

# Chitin derivatives of NAG and chitosan nanoparticles from marine disposal yards and their use for economically feasible fish feed development

Subramanian Kumaran<sup>a,\*</sup>, Antonyraj Matharasi Perianaika Anahas<sup>b,1</sup>,  
Nainangu Prasannabalaji<sup>c</sup>, Muthuramalingam Karthiga<sup>d</sup>, Selvaraj Bharathi<sup>c</sup>,  
Thirunavukkarasu Rajasekar<sup>a</sup>, Jerrine Joseph<sup>a</sup>, Srinivasan Guru Prasad<sup>e</sup>, Sivakumar Pandian<sup>f</sup>,  
Sampath Renuga Pugazhvendan<sup>g,h</sup>, Wilson Aruni<sup>d,i</sup>

<sup>a</sup> Centre for Drug Discovery and Development, Sathyabama Institute of Science and Technology, Chennai, 600119, Tamilnadu, India

<sup>b</sup> Department of Microbiology, Centre of Excellence in Life Sciences, Bharathidasan University, Palkalaiperur, Tiruchirappalli, 620024, Tamilnadu, India

<sup>c</sup> PG & Research Department of Microbiology, Sri Sankara Arts and Science College, Kanchipuram, 631561, Tamilnadu, India

<sup>d</sup> Department of Biotechnology, School of Bio and Chemical Engineering, Sathyabama Institute of Science and Technology, Chennai, 600119, Tamilnadu, India

<sup>e</sup> CAS in Marine Biology, Annamalai University, Cuddalore, 608502, Tamilnadu, India

<sup>f</sup> Pandit Deendayal Energy University, Gandhinagar, Gujarat, 382426, India

<sup>g</sup> Department of Zoology, Annamalai University, Annamalai Nagar, Cuddalore, 608002, Tamilnadu, India

<sup>h</sup> Department of Zoology, Arignar Anna Government Arts College, Cheyyar, 604407, Tamilnadu, India

<sup>i</sup> School of Medicine, Loma Linda University, CA, 92354, USA

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## ABSTRACT

Globally, the disposal of shellfishery waste is a major challenge and causes a risk to the coastal region. For potential development in aquaculture, the use of safe supplements to improve fish production and health is important. Chitosan (CS) used as feed additives for several fish species that enhanced production and immunity. The present study was intended to assess the effect of feed additives N-acetyl-D-glucosamine (NAG) loaded chitosan nanoparticles (CSNPs) on productivity, survival rate, and protein conversion efficiency of *Oreochromis niloticus* (L.). This is the first report on the effect of CSNPs and NAG loaded CSNPs as feed additives enhanced growth performance and non-specific immunity of *O. niloticus*. CSNPs and NAG loaded CSNPs were synthesized and characterized by scanning and transmission electron microscope, FT-IR, X-ray diffraction, particle size distribution, and zeta sizer. Fish (15.30 ± 0.23 g) administered diets fortified with 0.0, 0.25, 0.5, 1.0, and 2.0 g CSNPs/kg feed loaded with NAG for 45 d. The diets containing 1.0 g/kg NAG loaded CSNPs enhanced specific growth rate, weight gain, survival rate, respiratory burst, and lysozyme activities of tilapia compared control group. The data shows biologically active CSNPs and NAG loaded CSNPs are potent antimicrobial agents against selected bacterial pathogens. In conclusion, the findings suggested that the dietary supplement containing NAG loaded CSNPs significantly increased immune-modulatory properties, growth performance, and enhanced their disease resistance of Nile tilapia.

## 1. Introduction

Rising global population, waste generation are also increasing rapidly and a high portion of the by-products produced by contemporary foods remain underused, which can also contain high-value compounds (Yadav et al., 2019). Food waste is the major solid waste and its volume is increasing rapidly across the world (Morousek et al., 2020a). The share of renewables was also increasing rapidly, especially in developed

countries. Several researchers are interested in the recovery of wastewater and there is a huge development in theoretical knowledge. Novel and theoretically undemanding concepts are designed and techno-economically assessed on a pilot scale (Morousek et al., 2020a, 2020b, 2020c).

In 2018, global farmed shrimp production reached about 4 million tonnes and estimated to be growing at a rate of 6% annually (FAO, 2019). India has been the fourth major exporter since 2017, boosted by a

\* Corresponding author.

E-mail address: [kumarun23@gmail.com](mailto:kumarun23@gmail.com) (S. Kumaran).

<sup>1</sup> Equal contribution.

rapid rise in the production of farmed shrimp. Indian shrimp production (90% *Penaeus vannamei*) increased to approximately 700 000 tonnes (FAO, 2020). In specific, the rapid rise in shrimp industry sectors drastically pertains to tons of shrimp waste accounts for 58% of chitin (Kumaran et al., 2020). The shrimp waste also contains large amounts of chitin (15–40%), a polysaccharide with excellent inherent properties such as biodegradability, biocompatibility, antimicrobial, anticancer, and antioxidant properties (Shanmuganathan et al., 2019; Yadav et al., 2019). The characteristics of shrimp shell waste contain chitin (26.13%), moisture (28.21%), ash (21.67%), protein (37.84%) and mineral (36.03%).

Advanced processing and management strategies for these wastes are required to produce value byproducts and minimize ecological impacts; thus, the fisheries sector will have more economic development and environmental sustainability (Tsakanika et al., 2018; Sivaraman et al., 2019). *O. niloticus* (L.), the second most common fish species in the world contributes to its excellent production, fast adaptation, and increased marketing quality (Prabu et al., 2019). In modern aquaculture, intensified fish farming was considered the most effective strategy which could be disrupted by the poor quality of water, hypoxia, and bacterial infection would also suppress the immunity and increase the danger of aquaculture industries. Similarly, the production of nutritional feed additives to address any expected problems and to sustain the aquaculture industries environmentally safe have a relevant significance. To enhance the quality of the feed through the use of biological feed additives important to promote fish growth, immune response and sustainability are imperative (Hoseinifar et al., 2015, 2018).

CS has gained significant attention in the fishing industry for several fish species because of its prominent antimicrobial activities against a broad spectrum of pathogenic microorganisms (Ahmed et al., 2020). One of these dietary supplements CS, a linear polysaccharide of poly-( $\beta$ -1,4)-2-amino- 2-deoxy- D-glucopyranose units, produced from the exoskeletons of crustaceans and some fungi cell walls (Li et al., 2017). CS used as a dietary supplement in many European countries and generally recognized as safe (GRAS) as well as being endorsed as wound dressing by the FDA (USA) (Rampino et al., 2013).

The increasing worldwide restriction on antibiotic use and lack of an effective immunization strategy prompted fish aquaculturists to search for alternative, safe strategies for disease control. Currently, prebiotics, probiotics, synbiotics, and immunostimulants were widely utilized with appropriate amounts of commercial success for aquaculture production (Amenyogbe et al., 2020). In this context, CS being a natural non-toxic immunostimulant that enhances both humoral and cell-mediated immune response of various fish species by improving phagocytosis, alternative complements, respiratory bursts, lysozyme activity, and serum antibacterial peptide activity (Dawood et al., 2018).

The use of CS as an antimicrobial agent has received increasing importance in green chemistry (Abdel-Ghany and Salem, 2020). CS possesses a broad range of antimicrobial properties, due to its intracellular penetration by linking CS positive charge to the bacterial surface negative charge, resulting in impaired membrane integrity that triggers apoptosis (Ma et al., 2017). The unique properties of nano-sized materials possess excellent bioactivity and persist in the bloodstream for a long time (Nair et al., 2016). Udayangani et al. (2017) stated that the small size  $\beta$ -glucan has a greater immune response in fish and thus significantly increases disease resistance. Furthermore, nanosized materials are being used as antibiotics in fish farming (Dananjaya et al., 2016).

Existing conventional drugs have been replaced by using CSNPs with better immunostimulant characteristics could be a promising approach for enhancing fish production in the aquaculture sector. Hence, it is hypothesized that the use of NAG loaded CSNPs could enhance feed qualities, fish performance, and immune response. The present study aimed to assess the effect of NAG loaded CSNPs on survival rate, growth rate, and non-specific immune response of Nile tilapia. To develop a cost-effective feed formulation that escalates both disease resistance and

immune prophylaxis in fishes through polymeric nano-particle loaded with chitin derivatives. The present work will make a new era in the aquaculture sector by the development of economical feed for farm fishes.

## 2. Materials and methods

### 2.1. Sampling site

Shrimps shells used for the extraction of chitin and CS were collected from various locations of shrimp disposable area near Port Novo landing center located on the South-East coast of India (Parangipettai – Lat. 11°29' N, Long. 79°46' E).

### 2.2. Chitin extraction and CS production

Chitin extracted from shrimp shell waste was carried out according to the method described by Dima et al. (2015). About 1 g of chitin was dissolved in 25 mL of 50% (w/v) sodium hydroxide solution at 120 °C for 2 h. The prepared suspensions were filtered, rinsed with deionized water to obtain neutral pH, and dried at 65 °C for 24 h, to obtain CS particles.

### 2.3. Synthesis of CSNPs

The CSNPs were synthesized with slight modifications depending on the CS and TPP concentrations by the ionotropic gelation method according to Calvo et al. (1997) and Dima et al. (2015). The CS solution 1% (w/v) was produced by stirring up CS with aqueous acetic acid 1% (v/v) at 25 °C for 12 h. The solution was centrifuged at 9000 rpm for 30 min to separate the upper phase and filtered by pore size filters (1  $\mu$ m). Then, a 0.4% (w/v) TPP solution was prepared by incorporating the drop-by-drop TPP solution to CS through rapid magnetic stirring for 40 min. The synthesized CSNPs were harvested by centrifuge at 7000 rpm, 4 °C for 40 min, and washed with distilled deionized water. To obtain a relatively uniform suspension, ultrasonication (50 w) was performed by sonicator in an ice bath for 4 min and sonicate for every 0.7 s. The synthesized CSNPs samples were freeze-dried at –80 °C for 72 h using a lyophilizer.

### 2.4. Preparation of N-acetyl-D-glucosamine (NAG) loaded with CSNPs

NAG loaded with CSNPs was synthesized according to the modified method of Oushani et al. (2020). NAG (purity  $\geq$  99%) used in this study was purchased from Sigma-Aldrich (St. Louis, MO). For preparing NAG-loaded CSNPs, different concentrations of CS (0.25, 0.5, 1.0, and 2.0 g) were dissolved in 100 mL of distilled water (0.25–2.0 g based on the ratio) and stirred for 1 h at RT. NAG (0.2 g) was dissolved in the same amount of distilled water and kept at RT for 30 min. CS and NAG solutions were mixed, stirred for 24 h, and dried at 60 °C for 24 h.

