticles in SHK-1 cells [68]. Nevertheless, other studies report high efficacy for chitosan nanoparticles and chitosan/gamma-polyglutamic acid complexes in SILK and ZFL cell lines, with efficiencies up to 100% [92,93]. Table 2 provides a comparative overview of the efficacy of different transfection agents, including chitosan-based particles, in fish cell lines, alongside the RPS observed in vaccination-challenge trials in vivo. It is important to note that low transfection efficiency in vitro does not necessarily preclude a protective immune effect from the vaccine in vivo [68], suggesting that the immunoprotective mechanisms of chitosan and alginate-based particles involve complex pathways beyond simple transfection.

Examples of combined DNA vaccines utilizing chitosan and/or alginate in aquaculture are summarized in Table 3.

Table 2. Relationship between in vitro transfection efficiency in fish cell lines and relative percent survival (RPS) following challenge in vivo.

Cell Line	Plasmid	Gene	Agent	Transfection Efficacy, %	RPS, %	Reference
ABG HK7	pEGFP-N1	*GFP*	Lipofectamine 2000; X-tremeGENE HP; GeneJuice	5	n/d	[94]
CSTF	pEGFP	*GFP*	-	2	n/d	[95]
EPC RTG2	pMCV1.4-G pMCV1.4-bga pMCV1.4-EGFP	*β-gal* *GFP*	PEI,15 kDa	EPC–30 RTG2–6	n/d	[90]
Chinese sturgeon (*Acipenser sinensis*) head kidney cell lines	Plasmids containing the CMV promoter and GFP or RFP reporter gene	*GFP* *RFP*	X-tremeGENE HP DNA transfection reagent	40	n/d	[96]
EPC	pMCV1.4AE6- β-gal	*β-gal*	Fugene Trans IT LT1	30	n/d	[97]
EPC	G3pcDNAI/Amp	*GFP*	Lipofectin	Low	n/d	[98]
OCF	pMaxGFP	*GFP*	Lipofectamine 3000	7	n/d	[99]
FHM	pcDNA3.1-VP4C pcDNA3.1-VP56C	*GFP*	Chitosan-alginate microcapsules	2	34–54 (*per os*)	[91]
ZFL	pcDNA3-GFP	*GFP*	Nanoparticles from chitosan and Gamma-polyglutamic acid	100	n/d	[93]
SISK	pcDNA 3.1- OMP	*GFP*	Chitosan nanoparticles	100	46 (*per os*)	[92]
TK	pEGFP-N2-TRBIV-MCP	*GFP*	Chitosan nanoparticles	40	68 (*per os*)	[100]
SHK-1	EGFP-N1	*GFP*	Chitosan nanoparticles	Low	39–77 (*per os*)	[68]

Table 3. DNA vaccines for aquaculture utilizing chitosan-alginate delivery systems.