### 2.5. CSNPs and NAG loaded CSNPs characterization

#### 2.5.1. Ultraviolet–visible spectroscopy

The UV–visible spectrum of CSNPs and NAG loaded CSNPs was measured using UV–visible 1800 spectrophotometer (Shimadzu, Japan) wavelength range from 200 to 800 nm to confirm the synthesis of CSNPs (Vaezifar et al., 2013).

#### 2.5.2. Fourier-transform infrared (FTIR) analysis

The CSNPs and NAG loaded CSNPs were measured with frequency ranges from 4000 to 400  $\text{cm}^{-1}$  through FT-IR spectra (Bruker, France). Samples were processed by KBr to shape pellets for the CSNPs and NAG loaded CSNPs. Sixteen measurements were acquired for every spectrum, with a frequency of 4  $\text{cm}^{-1}$  (Hosseini et al., 2015).

### 2.5.3. Field emission scanning electron microscopic (FE-SEM) analysis

FE-SEM analysis was performed using Carl Zeiss NTS GMBH, SUPRA 55, Germany. A uniform thickness film was formed by positioning a tiny amount of NAG loaded CSNPs and CSNPs specimens on a carbon-coated copper filter. The excess specimen was removed by filter paper, and an SEM grid containing the film was placed under a mercury lamp intensity (100–600 W) for drying for 5 min. After that, the film was amplified at varying resolution intensity for nanoparticle interpretation (Shanmugasundaram et al., 2017).

### 2.5.4. High – resolution transmission electron microscope (HR-TEM) analysis

The morphological features of NAG loaded CSNPs and CSNPs were analyzed using an HR-TEM, Tecnai G20, Netherland. The suspensions were mounted on a carbon-coated copper grid (3.05 mm of diameter, grid size 200 mesh  $\times$  125  $\mu$ m pitch, 97  $\mu$ m of hole W), then the leftover moisture was drained by filter paper on the copper grid. Finally, the specimen was air-dried before analysis.

### 2.5.5. X-ray diffraction (XRD) analysis

CSNPs and NAG loaded CSNPs scattering patterns were measured using Siemens D5000 X-ray diffractometer, from a threshold angle of 5–50° at 0.04°/min of transition frequency (Hosseini et al., 2015).

### 2.5.6. Dynamic light scattering (DLS) and zeta potential analysis

The CSNPs and NAG loaded CSNPs particle size and polydispersity index (PDI) were measured by DLS (Malvern, United Kingdom) at 25 °C. The CSNPs and NAG loaded CSNPs prepared at pH 4.6 and pH 7.4, respectively was used to analyze the zeta potential value range from +100 mV to –100 mV through zeta sizer (Malvern, United Kingdom). The samples were kept at 25 °C to confirm the stability of synthesized nanoparticles and then samples were inverted multiple times before analysis (Shanmugasundaram et al., 2017).

## 2.6. Entrapment efficiency (EE) of NAG-loaded CSNPs

NAG was encapsulated by entrapping 0.2% (w/v) NAG in the CS-TPP matrix by ionic cross-linking with TPP. The efficacy of NAG encapsulated into CSNPs was assessed based on the extraction of CSNPs from the hydrophilic phase having pseudo-entrapped NAG by centrifuge (Beckman Coulter, CA) at 9000 rpm, 4 °C for 30 min. The UV–visible spectroscopy was used to determine the quantity of the non-entrapped free NAG in the clear solution. The percentage of entrapment efficiency in NAG loaded CSNPs was estimated based on Eq. (1) below according to Wang et al. (2008).

$$EE (\%) = \frac{\text{Total quantity of NAG loaded} - \text{free of NAG in supernatant}}{\text{Total quantity of NAG loaded}} \times 100 \quad (1)$$

## 2.7. Experimental design and diet preparation

Five dietary supplements incorporating 0.0 (control), 0.25, 0.5, 1.0, and 2.0 g of NAG-loaded CSNPs/kg feed was designed to provide 30% (w/w) of crude protein as shown in Table 1 (Abdel-Tawwab et al., 2019). Besides, various concentrations of the NAG-loaded CSNPs feed supplement were dissolved in 100 mL/kg feed and homogenized with the additional ingredients for 30 min to produce a fine consistency dough. The dough was processed through a mixer simultaneously and pelleted through a paste extruder with a diameter of approximately 1

**Table 1**

Chemical composition of feed supplements (% dry weight) containing various levels of N-acetyl-D glucosamine (NAG) loaded chitosan nanoparticles (CSNPs).

| Components                             | NAG loaded CSNPs (g/kg diet) |        |       |       |       |
|----------------------------------------|------------------------------|--------|-------|-------|-------|
|                                        | 0.0<br>(Control)             | 0.25   | 0.5   | 1.0   | 2.0   |
| Fish meal                              | 104                          | 104    | 104   | 104   | 104   |
| Soya bean meal                         | 330                          | 330    | 330   | 330   | 330   |
| Chicken meal                           | 150                          | 150    | 150   | 150   | 150   |
| Groundnut cake                         | 203                          | 203    | 203   | 203   | 203   |
| Wheat bran                             | 105                          | 104.75 | 104.5 | 104   | 103   |
| Codfish oil                            | 23                           | 23     | 23    | 23    | 23    |
| Corn oil                               | 15                           | 15     | 15    | 15    | 15    |
| Vitamins                               | 10                           | 10     | 10    | 10    | 10    |
| Minerals                               | 20                           | 20     | 20    | 20    | 20    |
| Starch                                 | 40                           | 40     | 40    | 40    | 40    |
| NAG loaded CSNPs                       | 0.0                          | 0.25   | 0.5   | 1.0   | 2.0   |
| Total                                  | 1000                         | 1000   | 1000  | 1000  | 1000  |
| Proximate chemical analysis (%)        |                              |        |       |       |       |
| Dry matter                             | 93.5                         | 92.7   | 92.8  | 93.1  | 92.5  |
| Crude protein                          | 32.17                        | 32.1   | 33.15 | 34.19 | 33.17 |
| Ether extract                          | 6.2                          | 6.4    | 6.4   | 6.3   | 6.3   |
| Total ash                              | 4.2                          | 4.1    | 5.4   | 5.9   | 5.3   |
| Crude fiber                            | 5.5                          | 5.7    | 5.8   | 6.6   | 6.8   |
| Nitrogen free extract <sup>a</sup>     | 49.92                        | 49.90  | 49.95 | 49.96 | 49.93 |
| Gross energy (Kcal/100 g) <sup>b</sup> | 445.5                        | 446.9  | 453.1 | 458.4 | 452.1 |

<sup>a</sup> Nitrogen free extract (NFE) = 100 – (protein % + lipid % + total ash % + crude fiber %).

<sup>b</sup> Gross energy was calculated according to NRC (1993) as 5.65, 9.45, and 4.11 kcal/g for protein, lipid, and carbohydrates, respectively.

mm. The feed pellets were dried at 55 °C for 24 h and preserved at –20 °C for future usage.

*Oreochromis niloticus* (L.) (hereafter referred to as *O. niloticus*) fish species were procured at ICAR-Central Institute of Brackishwater Aquaculture (CIBA), Chennai, India. Fishes were placed in an indoor oxygenated glass fiber tank for 2 weeks to adapt to the controlled environment and the light/dark cycle (12/12 h) was monitored under illumination. A total of 150 Nile tilapia fingerlings (15.30–15.83 g) were divided into five experimental groups (30 fish each) with three replicates (10 fish each) for each group. The NAG loaded CSNPs were provided to the control diet with different concentrations as 0.0, 0.25, 0.5, 1.0, and 2.0 g/kg diet for 45 d. Fishes consumed the feed twice daily at 9 a.m. and 6 p.m. for 45 d.

## 2.8. Feed consumption and growth rate measurements

After completion of the feeding trial analysis on the 45th d, the fishes fed with NAG-loaded CSNPs/kg feed from each tank were harvested, measured, and recorded. The survival rate of the fishes was also calculated by enumerating the individuals in each tank. The efficiency of fish productivity and diet consumption parameters includes weight gain (WG), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR) and protein efficiency ratio (PER) was determined based on Eq. (2) – (8) prescribed by Abdel-Tawwab et al. (2019).

$$\text{Weight gain (g)} = W_2 - W_1 \text{ (g)} \quad (2)$$

$$\text{Weight gain (\%)} = \frac{100(W_2 - W_1)}{W_1} \quad (3)$$

$$\text{Specific growth rate (SGR)} = 100 \frac{[\ln W_2(\text{g}) - \ln W_1(\text{g})]}{T} \quad (4)$$

$$\text{Feed intake (FI)} (\text{g feed / fish}) = \frac{\text{Summation of diets provided to fish throughout the number of the experiment}}{\text{fish number}} \quad (5)$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Feed consumption (g)}}{\text{Weight gain (g)}} \quad (6)$$

$$\text{Fish survival (\%)} = \frac{100 (\text{Fish number at final})}{\text{Fish number at initial}} \quad (7)$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Total wet weight gain (g)}}{\text{Crude protein fed (g)}} \quad (8)$$

where, W2 - final weight of the fish (gm), W1 - initial weight of the fish (gm), and T-total experiment duration (d).

### 2.8.1. Non-specific immune response in *O. niloticus*

Fish blood samples were aseptically obtained (every week) from the caudal vessels (100 IU/mL) in sodium heparin anticoagulant to assess the non-specific immunological parameters.

### 2.8.2. RBC and WBC count

For hematological parameters assessment, both RBC and WBC counts were counted using a Neubauer counting chamber (hemocytometer) based on Blaxhall (1972).

### 2.8.3. Respiratory burst activity and lysozyme activity

To determine the respiratory burst activity, the fish peripheral blood leucocytes were collected and assessed by NBT assay according to Esteban et al. (2001). Lysozyme activities were determined from serum samples using the turbidometric method according to Abu-Elala et al. (2013).

## 2.9. Antibacterial assay

The antibacterial efficacy of the synthesized CSNPs and NAG-loaded CSNPs were tested against the selected clinically pathogenic bacteria namely *Streptococcus agalactiae*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens* were procured from the Centre for Aquaculture, Sathyabama Institute of Science and Technology, Chennai, India. The standard agar well diffusion technique has been adopted to assess the antimicrobial property of the synthesized CSNPs (Soleimani et al., 2015). The bacterial strains were grown separately in LB broth and incubated for 24 h at 37 °C. The antimicrobial effect was evaluated using the Kirby-Bauer agar diffusion method and bacterial cultures grown overnight. The bacterial culture (0.5 McFarland OD) was swabbed as a uniform layer over the Petri dishes pre-seeded with the Mueller Hinton agar (MHA) medium. In all the MHA plates, four wells of 0.7 mm diameter were formed using a sterile cork borer. Each of the wells was labeled and loaded with 50 µL of CS, CSNPs, NAG loaded CSNPs along with negative control (saline), and positive control (ampicillin). The bacterial cultures were incubated for 24 h at 37 °C and the inhibitory effect was determined by inhibition zones formed around the wells.