Disease/Pathogen	Fish Model	Plasmid/Type	Polymers	Route	Dose/Fish	Survival Rate vs. Control	Reference
Nocardiosis (*Nocardia seriolae*)	Largemouth bass (*Micropterus salmoides*)	pcDNA-ABP	Mannosylated chitosan	i/p	20 µg	80/17	[101]
Viral nervous necrosis (Nodavirus)	Asian sea bass (*L. calcarifer*)	pcDNA3.1 modified with gene encoding for ORF of the major capsid protein	Low MW chitosan, Sigma-Aldrich	*per os*	-	85/50	[102]
Vibriosis (*V. anguillarum*)	Asian sea bass (*L. calcarifer*)	pcDNA3.1 modified with gene encoding OMP protein	Low MW chitosan, Sigma-Aldrich	*per os*	-	-	[103]
Vibriosis (*V. anguillarum*)	Asian sea bass (*L. calcarifer*)	pcDNA3.1 modified with gene encoding OMP38 protein	Chitosan DD 85%, Sigma-Aldrich	*per os*	50 µg	89/47	[92]
Spring viremia of carp (*Carp sprivivirus*)	Common carp (*Cyprinus carpio*)	*Lactobacillus plantarum* coexpressing glycoprotein of SVCV and ORF81 protein of koi herpesvirus	Chitosan/alginate	*per os*	50 µg	100/18	[104]
Edwardsiella septicemia (*Edwardsiella tarda*)	Rohu (*Labeo rohita)*	Bicistronic DNA vaccine (pGPD + IFN) containing *GAPDH* gene of *E. tarda* and *IFN*-ɤ gene of *L. rohita*	Chitosan, Sigma-Aldrich	imm	10 µg	-	[105]
-	Gilt-head bream (*Sparus aurata*)	pCMVβ modified with reporter gene encoding β -Galactosidase	Chitosan	*per os* or i/m	40 µg	-	[106]
-	Nile tilapia (*Oreochromis niloticus*)	pCMV-SPORT- β gal	Chitosan	i/b *per os* i/m	50 µg	-	[107]
-	Zebrafish (*Danio rerio*)	pcDNA3-GFP	Low MW chitosan MW 60 kDa, DD 85%	microinjection		-	[93]
Lymphocystis disease/ lymphocystis disease viruses (LCDV)	Olive flounder (*P. olivaceus*)	pEGFP-N2-modified with gene encoding virus protein LCDV	Chitosan, MW 1080 kDa, DD 80%	*per os*, gavage	30 mg	-	[108]
Edwardsiella septicemia (*E. tarda*)	Rohu (*L. rohita*)	pGPD + IFN containing *GAPDH* gene of *E. tarda* and *IFN*-ɤ gene of *L. rohita*	Chitosan	*per os* i/m imm		55/20%	[109]

Table 3. *Cont.*

Disease/Pathogen	Fish Model	Plasmid/Type	Polymers	Route	Dose/Fish	Survival Rate vs. Control	Reference
Vibriosis (*Vibrio parahaemolyticus*)	Black seabream (*Acanthopagrus schlegel*)	pEGFP-N2 modified with gene encoding protein of V. parahaemolyticus strain (OS4)	Chitosan DD 85% Sigma-Aldrich	*per os*	50 µg	73/ 20	[110]
Hemorrhagic septicemia. Fin and tail rot in fish (*Aeromonas hydrophila*)	Common carp (*Cyprinus carpio*)	pcDNA3.1-aerA	Chitosan MW 50 kDa, DD 93%, China. Hyaluronic acid, MW 5 kDa	*per os* gavage	5 µg	-	[111]
Aeromonas hydrophila infection	Rohu (*L. rohita*)	pVAX1 modified with gene encoding outer membrane protein and hemolysin	Chitosan DD 75–85%	*per os*	-	76/25	[112]
Cryptocaryonosis (*Cryptocaryon irritans*)	Orange-spotted grouper (*Epinephelus coioides*)	pcDNA3.1 modified with gene encoding immobilization antigen and heat shock protein	Chitosan MW 800 kDa, DD 85%	*per os*	20 µg	-	[113]
-	Gilthead sea bream (*Sparus aurata*) juveniles	pCMVβ	-	*per os*	40 µg or 125 µg	-	[114]
Nodaviridae family Betanodavirus genus	European seabass (*Dicentrarchus labrax*)	pcDNA3.1/V5-His-TOPO	Chitosan MW 390 kDa, Sigma-Aldrich	*per os*	10 µg	100/45	[115]
Turbot reddish body iridovirus (TRBIV)	Turbot (*S. maximus*)	pEGFP-N2-TRBIV-MCP	Chitosan MW 220 kDa, DD 85%, Sigma-Aldrich	*per os*	10 µg	89/28	[100]
Grass carp hemorrhagic disease caused by grass carp reovirus (GCRV)	Grass carp (*Ctenopharyngodon idella*)	pcDNA3.1-VP4C and pcDNA3.1-VP56C, respectively	Chitosan and alginate	*per os*	-	54/12	[91]
Infectious pancreatic necrosis virus (IPNV)	Rainbow trout (*Oncorhynchus mykiss*)	pcDNA3.1 contains VP2 gene	Chitosan and alginate	*per os*	10 or 15 µg	57/10	[116]

Abbreviations: pcDNA—plasmid DNA, DD—degree of deacetylation, MW—molecular weight, *per os*—oral vaccination, i/m—intramuscular, i/p—intraperitoneal, i/b—intrabuccal, imm—immersion.