## 2.10. Statistical analysis

All the analyses were carried out in triplicate, and the results were represented as the mean ± standard error. All the obtained data were subjected to one-way ANOVA analysis using SPSS software (version 17.0, SPSS Inc., USA). ANOVA was used to detect differences among groups, and then the difference in means was determined using Duncan's multiple range test.

## 3. Results

### 3.1. Synthesis of CSNPs and NAG loaded CSNPs

In this study, ionotropic gelation of CSNPs was synthesized with drop

by drop adding of TPP to CS solution. The synthesized CSNPs showed monodispersed nanoparticle (<100 nm) size, consistently with low polydispersity index (PDI) values. The PDI was used as a measure of nanoparticle stability and structural homogeneity. The PDI value represents the distribution of nanoparticle size; samples have significantly greater PDI values with a greater range of particle sizes, whereas samples composed of equally sized particles have relatively low PDI values.

In the present study, low PDI values indicate more monodisperse CSNPs and higher particle stability, whereas greater values indicate less particle stability and aggregation of CSNPs. The occurrence of opalescence confirmed the efficient synthesis of CSNPs was characterized for physicochemical characteristics such as particle size, zeta potential, ultrastructural morphology, and crystalline structure.

### 3.2. Characterization of CSNPs and NAG loaded CSNPs

#### 3.2.1. UV-vis absorption spectrum

UV-vis absorption spectrum was used to study the optical characteristics of CSNPs and NAG loaded CSNPs shown in Fig. S1. The CSNPs and NAG loaded CSNPs were detectable in the spectrum wavelength between 200 and 300 nm. A UV-visible CSNPs spectrum acquired sharp absorption band intensity at 226 nm. UV-visible spectroscopy confirmed the NAG loaded CSNPs formation by encapsulation of NAG into CSNPs.

Analysis of the UV-vis absorption spectra indicated the characteristic absorption peak of NAG loaded CSNPs at 230 nm (Fig. S1). On the other hand, NAG loaded CSNPs showed significantly greater absorption spectra reported than CSNPs, due to NAG formation. Furthermore, there has been no peak was observed from the range of 250–800 nm visible spectrum for NAG loaded CSNPs solution, validating NAG formation.

#### 3.2.2. FT-IR spectra analysis

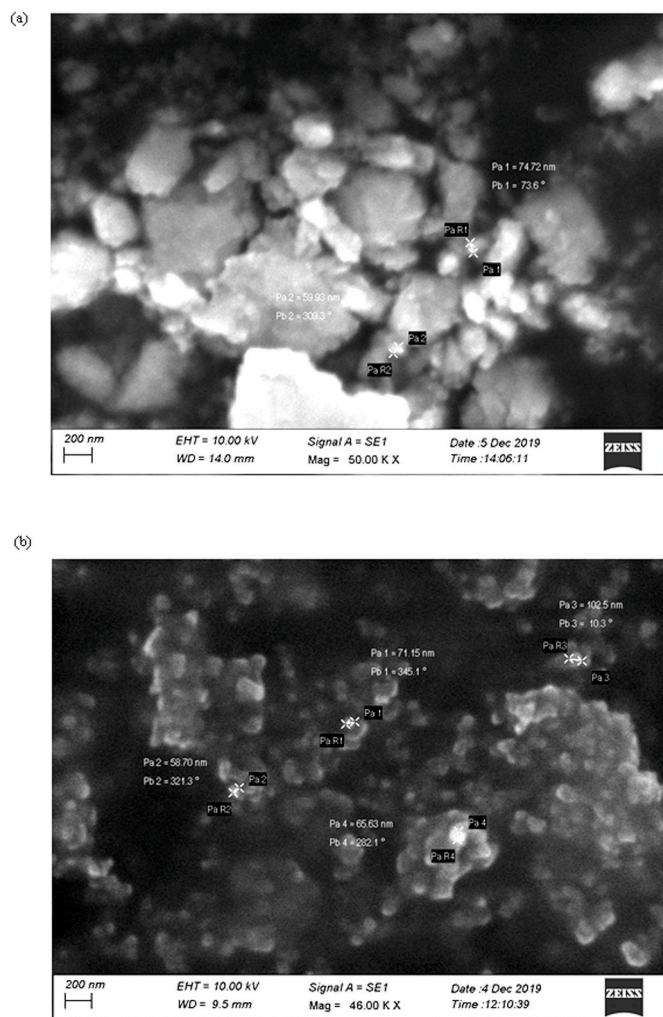
FT-IR analyses were used to assess the molecular structure of CSNPs and NAG loaded CSNPs were shown (Fig. S2). In this study, CSNPs exhibited stretching vibration bands at 3445 cm<sup>-1</sup> (-OH and -NH<sub>2</sub> stretch bends), 2621 cm<sup>-1</sup> (-OH stretching), 2063 cm<sup>-1</sup> (C=C asymmetric stretching), 1715 cm<sup>-1</sup> (C=O asymmetric stretch), 1640 cm<sup>-1</sup> (amide I band), 1388 cm<sup>-1</sup> (CH<sub>3</sub> stretch), 1276 cm<sup>-1</sup> (C=O stretch), 1050–1075 cm<sup>-1</sup> (C-O vibrations), 886 cm<sup>-1</sup> (C=CH<sub>2</sub> stretch vibration) and 623 cm<sup>-1</sup> (-CH stretching) (Fig. S2).

The strong broad peak at 3445 cm<sup>-1</sup> indicated the characteristic of the hydrogen-bonded OH stretching band. This indicates that certain proteins and metabolites such as sterols and alkaloids that have hydroxyl, amines, alcohols, phenol, and carboxylic acid functional groups surrounded by CS. Also, the band at 1276 cm<sup>-1</sup> decreased significantly and a new vibration band was developed at 1150 cm<sup>-1</sup> (C-O-C stretch). The strong peaks can be attributed to the symmetrical CH<sub>3</sub> vibration bend at 1380 cm<sup>-1</sup> and 1419 cm<sup>-1</sup>. After synthesis of NAG-loaded CSNPs, a new band (amide II stretching) occurred at 1461 cm<sup>-1</sup> (Fig. S2) whereas, the amide I band was transitioned from 1640 to 1633 cm<sup>-1</sup>.

#### 3.2.3. FE-SEM analysis

SEM analysis was employed to characterize the microstructure of particles, such as the size, shape, and dispersion patterns of synthesized CSNPs and NAG-loaded CSNPs. The SEM micrographs of CSNPs and NAG-loaded CSNPs are shown (Fig. 1). In general, the CSNPs demonstrated dense spherical particle bodies and uniform nanostructure, while the NAG-loaded CSNPs exhibited relatively homogeneous microstructure with a spherical shape, highly dispersed, and small particles.

Similar appearances of CSNPs were observed in previous studies (Hosseini et al., 2013). The different surface characteristics and spherical morphology of CSNPs indicated that nano-sized CSNPs were formed by cross-linking of CS and TPP molecules. The results indicated that the CSNPs represent a highly porous surface structure due to aggregation characteristics. The SEM micrographs of CSNPs distinctly showed a relatively dense surface morphology, because of its aggregation characteristics.



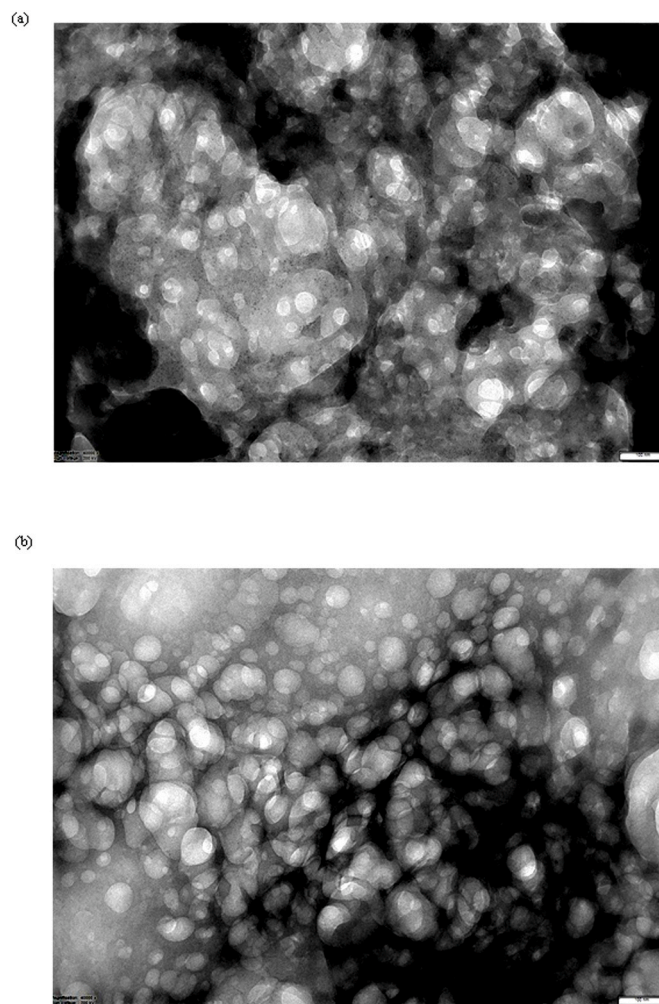
**Fig. 1.** SEM micrographs of (a) chitosan nanoparticles (CSNPs) showing dense spherical shaped bodies and uniform microstructure (b) N-acetyl-D glucosamine (NAG) loaded chitosan nanoparticles (CSNPs) showing homogenous morphology with a spherical shape, highly dispersed, and small-sized particles.

### 3.2.4. HR-TEM analysis

HR-TEM measurement confirmed the accurate particle size and microstructure of NAG loaded with CSNPs. The microstructure of CSNPs and NAG-loaded CSNPs synthesized under optimum conditions has been shown (Fig. 2). The CSNPs showed a spherical morphology and a nano-sized distribution of particles with a diameter of 60–70 nm. The TEM micrographs showed sample lattice fringes indicate a high degree of crystallinity. The CSNPs were formed by the interaction between positive charge amino groups of CS and negative charge of TPP ions from CS and TPP. HR-TEM provides an accurate size of CSNPs in a dry state.

### 3.2.5. XRD measurements

XRD was employed to assess the degree of crystallite size and the physical state of CSNPs loaded into the NAG. The XRD patterns of CSNPs and NAG loaded CSNPs are shown (Fig. S3). CSNPs showing a diffraction peak at  $2\theta$  range with a  $20^\circ$ , the greater diffraction angle was shifted slightly and revealed the excellent crystalline nature of the CSNPs. A wide diffraction peak at  $20^\circ$  was assigned to be the significant diffraction peak (110) of both CSNPs and NAG loaded CSNPs spectra. At the same time, CS contained only one broad diffraction peak centered at  $2\theta = 20.20^\circ$ . Interestingly, when NAG was loaded with CSNPs, four new diffraction peaks were observed which can be designated to 111, 200, 220, and 311 CSNPs face center cubic (fcc) lattice at  $2\theta = 30.1^\circ$ ,  $44.6^\circ$ ,



**Fig. 2.** TEM microstructure of (a) chitosan nanoparticles (CSNPs) and (b) N-acetyl-D glucosamine (NAG) loaded chitosan nanoparticles (CSNPs) exhibiting spherical particles with a smooth surface and homogeneous distribution of size.

$64.6^\circ$  and  $77.5^\circ$ , respectively.

### 3.2.6. Particle size distribution by DLS

The DLS analysis has often been used to validate the average particle size dispersion of NPs. Fig. S4 shows a characteristic distribution pattern of CSNPs and NAG loaded CSNPs with a mean particle size of 44 nm and 106 nm, respectively. The differences in surface characteristics of CSNPs from CS and TPP molecules were significant, as nano-sized CSNPs were formed through cross-linking between CS and TPP molecules at a specific volume ratio as exhibited by DLS data.

### 3.2.7. Zeta potential

The surface charges an important criterion to determine the stability and conductivity of particle systems to biological surfaces. Therefore, the investigation of zeta potential a crucial component for the characterization of nanoparticles. Hence, the CSNPs and NAG loaded CSNPs were evaluated by zeta potential and compared with the precursor CS. In this study, both the CSNPs and the NAG loaded CSNPs exhibited a positive zeta potential value, depending on its surface charge (Fig. S5). When CS and TPP were combined, rapidly generated compact structures with a relatively positive surface charge and confirmed by the measurement of zeta potential values.

The CSNPs and NAG loaded CSNPs prepared at pH 4.6 and pH 7.4 showed the zeta potential value of +5.37 and +11.3 mV, respectively. The present findings indicate that synthesized CSNPs and NAG loaded

CSNPs were more stable with positive zeta potential value. This positive zeta potential charge is consistent with previous findings. In this study, the lower value of zeta potential demonstrated the formation of the increased second peak due to the aggregation of nanoparticles.

### 3.3. Entrapment efficiency of NAG

In this study, the loading capacity and trapping efficiency were evaluated for the synthesis of NAG and CSNPs. In this study, loading capacity (LC) of  $89.7 \pm 1.2\%$  and high encapsulation capacity of  $95.7 \pm 0.12\%$  was found between NAG and CSNPs. The electrophoretic mobility of NAG was assessed before and after loading onto CSNPs, clearly indicates that the NAG was well trapped. Several studies have assessed the encapsulation of CS with nanoparticles, enhanced their deliverability inside the fish body. For example, Alishahi et al. (2011) demonstrated the encapsulation of vitamin C with CSNPs help to protect it from adverse conditions and to control its release through the gastrointestinal tract of *Oncorhynchus mykiss*.

### 3.4. Growth and feed conversion efficiency of *O. niloticus*

Though, CSNPs possess diverse biological effects, very little is known about the effects of various concentrations of CSNPs on productivity and survival rate of *O. niloticus*. It was suggested that the dietary NAG loaded CSNPs had a positive impact on growth efficiency and feed conversion ratio when fish supplemented with CSNPs enriched diets than control basal feed. In this study, the effects of oral NAG loaded CSNPs administered to *O. niloticus* on the growth efficiency was investigated. Table 2 shows that the optimal NAG loaded CSNP dose for *O. niloticus* was 1.0 g/kg of feed compared to control and other treatment conditions.

In comparison, fish fed on dietary supplementation contain 1.0 and 2.0 g CSNPs/kg diet consumed more feed  $40.61 \pm 0.55$  and  $40.06 \pm 0.43$  g/fish, respectively than control basal feed 15.1 g/fish (Table 2;  $p < 0.05$ ). On the other hand, no significant variations were found in the feed conversion ratio with a range from  $1.86 \pm 0.03$  to  $3.14 \pm 0.29$  (g/g) (Table 2;  $p > 0.145$ ). During the experimental period, control and treatment groups exhibited excellent health as inferred from their growth efficiency. The survival rate of fish was 100%, with no significant variation between the control and treatment groups (Table 2;  $p < 0.05$ ), which indicates that the dietary NAG loaded CSNPs do not have adverse effects on fishes. Administration of optimum levels of oral NAG loaded CSNP (1.0 g/kg) significantly increased the body weight gain, SGR, feed intake and decreased FCR (Table 2;  $p < 0.05$ ).

### 3.5. Non-specific immune response in *O. niloticus*

#### 3.5.1. Haematological parameters

The effect of NAG loaded CSNPs diet supplemented with fish significantly increased RBC and WBC count from 1st week to 3rd week in the control group (Fig. 3). The number of red blood cells decreased in a

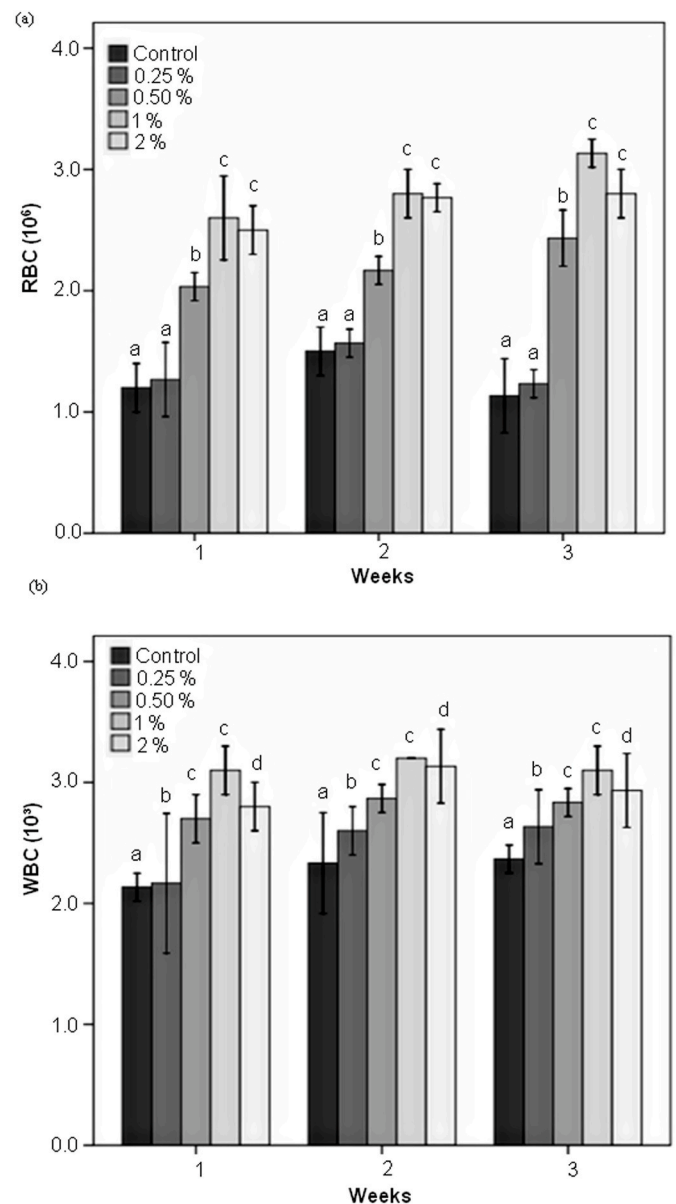


Fig. 3. Effect of different concentration of N-acetyl-D glucosamine (NAG) loaded chitosan nanoparticles (CSNPs) (0.0, 0.25, 0.50, 1.0 and 2.0%) supplemented diets for *O. niloticus* showing (a) RBC count and (b) WBC count. Vertical bars represented the mean  $\pm$  SE. Different superscript letters indicate significant differences between the control and treatment groups ( $p < 0.05$ ). Values are the mean  $\pm$  SE ( $n = 3$ ).

Table 2

Growth and feed consumption efficiency of *O. niloticus* supplemented with varying quantities of N-acetyl-D glucosamine (NAG) loaded chitosan nanoparticles (CSNPs).

| Parameters                           | NAG loaded CSNPs (g/kg diet)  |                                |                                |                                  |                                  | P-value |
|--------------------------------------|-------------------------------|--------------------------------|--------------------------------|----------------------------------|----------------------------------|---------|
|                                      | 0.0 (Control)                 | 0.25                           | 0.5                            | 1.0                              | 2.0                              |         |
| Initial weight (g)                   | 15.30 $\pm$ 0.23              | 15.63 $\pm$ 0.14               | 15.56 $\pm$ 0.08               | 15.83 $\pm$ 0.17                 | 15.73 $\pm$ 0.08                 | <0.046  |
| Final weight (g)                     | 24.33 $\pm$ 0.43 <sup>a</sup> | 30.73 $\pm$ 0.44 <sup>b</sup>  | 34.80 $\pm$ 0.20 <sup>c</sup>  | 37.3 $\pm$ 0.56 <sup>d</sup>     | 37.17 $\pm$ 0.20 <sup>d</sup>    | <0.0001 |
| Weight gain (g)                      | 9.033 $\pm$ 0.66 <sup>a</sup> | 15.37 $\pm$ 0.37 <sup>b</sup>  | 19.23 $\pm$ 0.23 <sup>c</sup>  | 21.47 $\pm$ 0.58 <sup>c,d</sup>  | 21.43 $\pm$ 0.24 <sup>d</sup>    | <0.0001 |
| Weight gain (%)                      | 59.19 $\pm$ 5.23 <sup>a</sup> | 98.35 $\pm$ 3.22 <sup>b</sup>  | 123.56 $\pm$ 1.91 <sup>c</sup> | 135.63 $\pm$ 4.33 <sup>c,d</sup> | 136.24 $\pm$ 2.07 <sup>d,e</sup> | <0.0001 |
| Specific growth rate (SGR) (%)       | 3.17 $\pm$ 0.018 <sup>a</sup> | 3.38 $\pm$ 0.01 <sup>b</sup>   | 3.53 $\pm$ 0.12 <sup>c</sup>   | 3.57 $\pm$ 0.15 <sup>d</sup>     | 3.28 $\pm$ 0.097 <sup>d</sup>    | <0.0001 |
| Total food intake (TFI) (g)          | 28.03 $\pm$ 0.94 <sup>a</sup> | 32.21 $\pm$ 0.98 <sup>b</sup>  | 36.37 $\pm$ 0.68 <sup>c</sup>  | 40.61 $\pm$ 0.55 <sup>d</sup>    | 40.06 $\pm$ 0.43 <sup>d</sup>    | <0.0001 |
| Feed conversion ratio (FCR) (g/g)    | 3.14 $\pm$ 0.29 <sup>a</sup>  | 2.10 $\pm$ 0.11 <sup>a,b</sup> | 1.89 $\pm$ 0.03 <sup>a,b</sup> | 1.89 $\pm$ 0.02 <sup>a,b</sup>   | 1.86 $\pm$ 0.03 <sup>b</sup>     | >0.145  |
| Protein efficiency ratio (PER) (g/g) | 2.17 $\pm$ 0.09 <sup>a</sup>  | 2.32 $\pm$ 0.04 <sup>a,b</sup> | 2.49 $\pm$ 0.11 <sup>b</sup>   | 2.54 $\pm$ 0.23 <sup>b</sup>     | 2.49 $\pm$ 0.09 <sup>b</sup>     | <0.029  |
| Survival rate (%)                    | 100 $\pm$ 0.00                | 100 $\pm$ 0.00                 | 100 $\pm$ 0.00                 | 100 $\pm$ 0.00                   | 100 $\pm$ 0.00                   | –       |