4. Advanced Vaccine Approaches

4.1. Hybrid Particles

Viral vectors represent an alternative method for DNA delivery, though often at a higher production cost [12]. Technologies for generating recombinant baculoviruses, lentiviruses, and adenoviruses are increasingly sophisticated and widely utilized. Baculovirus systems have been explored for vaccine development against carp reovirus and infectious spleen and kidney necrosis virus [91,117]. The recombinant adenovirus platform has been successfully applied to create vaccines against the bacterial pathogen *A. salmonicida* in rainbow trout (*O. mykiss*) and against infectious hematopoietic necrosis virus [118,119]. The use of recombinant adenoviruses has grown considerably; their high safety profile and versatility make them an attractive platform for viral-vectored vaccines in veterinary medicine [120,121]. Nonetheless, regulatory restrictions on genetically modified organisms limit the application of DNA vaccines in some countries [122].

Incorporating adjuvants into viral vaccines or encapsulating viral vectors within particulate systems can protect them from rapid inactivation in the aquatic environment, thereby enhancing vaccine efficacy [123]. Research on such hybrid systems for aquaculture is ongoing, building on encouraging results from mammalian gene therapy. For instance, hexanoyl-chitosan-stabilized magnetic iron particles complexed with an adenoviral transgene demonstrated significantly enhanced activity under a magnetic field both in vitro and in vivo [124]. Furthermore, oncolytic adenoviruses coated with chitosan-PEG-folate proved effective against epithelial carcinoma, reducing tumor growth without increasing systemic toxicity [125].

Currently, hybrid systems combining antigens, lipids, and chitosan (chitosomes) are also being developed for mammalian applications [126]. These particles typically possess a smaller size and higher zeta potential, leading to improved transfection efficiency in both in vitro and in vivo mouse models [127]. The addition of functional peptides, such as those enhancing mucoadhesion or cell penetration, can further increase vaccine efficacy [128,129]. However, the elevated cost associated with these sophisticated systems may constrain their widespread implementation.

4.2. Artificial Intelligence for Vaccine Optimization

Recently, quantitative structure-activity relationship analysis using artificial intelligence (AI) has emerged as a novel approach in polysaccharide research. Lu et al. [130] utilized AI to analyze the adjuvant activity of raspberry pulp polysaccharides based on their structural information. The results indicated that MW and the content of arabinose, galactose, and galacturonic acid were the primary factors significantly influencing the polysaccharides' adjuvant properties.

Various AI-based methodologies can also be employed to develop new orotransmucosal vaccine delivery platforms [131,132], offering considerable potential for more convenient vaccine administration.

In one application, Hosney et al. [27] used AI to optimize both the modification and yield of chitosan during purification. The authors identified deacetylation alkali concentration as the most crucial parameter, followed by the acid and alkali concentrations used in demineralization and deproteinization, respectively. Baharifar and Amani utilized AI to optimize the size, loading efficiency, and cytotoxicity of chitosan nanoparticles loaded with a model antigen (bovine serum albumin) [133]. Other studies have further validated this computational approach [134,135]. Such machine learning algorithms present a promising tool for optimizing the adjuvant activity and particle characteristics of chitosan/alginate-based systems, opening new avenues for rational vaccine design.

5. Possible Mechanisms of Action of Chitosan in Vaccines

The immune system of fish relies more heavily on innate immunity than that of mammals, while simultaneously exhibiting greater efficiency in this regard. The first line of defense in fish involves mucosal surfaces, collectively termed Mucosal-Associated Lymphoid Tissues (MALTs). In fish, this includes six distinct types: gills (GIALT), skin (SALT), nose (NALT), gut (GALT), pharynx, and buccal mucosa [136]. These MALTs protect against mechanical injury and pathogen invasion [137]. Mucus contains abundant mucin, antimicrobial peptides, lysozyme, lectins, and immunoglobulins [137–139]. Mucin determines the viscous and structural properties of mucus. All mucosal surfaces carry a negative charge due to sialic acid residues in mucin, whose carboxyl groups are exposed on the epithelial surface [140]. The polycationic chitosan interacts with these sialic acids, forming complexes via electrostatic attraction.