Values within the same row having different superscripts are significantly different ( $P < 0.05$ ). Data were presented as mean  $\pm$  SE ( $n = 3$ ). <sup>a,b,c,d,e</sup> superscripts represents significant variation based on DMT (Duncan multiple test range).

control group without supplementation of feed enriched with NAG loaded CSNPs. After feeding with an enriched diet, fishes RBC counts dramatically increased from 1st week and the RBC level improved the blood system due to the NAG loaded CSNPs diet.

The control group showed gradual value, but the fish fed with different concentrations of 0.25, 0.50, 1.0, and 2.0 g CSNPs/kg diet has significantly increased RBC and WBC count in treatment groups. In comparison, fish fed on dietary supplementation contain 1.0 g CSNPs/kg diet exhibited increased RBC and WBC count than other treatment groups. Fish fed diets contain NAG loaded CSNPs experienced an increased level of blood parameters.

### 3.5.2. Respiratory burst activity

Studies on neutrophil activity showed the enhancing effect of NAG loaded CSNPs dietary supplements on neutrophil respiratory burst (RB) activities as evidenced by the increased NBT reduction (Fig. 4). In comparison, fish fed on dietary supplementation contains 1.0 g CSNPs/

**Table 3**

Antimicrobial assay of chitosan (CS), chitosan nanoparticles (CSNPs), and N-acetyl-D glucosamine (NAG) loaded chitosan nanoparticles (CSNPs) against selected microbial strains.

| S. No | Microorganisms                  | Antimicrobial activity |            |                       |
|-------|---------------------------------|------------------------|------------|-----------------------|
|       |                                 | CS (mm)                | CSNPs (mm) | NAG loaded CSNPs (mm) |
| 1.    | <i>Pseudomonas aeruginosa</i>   | 12 ± 0.3               | 15 ± 0.2   | 21 ± 0.3              |
| 2.    | <i>Streptococcus agalactiae</i> | 14 ± 0.2               | 19 ± 0.1   | 27 ± 0.2              |
| 3.    | <i>Aeromonas hydrophila</i>     | 10 ± 0.3               | 14 ± 0.3   | 24 ± 0.4              |
| 4.    | <i>Pseudomonas fluorescens</i>  | 11 ± 0.1               | 13 ± 0.3   | 19 ± 0.2              |
| 5.    | Control                         | NA                     | NA         | NA                    |

Zone of inhibition; NA = No activity; CS - Chitosan; CSNPs – Chitosan nanoparticles; NAG – N-acetyl – D-glucosamine. Data were presented as mean ± SE (n = 3).

kg diet exhibited enhanced respiratory burst activity at 3rd week than other treatment groups.

### 3.5.3. Lysozyme activity

The effect of NAG loaded CSNPs diet supplemented with fish significantly increased lysozyme activity from 1st week to 3rd week in treatment and control group (Fig. 4). In comparison, fish fed on dietary supplementation contain 1.0 g CSNPs/kg diet enhanced the lysozyme activity at 3rd week than other treatment groups.

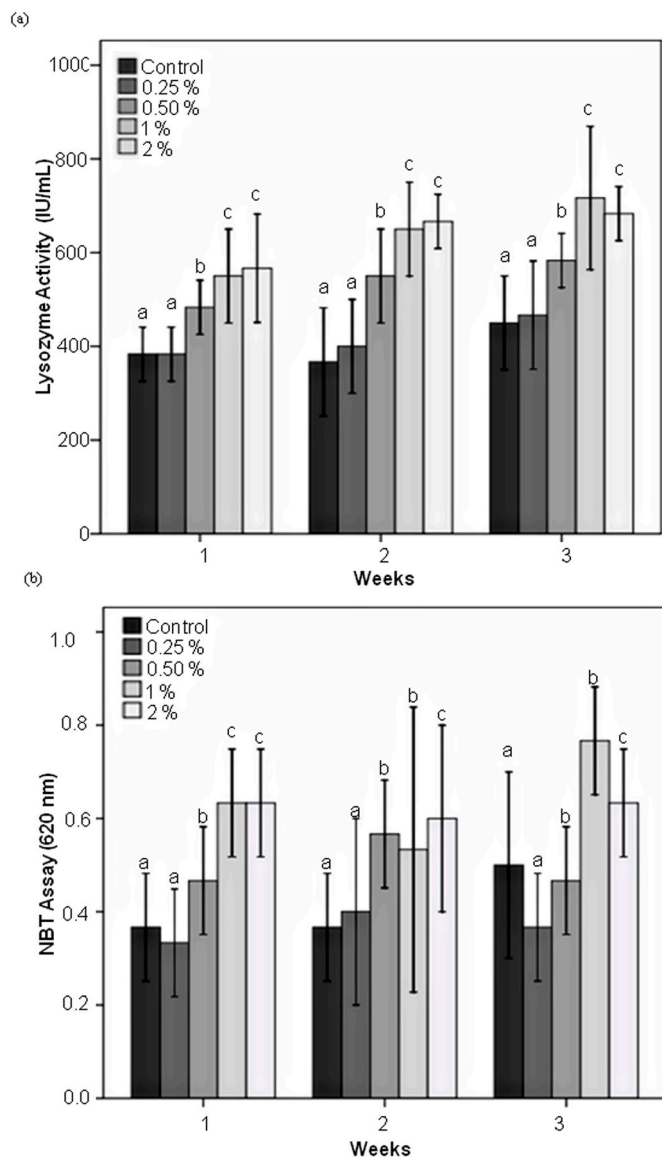
### 3.6. Antimicrobial efficacy of CSNPs and NAG-loaded CSNPs

The antimicrobial efficacy of CSNPs and NAG-loaded CSNPs was evaluated using the agar well diffusion technique against *Streptococcus agalactiae*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens*. The data obtained for antimicrobial assay of CSNPs and NAG-loaded CSNPs are shown in Table 3. Among the tested bacterial strains *Streptococcus agalactiae*, exhibited higher inhibitory activity with a maximum inhibition zone of 27 mm for NAG-loaded CSNPs. While, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens* exhibited inhibitory activity of 24 mm, 21 mm, and 19 mm, respectively. In this study, NAG-loaded CSNPs exhibited the highest antimicrobial properties compared to CSNPs and CS implying moderate to lower inhibitory effect against tested pathogens (Table 3).

## 4. Discussion

CSNPs can be synthesized using different methods, including ionic cross-linking, complex-coacervation, modified emulsification, precipitation, and ionotropic gelation (Samrot et al., 2016). In the current study, the ionic gelation method was followed to obtain CS nanosuspension. Synthesis of CSNPs using the aforementioned techniques is beneficial because of its fast and mild process, without the use of organic solvents and high temperatures (Rampino et al., 2013). It depends on the ionic interaction between the positively charged amino groups of the acidic CS solution and the negatively charged polyanions of the aqueous STPP solution. The continuous magnetic stirring allows the formation of intermolecular crosslinking between the oppositely charged molecules (Ahmed et al., 2020).

The synthesized NAG loaded CSNPs and CSNPs were analyzed for physicochemical characteristics includes particle size, zeta potential, ultrastructural morphology, and crystalline structure. The optical characteristics of CSNPs and NAG loaded CSNPs were detected through a UV-vis absorption spectrum wavelength range between 200 and 300 nm. The present results were consistent with earlier findings documented by Govindan et al. (2012) and Akmaz et al. (2013).



**Fig. 4.** Effect of different concentration of N-acetyl-D glucosamine (NAG) loaded chitosan nanoparticles (CSNPs) (0.0, 0.25, 0.50, 1.0 and 2.0%) supplemented diets for *O. niloticus* showing (a) lysozyme activity and (b) respiratory burst activity. Vertical bars represented the mean ± SE. Different superscript letters indicate significant differences between the control and treatment groups (p < 0.05). Values are the mean ± SE (n = 3).

The synthesized CSNPs exhibited IR stretching vibration bands were observed at  $3445\text{ cm}^{-1}$  ( $-\text{OH}$  and  $-\text{NH}_2$  stretch bends),  $1640\text{ cm}^{-1}$  (amide I band),  $1276\text{ cm}^{-1}$  ( $\text{C}=\text{O}$  stretch),  $623\text{ cm}^{-1}$  ( $-\text{CH}$  stretching), and  $1419\text{ cm}^{-1}$  (strong peaks attributed to the symmetrical  $\text{CH}_3$  vibration bend at  $1380\text{ cm}^{-1}$  and  $1419\text{ cm}^{-1}$ ). Similarly, Mohammadpour et al. (2012) and Ferreira et al. (2018) also revealed a significant identical spectral range of infrared bands were observed for CSNPs.

The CSNPs showed the amide I band was transitioned from  $1640$  to  $1633\text{ cm}^{-1}$ , confirms a dynamic electrostatic attraction between  $\text{NH}_3^+$  CS groups and TPP Polyphosphoric groups to enhance intermolecular and intramolecular interactions (Dudhani and Kosaraju, 2010; Jingou et al., 2011). FT-IR spectra for CSNPs exhibit a characteristic absorption peak at  $1217\text{ cm}^{-1}$  implying PO stretching (Qi and Xu, 2004). The CS shows a band at  $3367\text{ cm}^{-1}$  broadened and shifted to  $3100\text{ cm}^{-1}$  in CSNPs signifying strengthened hydrogen bonding (Wu et al., 2005; Xu and Du, 2003).

Further, the SEM micrographs of CSNPs distinctly showed a relatively dense surface morphology, because of its aggregation characteristics. A similar morphological structure has been observed for CS and CSNPs by Oh et al. (2019). The porous structure and aggregation of the CSNPs would be used as an essential CS-based bionanopesticide (Saharan et al., 2015). Aggregation was considered to be the principal phenomenon for the synthesis of novel CSNPs for pharmaceutical and nanomedicine applications (Ghadi et al., 2014).