During immersion immunization, chitosan-adjuvanted vaccines primarily bind to the skin and gill mucosa, with lesser attachment to other mucosal surfaces. Skin mucus is readily washed off in water, a mechanism that protects against parasites but can reduce vaccine uptake. Evolutionarily, both fish and mammals possess mechanisms for capturing pathogens or their fragments from mucosal tissues for presentation to immune cells, enabling protective immunity. This transfer can occur via transcytosis through epithelial cells or via binding to mucosal immunoglobulins (IgA in mammals; its evolutionary precursor, IgT, in fish) [141].

During oral immunization, the vaccine is captured by buccal ALT and GALT. The resulting pathogen-mucus complex is then passed to the stomach, where digestive enzymes may inactivate the pathogen. Fragments of the inactivated pathogen can subsequently be transported by intestinal M-cells to immune cells via transcytosis [142]. The mucoadhesive properties of chitosan were demonstrated by Kitiyodom et al. [66] using an inactivated vaccine against *F. columnare* in red tilapia. The authors showed that encapsulating inactivated bacteria in chitosan significantly enhanced mucoadhesion to gills during immersion immunization and increased the survival rate of red tilapia three-fold (from 30% to 90%) compared to non-encapsulated bacteria, and nine-fold compared to the control group after challenge [66]. The same research group developed an immersion nanovaccine against *F. oreochromis* in red tilapia using inactivated bacteria encapsulated in a cationic lipid [55]. This lipid encapsulation increased the survival rate from 60% to 90%, compared to 35% in the control. These results suggest an advantage of chitosan over cationic lipid adjuvants.

A different scenario occurs with i/m or i/p immunization, which bypasses mucosal tissues. In this case, resident innate immune cells are directly activated, recruiting cells of the adaptive immune system. As a polycation, chitosan binds not only to sialic acids in mucus but also to negatively charged cell membranes. Numerous studies have demonstrated chitosan's ability to enhance the immune response to protein antigens administered via various routes [71].

We have previously shown that chitosan binds rapidly to cell surfaces but is poorly internalized, unlike negatively charged succinyl chitosan derivatives [143,144]. Chitosan binding to cell membranes can stimulate cells to shed the polymer along with fragments of the membrane. This phenomenon underpins the ability of chitosan (and other polycations) to temporarily open tight junctions between epithelial cells, thereby enhancing vaccine penetration [145–147].

Conversely, macrophages are specialized phagocytes capable of engulfing diverse materials—including bacteria, allergens, apoptotic bodies, and positively charged particles. Our unpublished data (Figure 2) illustrate the interaction of chitosan nanoparticles with macrophages and epithelial cells. Briefly, for Figure 2, chitosan nanoparticles were prepared by ionotropic gelation with tripolyphosphate using rhodamine-labeled Der f 2 dust mite

protein as a model antigen. J774 murine macrophages and COLO357 human pancreatic epithelial cells were incubated with the nanoparticles for 3–4 h. Following fixation, cells were stained with LysoTracker Green (J774) or LysoTracker Red (COLO357) and DAPI, and visualized by confocal microscopy. Macrophages efficiently internalize nanoparticles loaded with fluorescently labeled protein and transport them to lysosomes (Figure 2a, indicated by yellow co-localization of green and red signals), a process essential for antigen presentation to immune cells. In contrast, chitosan does not significantly penetrate epithelial cells and is largely shed from their surface (Figure 2b, absence of co-localization).