The HR-TEM analysis of synthesized CSNPs showed a spherical morphology and a nano-sized distribution of particles with a diameter of  $60\text{--}70\text{ nm}$ . Previously, Dananjaya et al. (2017) revealed that TEM analysis results exhibiting that the particle size of CSNPs ranged from  $15$  to  $30\text{ nm}$ . The variation in the size of the CSNPs between DLS and TEM could be resulting due to CSNPs increased in aqueous solution and the DLS providing the NPs hydrodynamic size, while the TEM provides the exact size of the NPs in dried samples.

The aggregation of CS/TPP NPs possible to be because hydrogen bonding interacts with CSNPs progressively becomes prominent in the drying process. It could be observed that NPs in the center and surface have a dark appearance, reflecting significantly greater dispersion of electron density. Even though, TPP contains phosphorus elements, provide a relatively high electron density than CS, it could be deduced that CS has a high level of hydrogen bonding interaction with TPP in this field.

XRD patterns of NAG loaded CSNPs, four new diffraction peaks were observed which can be designated to  $111$ ,  $200$ ,  $220$ , and  $311$  CSNPs face center cubic (fcc) lattice at  $2\theta = 30.1^\circ$ ,  $44.6^\circ$ ,  $64.6^\circ$  and  $77.5^\circ$ , respectively. The data were consistent with the earlier findings reported by Jingou et al. (2011) and Ali et al. (2011). Yoksan et al. (2010) found that characteristic peak was not detected in CSNPs after it bonded with TPP, which represents the disruption of the unique binding mechanism of CS.

Similarly, Rokhade et al. (2006) also reported that the diffraction pattern of CSNPs would influence the hydrogen bonding interaction between CS and TPP, interrupting the CS crystalline structure. Besides, CSNPs reflect a reduced crystalline structure due to freeze-drying (Qi et al., 2004). Jingou et al. (2011) evident the circumference of the diffractogram was influenced by the size of the crystal, and the enlarged band indicates the imprecise crystal structure.

The DLS analysis showed a characteristic distribution pattern of CSNPs and NAG-loaded CSNPs with an average particle size of  $44\text{ nm}$  and  $106\text{ nm}$ , respectively. The particle dimensions measured in the hydrophilic state are observed to be significantly greater than in dry conditions. As a result, the relatively large size could be produced due to the hydration of the CS layer envelopes particles and agglomeration of NPs immersed in water (Yoksan et al., 2010).

Zeta potential was already proposed as a significant factor attributed to the antimicrobial activities of CS by interacting with the negatively charged bacterial surface (Saharan et al., 2015). Further, the CSNPs and the NAG-loaded CSNPs exhibited a positive zeta potential value of  $+5.37$  and  $+11.3\text{ mV}$ , respectively depending on its surface charge. It has been interpreted that the zeta potential could significantly impact the

stability of the particle in the suspension by electrostatic interaction between the particles (Cheng et al., 2012). When CS and TPP were combined, rapidly generated compact structures with a relatively positive surface charge and confirmed by the measurement of zeta potential values.

The positive zeta potential value indicates that CSNPs are highly stable. It would appear that the lengthy amino groups affect the bio-sorption of anion and maintain the greater value of density of the electrical dual film, inferring the reduction of agglomeration (Dima et al., 2015). Chen and Zeng (2018) have reported a similar range of zeta potential for CSNPs. A similar phenomenon for positive zeta potential has been evidenced by the synthesis of copper-charged CSNPs (Du et al., 2009).

Previously, Dudhani and Kosaraju (2010), and Liu et al. (2011) revealed that the positive zeta potential value was observed on the CSNPs surface, resulting due to cationic properties of the CS chain, as well as the CS/TPP nanoparticles. Lorevice et al. (2012) observed that CSNPs value differs from  $14.5$  to  $37.5\text{ mV}$  based on the amount of CS used.

The antimicrobial efficacy of CSNPs and NAG-loaded CSNPs was assessed against gram-positive bacteria *Streptococcus agalactiae*, gram-negative bacteria *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens*. Among the tested bacterial strains *Streptococcus agalactiae*, exhibited higher inhibitory activity than other strains. This study revealed CSNPs exhibited less antimicrobial properties compared to NAG-loaded CSNPs, implying moderate to lower inhibitory effect against tested pathogens.

Numerous studies have demonstrated that CSNPs are highly effective in hindering the growth of *S. aureus*, *C. albicans*, *S. cerevisiae*, *E. coli*, and *F. culmorum* (Jung et al., 2002; Yun et al., 1999). Growth-enhancing impacts of CSNPs could also have been significantly linked to their potent antibacterial activity, as evidenced by a significant dose-dependent inhibitory activity of total intestinal aerobic and anaerobic bacterial species in fish (Huang et al., 2009; De Paz et al., 2011; Kaur et al., 2013).

Due to its polycationic nature, the antibacterial activity of CSNPs specifically linked to primary amine groups. Such amine groups bind to the bacterial cell wall, significantly alter the cell membrane fluidity, disrupt the cell, and attach to DNA, resulting in the inactivation of DNA replication and induce apoptosis (Divya et al., 2017; Nagy et al., 2011). Sathiyavimal et al. (2020) reported that the synthesized CS-CuO nanocomposite has promising inhibitory activity against gram-negative bacteria *E. coli* and *P. mirabilis*. The positive charge CSNPs have received great interest as feasible and effective targeted drug delivery applications, due to excellent trapping efficiency for negative macromolecules (Kang et al., 2014).

The loading capacity and high encapsulation efficiency between NAG and CSNPs were evaluated in this study. The electrophoretic mobility of NAG was assessed before and after loading onto CSNPs, clearly indicates that the NAG was well trapped. Complex stability of nanoparticles is key criterion for evaluating the efficacy of NAGs delivery.

When the amount of CS increased, the viscosity of the solution also increased which inhibiting an efficient ionic interaction between TPP and CS solution that increased the size and percentage of NAG loaded CSNPs was found to be above  $90\%$ . Wu et al. (2005) demonstrated that the concentration of CS ( $1.44\text{ mg/mL}$ ) and TPP ( $0.6\text{ mg/mL}$ ) used to enhance the entrapment potential ( $85\%$ ) and loading capacity ( $7.9\%$ ).

The effects of oral NAG loaded CSNPs administered to *O. niloticus* on the growth performance and feed consumption efficiency were investigated in this study. The optimal NAG loaded CSNPs dose for *O. niloticus* was  $1.0\text{ g/kg}$  of feed. Abdel-Tawwab et al. (2019) also stated that the optimal CSNPs concentration was  $1.0\text{ g/kg}$  feed for *O. niloticus*. During the experimental period, control and treatment groups exhibited excellent health as inferred from their growth efficiency and survival rate indicates that the dietary NAG loaded CSNPs do not have adverse

effects on fishes.

The results are consistent with Dawood et al. (2020) also reported that *L. ramada* fed with CSNPs significantly enhanced the final body weight, weight gain, and specific growth rate compared to the control, whereas feed conversion ratio was decreased in fish fed with 0.5, 1.0, and 2.0 g/kg of CSNPs. Similarly, Panalikul et al. (2017) also reported that the incorporation of 2% CS to the feed enhances *Esanthelphusa dugasti* productivity through gross body mass, SGR, and rate of survival. Kamali Najafabad et al. (2016) have shown that CS-loaded feed supplemented for *Caspian kutum* would boost immune response, FCR, and tolerance to certain adverse ecological conditions.

Niu et al. (2015) also found that feed supplement with 0.1–0.2% of CS enhanced the productivity of *Penaeus monodon* while the dietary intake was higher than 0.3% and 0.4%. Abd El-Naby et al. (2019) revealed that *O. niloticus* administered with basal control feed containing different CSNPs concentrations (1, 3, and 5 g/kg diet). In particular, the concentration of CSNPs at a diet of 5 g/kg treatment group fishes substantially increased body mass, SGR, PER, and consumption of feed than the control group.

The present findings demonstrated that NAG loaded CSNPs would be utilized as promising feed additives with an optimal amount of 1.0 g CSNPs/kg feed to boost immune response and productivity of *O. niloticus*. CS acts as an excellent growth stimulant and would be recognized as a significant factor in the growth of aquatic organisms (Abdel-Ghany and Salem, 2020). Enhancement of fish growth would be due to progression in intestinal villi and greater nutrients uptake, SGR, and diet consumption of fish. Zaki et al. (2015) observed that dietary supplementation of CS concentrations at 1.0 and 2.0 g/kg feed improved *Dicentrarchus labrax* productivity and feed conversion efficiency.

Hematological parameters analysis is an efficient technique in monitoring fish health (Jawad et al., 2004). The present findings revealed that the dietary supplementation with the different levels of NAG loaded CSNPs significantly increased the RBC and WBC counts in treatment groups of tilapia. This may probably be linked to improved macro and micro-nutrient absorption. Dawood et al. (2020) also reported that RBCs were significantly enhanced in *Liza ramada* fed with 1.0 and 2.0 g/kg of CSNPs compared control.

Recently, CS has been proved as an efficient immunostimulant capable of eliciting non-specific immune responses in various fish species (Abdel-Ghany and Salem, 2020). The NAG loaded CSNPs significantly enhanced the lysozyme and RB activities were observed in fish fed with dietary supplementation contain 1.0 g NAG loaded CSNPs/kg than other treatment groups. Similarly, Dawood et al. (2020) also reported that the lysozyme activity and phagocytic index were significantly increased in *Liza ramada* fed with CSNPs at 0.5, 1.0, and 2.0 g/kg ( $p < 0.05$ ), whereas highest lysozyme activity and phagocytic index were observed in fish fed with 1.0 g/kg CSNPs.

Both the lysozyme and RB functions are known to play major functions in non-specific immune response (Grinde, 1989; Ellis, 1990). The bacterial cell walls containing  $\beta$ -1, 4 glycosidic bonds between NAM and NAG were cleaved by lysozyme (Alexander and Ingram, 1992).

To attack invasive pathogens during phagocytosis, RB is produced by phagocytes and has been extensively used to assess the defense capability against pathogens (Secombes et al., 1990). Increased lysozyme and RB activity evidenced an improved level of defense mechanism for fish against bacterial infections. Harikrishnan et al. (2012) stated that the immunologic parameters of *Epinephelus bruneus* were enhanced by dietary CS supplementation.