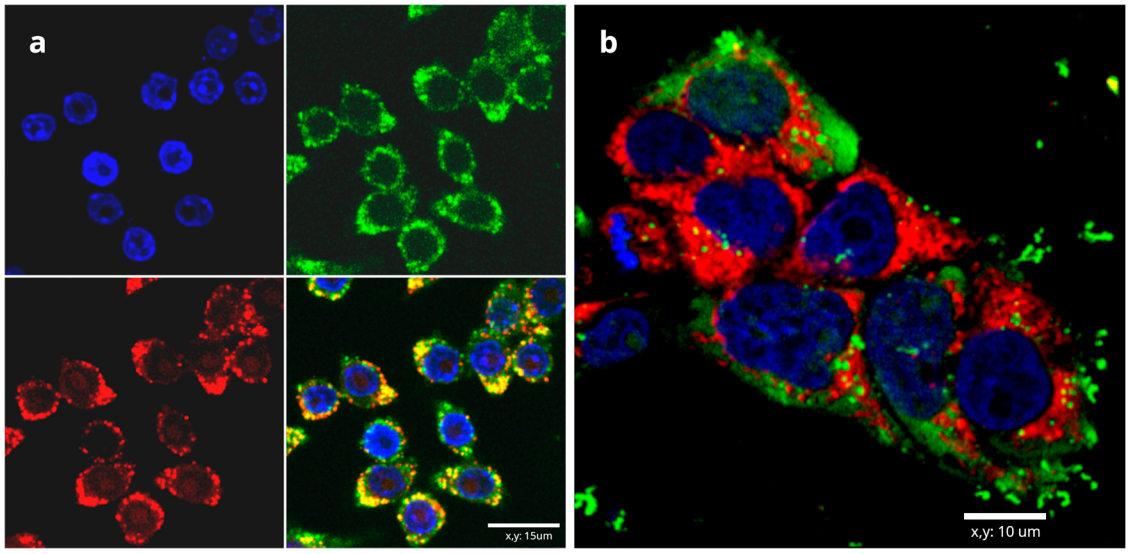

Figure 2. Confocal microscopy of chitosan nanoparticles interaction with macrophages and epithelial cells. (**a**) House dust mite protein Der f 2, labeled with rhodamine (red), was encapsulated in chitosan nanoparticles and incubated with J774 macrophage cell line for 3 h. After fixation, cells were stained with LysoTrackerGreen (green). Split channels are shown. (**b**) COLO357 epithelial cells were incubated with FITC-labeled chitosan (green) for 4 h. After fixation, cells were stained with LysoTrackerRed (red). Nuclei are stained with DAPI (blue). This figure presents original, unpublished data from the authors.

Another significant property of chitosan is its capacity to form nanoparticles of various sizes. Macrophages efficiently phagocytose particulate matter, including bacteria, viruses, apoptotic bodies, and nanoparticles. Varying the MW of chitosan allows the formation of nano- and microparticles or complexes ranging from 100 to 800 nm. Vaccine development requires optimizing particle size to balance efficient transcytosis across epithelial barriers (favored by smaller particles) and effective uptake by macrophages (favored by larger particles).

The effect of chitosan on systemic immune responses in aquatic species remains insufficiently studied, partly due to substantial differences between mammalian and teleost immune systems. In mammals, effective vaccination relies on adaptive immunity generated in lymph nodes, where T and B cell activation occurs through cognate interactions with follicular dendritic cells (FDCs) unique to these structures. Fish lack lymph nodes and FDCs, resulting in a comparatively reduced adaptive immune response. Teleost immunoglobulins include IgM, IgD, and IgT/Z, with B cell repertoire generation occurring via somatic hypermutation in the head kidney. Differentiated B cells do not undergo immunoglobulin heavy chain class switching [148], resulting in T-cell-independent humoral responses [149]. Nevertheless, IgM titers and affinity in fish increase steadily post-immunization, though maximum antibody titers (100–1000 fold increase) remain substantially lower than in mammals (up to 10^6).

Fish T cells can differentiate into Th1 or Th2 phenotypes and produce cytokines characteristic of various mammalian T helper subsets (Th17, Th22, Th9, Th6, TFH, Tregs) [150]. However, identifying specific T cell subpopulations in fish is constrained by the limited availability of fish T cell-specific antibodies. While T cell involvement in transplant immunity has been demonstrated [151], their role in vaccine responses remains unclear.

In conclusion, fish vaccination is feasible, but an efficient antigen-specific response is reminiscent of an innate type of immunity, enhanced by increased proliferation of pre-existing antigen-specific T and B cells, IgM and IgT multimerization, and TLR-mediated innate immunity stimulation. Apparently, multiple booster immunizations are required to achieve high titers of antigen-specific cells and antibodies. Due to the absence of germinal centers, a much longer time (up to 15 weeks) is required to achieve sufficient antibody titers [152], compared to 2–4 weeks for the mammalian immune response.