Similarly, Abu-Elala et al. (2015) also reported that CS diet exhibited immune-modulating effects on some non-specific immune responses. The exact mechanism of the immunostimulatory effect of CS inside fish remains unclear. The immune-modulating effect of CS for *Paralithchys olivaceus* was attributed to inducing non-specific defense mechanisms in fish through its amino moieties (Cha et al., 2008). In conclusion, *O. niloticus* fed with 1% NAG loaded CSNPs containing diets as therapeutic or preventive regimes have substantially improved the immune

response.

To develop an efficient, cost-effective, and disease resistance feed formulation for Nile tilapia using NAG loaded CSNPs for aquaculture production. Statistically, nearly more than lakhs (INR) are payable by currently growing fish farmers as importer feed costs for the single pond. This cost-effective cheaper feed product has no distrust to reach the aqua farmers and will get the top position in the global and Indian aquaculture feed market.

Current research focuses to develop cost-effective and immunostimulatory marine waste feed with chitin derivatives loaded with nanoparticles to enhance the immune status and disease resistance of commercial Nile tilapia. CS has demonstrated effective removal of different pollutants from aquaculture wastewater through coagulation and adsorption process. Besides, CSNPs act as an eco-sustainable resource for wastewater treatment. Though CS provides a robust approach for wastewater treatment in aquaculture, the plausible application of research data in practical fish farming required further studies, and it could be a significant source in the future for the development of value-added products from shrimp shell waste.

## 5. Conclusions

The present findings suggest that feed supplemented with NAG loaded CSNPs synthesized from shrimp disposal waste positively influences the economically important fish *O. niloticus*. Administration of moderate levels of oral NAG loaded CSNPs (1.0 g/kg) were significantly increased growth performance, feed intake, survival rate, non-specific immune response, and decreased feed conversion ratio. This is the first comparative study examining the effect of CSNPs and NAG loaded CSNPs as dietary supplement for *O. niloticus*. Fish fed with 1% NAG loaded CSNPs containing diets as therapeutic and preventive regimes have remarkably enhanced the growth efficiency. *In-vitro* and *in-vivo* results strongly confirm that CSNPs and NAG loaded CSNPs from shrimp disposal waste could be used as a cost-effective, value-added dietary supplement for aquaculture industry. This opens up new research to develop an effective combination of NAG loaded CSNPs as fish feed to enhance growth performance and immune response in commercial aquaculture production.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2021.130746>.

## Credit author statement

Subramanian Kumaran, Antonyraj Matharasi Perianaika Anahas, Nainangu Prasannabalaji: Conceptualization, Data curation, Visualization, Formal analysis and Investigation, Methodology, Resources and Software, Writing – original draft, Writing – review & editing. Muthuramalingam Karthiga, Selvaraj Bharathi, Thirunavukarasu Rajasekar, Jerrine Jospeh: Conceptualization, Data curation, Visualization,

Methodology, Resources and Software. Srinivasan Guru Prasad, Siva Kumar Pandian, Sampath Renuka Pugazhendian, Wilson Aruni - Supervision, Project administration, Writing – review & editing, Resources and Software.

## References

- Abd El-Naby, F.S., Naiel, M.A., Al-Sagheer, A.A., Negm, S.S., 2019. Dietary chitosan nanoparticles enhance the growth, production performance, and immunity in *Oreochromis niloticus*. *Aquaculture* 501, 82–89.
- Abdel-Ghany, H.M., Salem, M.E.S., 2020. Effects of dietary chitosan supplementation on farmed fish: a review. *Rev. Aquacult.* 12, 438–452.
- Abdel-Tawwab, M., Razeq, N.A., Abdel-Rahman, A.M., 2019. Immunostimulatory effect of dietary chitosan nanoparticles on the performance of Nile tilapia, *Oreochromis niloticus* (L.). *Fish Shellfish Immunol.* 88, 254–258.
- Abu-Elala, N.M., Mohamed, S.H., Zaki, M.M., Eissa, A.E., 2015. Assessment of the immunomodulatory and antimicrobial effects of dietary chitosan on Nile tilapia (*Oreochromis niloticus*) with special emphasis to its bio-remediating impacts. *Fish Shellfish Immunol.* 46, 678–685.
- Abu-Elala, N., Marzouk, M., Moustafa, M., 2013. Use of different *Saccharomyces cerevisiae* biotic forms as immune-modulator and growth promoter for *Oreochromis niloticus* challenged with some fish pathogens. *Int. J. Veterinary Sci. Med.* 1, 21–29.
- Ahmed, F., Soliman, F.M., Adly, M.A., Soliman, H.A., El-Matbouli, M., Saleh, M., 2020. *In vitro* assessment of the antimicrobial efficacy of chitosan nanoparticles against major fish pathogens and their cytotoxicity to fish cell lines. *J. Fish. Dis.* 43, 1049–1063.
- Akmaz, S., Dilaver Adigüzel, E., Yasar, M., Ergüven, O., 2013. The effect of Ag content of the chitosan-silver nanoparticle composite material on the structure and antibacterial activity. *Ann. Mater. Sci. Eng.* 6.
- Alexander, J.B., Ingram, G.A., 1992. Non-cellular non-specific defence mechanisms of fish. *Annu. Rev. Fish Dis.* 2, 249–279.
- Ali, S.W., Rajendran, S., Joshi, M., 2011. Synthesis and characterization of chitosan and silver loaded chitosan nanoparticles for bioactive polyester. *Carbohydr. Polym.* 83, 438–446.
- Amenyogbe, E., Chen, G., Wang, Z., Huang, J., Huang, B., Li, H., 2020. The exploitation of probiotics, prebiotics and synbiotics in aquaculture: present study, limitations and future directions : a review. *Aquacult. Int.* 1–25.
- Alishahi, A., Mirvaghefi, A., Tehranib, M.R., Farahmandi, H., Koshic, S., Dorkoosh, F. A., Elsaabee, M.Z., 2011. Chitosan nanoparticle to carry vitamin C through the gastrointestinal tract and induce the non-specific immunity system of rainbow trout (*Oncorhynchus mykiss*). *Carbohydr. Polym.* 86, 142–146.
- Blaxhall, P.C., 1972. The haematological assessment of the health of freshwater fish. A review of selected literature. *J. Fish. Biol.* 4, 593–604.
- Calvo, P., Remünán-López, C., Vila-Jato Alonso, M.J., 1997. Novel hydrophilic chitosan polyethylene oxide nanoparticles as protein carrier. *J. Appl. Polym. Sci.* 63, 125–132.
- Cha, S.H., Lee, J.S., Song, C.B., Lee, K.J., Jeon, Y.J., 2008. Effects of chitosan-coated diet on improving water quality and innate immunity in the olive flounder, *Paralichthys olivaceus*. *Aquaculture* 278, 110–118.
- Chen, K.Y., Zeng, S.Y., 2018. Fabrication of quaternized chitosan nanoparticles using tripolyphosphate/genipin dual cross-linkers as a protein delivery system. *Polymers* 10, 1226.
- Cheng, Y.C., Yu, C.C., Lo, T.Y., Liu, Y.C., 2012. Size-controllable synthesis of catalyst of gold nanoparticles with capping agents of natural chitosan. *Mater. Res. Bull.* 47, 1107–1112.
- Dananjaya, S.H.S., Erandani, W.K.C.U., Kim, C.H., Nikapitiya, C., Lee, J., De Zoysa, M., 2017. Comparative study on antifungal activities of chitosan nanoparticles and chitosan silver nano composites against *Fusarium oxysporum* species complex. *Int. J. Biol. Macromol.* 105, 478–488.
- Dananjaya, S.H.S., Godahewa, G.I., Jayasooriya, R.G.P.T., Lee, J., De Zoysa, M., 2016. Antimicrobial effects of chitosan silver nano composites (CAGNCs) on fish pathogenic *Aliivibrio (Vibrio) salmonicida*. *Aquaculture* 450, 422–430.
- Dawood, M.A., Koshio, S., Esteban, M.Á., 2018. Beneficial roles of feed additives as immunostimulants in aquaculture: a review. *Rev. Aquacult.* 10, 950–974.
- Dawood, M.A., Gewailly, M.S., Soliman, A.A., Shukry, M., Amer, A.A., Younis, E.M., Abdel-Warith, A.W.A., Van Doan, H., Saad, A.H., Aboubakr, M., Abdel-Latif, H.M., 2020. Marine-derived chitosan nanoparticles improved the intestinal histomorphometrical features in association with the health and immune response of Grey Mullet (*Liza ramada*). *Mar. Drugs* 18, 611.
- De Paz, L.E.C., Resin, A., Howard, K.A., Sutherland, D.S., Wejse, P.L., 2011. Antimicrobial effect of chitosan nanoparticles on *Streptococcus mutans* biofilms. *Appl. Environ. Microbiol.* 77, 3892–3895.
- Dima, J.B., Sequeiros, C., Zaritzky, N.E., 2015. Hexavalent chromium removal in contaminated water using reticulated chitosan micro/nanoparticles from seafood processing wastes. *Chemosphere* 141, 100–111.
- Divya, K., Vijayan, S., George, T.K., Jisha, M., 2017. Antimicrobial properties of chitosan nanoparticles: mode of action and factors affecting activity. *Fibers Polym.* 18, 221–230.
- Du, W.L., Niu, S.S., Xu, Y.L., Xu, Z.R., Fan, C.L., 2009. Antibacterial activity of chitosan tripolyphosphate nanoparticles loaded with various metal ions. *Carbohydr. Polym.* 75, 385–389.
- Dudhani, A.R., Kosaraju, S.L., 2010. Bioadhesive chitosan nanoparticles: preparation and characterization. *Carbohydr. Polym.* 81, 243–251.
- Ellis, A.E., 1990. Lysozyme assays, tech. *Fish Immunol* 1, 101–103.
- Esteban, M.A., Cuesta, A., Ortuno, J., Meseguer, J., 2001. Immunomodulatory effects of dietary intake of chitin on gilthead seabream (*Sparus aurata* L.) innate immune response. *Fish Shellfish Immunol.* 11, 303–315.
- FAO, 2019. Globefish Highlights-A Quarterly Update on World Seafood Markets.
- FAO, 2020. Globefish Highlights-A Quarterly Update on World Seafood Markets.
- Ferreira Tomaz, A., Sobral de Carvalho, S.M., Cardoso Barbosa, R., L Silva, S.M., Sabino Gutierrez, M.A., B de Lima, A.G., L Fook, M.V., 2018. Ionically crosslinked chitosan membranes used as drug carriers for cancer therapy application. *Materials* 11, 2051–2069.
- Ghadi, A., Mahjoub, S., Tabandeh, F., Talebnia, F., 2014. Synthesis and optimization of chitosan nanoparticles: potential applications in nanomedicine and biomedical engineering. *Caspian J. Intern. Med.* 5, 156–161.
- Govindan, S., Nivethaa, E.A.K., Saravanan, R., Narayanan, V., Stephen, A., 2012. Synthesis and characterization of chitosan-silver nanocomposite. *Appl. Nanosci.* 2, 299–303.
- Grinde, B., 1989. Lysozyme from rainbow trout, *Salmo gairdneri* Richardson, as an antibacterial agent against fish pathogens. *J. Fish. Dis.* 12, 95–104.
- Hoseinifar, S.H., Esteban, M.Á., Cuesta, A., Sun, Y.Z., 2015. Prebiotics and fish immune response: a review of current knowledge and future perspectives. *Rev. Fish. Sci. Aquac.* 23, 315–328.
- Hoseinifar, S.H., Sun, Y.Z., Wang, A., Zhou, Z., 2018. Probiotics as means of diseases control in aquaculture, a review of current knowledge and future perspectives. *Front. Microbiol.* 9, 2429.
- Hosseini, S.F., Zandi, M., Rezaei, M., Farahmandghav, F., 2013. Two-step method for encapsulation of oregano essential oil in chitosan nanoparticles: preparation, characterization and in vitro release study. *Carbohydr. Polym.* 95, 50–56.
- Hosseini, S.F., Rezaei, M., Zandi, M., Farahmandghavi, F., 2015. Fabrication of bio-nanocomposite films based on fish gelatin reinforced with chitosan nanoparticles. *Food Hydrocolloids* 44, 172–182.
- Huang, L., Cheng, X., Liu, C., Xing, K., Zhang, J., Sun, G., Li, X., Chen, X., 2009. Preparation, characterization, and antibacterial activity of oleic acid-grafted chitosan oligosaccharide nanoparticles. *Front. Biol.* 4, 321–327.
- Jingou, J., Shilei, H., Weiqi, L., Danjun, W., Tengfei, W., Yi, X., 2011. Preparation, characterization of hydrophilic and hydrophobic drug in combine loaded chitosan/cyclodextrin nanoparticles and *in vitro* release study. *Colloids Surf. B Biointerfaces* 83, 103–107.
- Jung, B.O., Chung, S.J., Lee, G.W., 2002. Effect of molecular weight of chitosan on its antimicrobial activity. *J. Chitin Chitosan.* 7, 231–236.
- Kang, J.H., Battogtokh, G., Ko, Y.T., 2014. Folate-targeted liposome encapsulating chitosan/oligonucleotide polyplexes for tumor targeting. *AAPS PharmSciTech* 15, 1087–1092.
- Kaur, P., Choudhary, A., Thakur, R., 2013. Synthesis of chitosan-silver nanocomposites and their antibacterial activity. *Int. J. Sci. Eng. Res.* 4, 869.
- Kumaran, S., Balamurugan, S., Wilson, A., Alagappan, K., Rajasekar, T., Prasad, S.G., Selvaraj, B., Prasannabalaji, N., Sampath, R.P., Anandajothi, E., Devisagamani, B., 2020. Bioconversion of chitin and concomitant production of chitinase and N-acetylglucosamine by novel *Achromobacter xylosoxidans* isolated from shrimp waste disposal area. *Sci. Rep.* 10, 11898.
- Li, S.W., He, H., Zeng, R.J., Sheng, G.P., 2017. Chitin degradation and electricity generation by *Aeromonas hydrophila* in microbial fuel cells. *Chemosphere* 168, 293–299.
- Liu, Y., Sun, Y., Li, Y., Xu, S., Tang, J., Ding, J., Xu, Y., 2011. Preparation and characterization of  $\alpha$ -galactosidase-loaded chitosan nanoparticles for use in foods. *Carbohydr. Polym.* 83, 1162–1168.
- Lorevice, M.V., Moura, M.R.D., Aouada, F.A., Mattoso, L.H., 2012. Development of novel guava puree films containing chitosan nanoparticles. *J. Nanosci. Nanotechnol.* 12, 2711–2717.
- Ma, Z., Garrido-Maestu, A., Jeong, K.C., 2017. Application, mode of action, and *in vivo* activity of chitosan and its micro- and nanoparticles as antimicrobial agents: a review. *Carbohydr. Polym.* 176, 257–265.
- Maroušek, J., Strunecký, O., Kolář, L., Vochozka, M., Kopecký, M., Maroušková, A., Batt, J., Poliak, M., Soch, M., Bartoš, P., Kliestik, T., 2020a. Advances in nutrient management make it possible to accelerate biogas production and thus improve the economy of food waste processing. *Energy Sources Part A* 1–10.
- Maroušek, J., Bartoš, P., Filip, M., Kolář, L., Konvalina, P., Maroušková, A., Moudrý, J., Peterka, J., Šál, J., Soch, M., Stehel, V., 2020b. Advances in the agrochemical utilization of fermentation residues reduce the cost of purpose-grown phytomass for biogas production. *Energy Sources Part A* 1–11.
- Maroušek, J., Kolář, L., Strunecký, O., Kopecký, M., Bartoš, P., Maroušková, A., Čudlínová, E., Konvalina, P., Soch, M., Moudrý Jr., J., Vaníčková, R., 2020c. Modified biochars present an economic challenge to phosphate management in wastewater treatment plants. *J. Clean. Prod.* 272, 123015.
- Mohammadpour Dounighi, N., Eskandari, R., Avadi, M.R., Zolfagharian, H., Mir Mohammad Sadeghi, A., Rezayat, M., 2012. Preparation and *in vitro* characterization of chitosan nanoparticles containing *Mesobuthus eupeus* scorpion venom as an antigen delivery system. *J. Venom. Anim. Toxins Incl. Trop. Dis.* 8, 44–52.
- Nagy, A., Harrison, A., Sabbani, S., Munson Jr., R.S., Dutta, P.K., Waldman, W.J., 2011. Silver nanoparticles embedded in zeolite membranes: release of silver ions and mechanism of antibacterial action. *Int. J. Nanomed.* 6, 1833–1852.
- Nair, M., Jayant, R.D., Kaushik, A., Sagar, V., 2016. Getting into the brain: potential of nanotechnology in the management of NeuroAIDS. *Adv. Drug Deliv. Rev.* 103, 202–217.
- Najafabad, M.K., Imanpoor, M.R., Taghizadeh, V., Alishahi, A., 2016. Effect of dietary chitosan on growth performance, hematological parameters, intestinal histology and stress resistance of Caspian kutum (*Rutilus frisii kutum Kamenskii*, 1901) fingerlings. *Fish Physiol. Biochem.* 42, 1063–1071.