Thus, chitosan in vaccines has several effects. It increases the adhesion to mucus, which retains the antigen on the mucous membrane longer and stimulates transcytosis. At the same time, chitosan promotes the opening of tight junctions, also increasing the penetration of the vaccine into the submucosal tissues. Finally, the particulate nature of the vaccine formulation stimulates phagocytosis by resident MALTs macrophages. Simultaneously, the vaccine exhibits limited entry into non-phagocytic cells due to the ability of cells to shed positively charged molecules from their membrane, thereby focusing antigen capture on macrophages, which in fish are the primary antigen-presenting cells.

6. Regulatory Pathways and Ecological Safety Aspects

To date, a substantial body of data has accumulated on the use of chitosan in experimental medicine and veterinary practice. However, the variety of chitosan sources and batch-to-batch variability in its properties complicate its translation into clinical practice [29,30]. There is a consequent urgent need for standardized methods for chitosan and its derivatives. Recent results demonstrating similar biological properties across six commercial medium-molecular-weight chitosan samples are promising in this regard [153]. The current lack of standards hinders the consistent application of the Safe-by-Design concept, which is essential for ensuring the safety and efficacy of nanomedicines [154].

Currently, both chitosan and alginate are classified by the U.S. Food and Drug Administration as Generally Recognized as Safe. Both polysaccharides are approved for oral administration: alginate in antacids and rectal suppositories, and chitosan as a dietary supplement [155] or in hemostatic and wound dressing agents [156].

The development of parenteral vaccines necessitates high-purity preparations with minimal endotoxin, heavy metal, and microbial contamination [157]. In contrast, the requirements for immersion and *per os* fish vaccines can be less stringent, as similar impurities are commonly present in the aquatic environment. Importantly, the use of chitosan and alginate in oral and immersion vaccines is considered environmentally safe. Numerous studies have confirmed that these biodegradable, natural-origin polysaccharides are low-toxicity substances with a high safety profile [158,159].

Presently, several dozen chitosan-based pharmaceuticals are in various stages of clinical trials, indicating significant and growing interest in these delivery systems [160].

7. Conclusions

In summary, marine polysaccharides, specifically alginate and chitosan, represent a promising strategy for developing novel aquaculture vaccines. These polymers are exceptional candidates for antigen encapsulation due to their natural origin, low cost, minimal toxicity, biodegradability, and biocompatibility. Encapsulation systems enhance existing vaccine efficacy by improving stability under physiological conditions and providing

protection from environmental degradation. Chitosan and alginate can function both as standalone adjuvants and as components of delivery systems for various vaccine types. The modest but reliable transfection efficiency of chitosan is counterbalanced by its low cost, high biocompatibility, and intrinsic adjuvant activity.

The opposing charges of chitosan and alginate enable the fabrication of micro- and nanoparticles for encapsulating proteins, peptides, and nucleic acids (DNA/RNA). Notably, the production methods for these systems are straightforward and cost-effective, a crucial consideration for aquaculture applications. The high production costs of novel vaccines often hinder their commercial competitiveness, which explains why most commercially available aquaculture vaccines remain traditional inactivated formulations.

It is important to recognize that chitosan and alginate are natural products whose properties vary depending on source, impurity profile, and MW. Therefore, considerable attention must be paid to standardizing these materials. Such standardization will facilitate identification of correlations between polymer characteristics (e.g., MW, modification type, quantity) and biological activity. Meanwhile, the use of chitosan and alginate in immersion or oral vaccines can proceed under current regulations, as these marine-derived compounds are generally recognized as safe. Since both polysaccharides are already approved for oral use in mammals, immersion and oral aquatic vaccines need not meet the stringent requirements for parenteral formulations.

Although several successful chitosan/alginate-based aquaculture vaccines have been developed, their mechanisms of action remain incompletely understood—particularly the observed protection in the absence of specific IgM/IgT production. Vaccine design must account for the distinctive features of piscine immunity, where innate immune responses play a dominant protective role alongside adaptive immunity. Consequently, future research should focus on various aspects of vaccinating aquatic species, including fish and invertebrates, to ultimately introduce new effective technologies for sustainable aquaculture.

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