- Niu, J., Li, C.H., Tian, L.X., Liu, Y.J., Chen, X., Wu, K.C., Jun, W., Huang, Z., Wang, Y., Lin, H.Z., 2015. Suitable dietary chitosan improves the growth performance, survival and immune function of tiger shrimp, *Penaeus monodon*. *Aquacult. Res.* 46, 1668–1678.
- Oh, J.W., Chun, S.C., Chandrasekaran, M., 2019. Preparation and *in vitro* characterization of chitosan nanoparticles and their broad-spectrum antifungal action compared to antibacterial activities against phytopathogens of tomato. *Agronomy* 9, 21.
- Panalikul, P., Wongmaneeprateep, S., Doolgindachbaporn, S., 2017. Effects of chitosan Levels on growth performance, feed utilization and survival rate of rice-field crab (*Esanthelphusa dugasti*). *J. Pure Appl. Microbiol.* 11, 1357–1364.
- Prabu, E., Rajagopalsamy, C.B.T., Ahilan, B., Jeevagan, I.J.M.A., Renuhadevi, M., 2019. Tilapia—an excellent candidate species for world aquaculture: a review. *Annu. Res. Rev. Biol.* 1–14.
- Qi, L., Xu, Z., 2004. Lead sorption from aqueous solutions on chitosan nanoparticles. *Colloids Surf. A Physicochem. Eng. Asp.* 251, 183–190.
- Qi, L., Xu, Z., Jiang, X., Hu, C., Zou, X., 2004. Preparation and antibacterial activity of chitosan nanoparticles. *Carbohydr. Res.* 339, 2693–2700.
- Rampino, A., Borgogna, M., Blasi, P., Bellich, B., Cesaro, A., 2013. Chitosan nanoparticles: preparation, size evolution and stability. *Int. J. Pharm.* 455, 219–228.
- Rokhade, A.P., Agnihotri, S.A., Patil, S.A., Mallikarjuna, N.N., Kulkarni, P.V., Aminabhavi, T.M., 2006. Semi-interpenetrating polymer network microspheres of gelatin and sodium carboxymethyl cellulose for controlled release of ketorolac tromethamine. *Carbohydr. Polym.* 65, 243–252.
- Saharan, V., Sharma, G., Yadav, M., Choudhary, M.K., Sharma, S.S., Pal, A., Raliya, R., Biswas, P., 2015. Synthesis and *in vitro* antifungal efficacy of Cu–chitosan nanoparticles against pathogenic fungi of tomato. *Int. J. Biol. Macromol.* 75, 346–353.
- Samrot, A.V., Jahnvi, T., Padmanaban, S., Philip, S.A., Burman, U., Rabel, A.M., 2016. Chelators influenced synthesis of chitosan–carboxymethyl cellulose microparticles for controlled drug delivery. *Appl. Nanosci.* 6, 1219–1231.
- Sathiyavimal, S., Vasantharaj, S., Kaliannan, T., Pugazhendhi, A., 2020. Eco-biocompatibility of chitosan coated biosynthesized copper oxide nanocomposite for enhanced industrial (Azo) dye removal from aqueous solution and antibacterial properties. *Carbohydr. Polym.* 116243.
- Secombes, C.J., 1990. Isolation of salmonid macrophages and analysis of their killing activity. *Tech. Fish Immunol.* 1, 137–154.
- Shanmuganathan, R., Edison, T.N.J.L., LewisOscar, F., Kumar, P., Shanmugam, S., Pugazhendhi, A., 2019. Chitosan nanopolymers: an overview of drug delivery against cancer. *Int. J. Biol. Macromol.* 130, 727–736.
- Shanmugasundaram, T., Radhakrishnan, M., Gopikrishnan, V., Kadirvelu, K., Balagurunathan, R., 2017. *In vitro* antimicrobial and *in vivo* wound healing effect of actinobacterially synthesised nanoparticles of silver, gold and their alloy. *RSC Adv.* 7, 51729–51743.
- Sivaraman, I., Krishnan, M., Radhakrishnan, K., 2019. Better management practices for sustainable small-scale shrimp farming. *J. Clean. Prod.* 214, 559–572.
- Soleimani, N., Mobarez, A.M., Jafari Olia, M.S., Atyabi, F., 2015. Synthesis, characterization and effect of the antibacterial activity of chitosan nanoparticles on vancomycin-resistant *Enterococcus* and other gram negative or gram positive bacteria. *Int. J. Pure Appl. Sci. Technol.* 26.
- Tsakanika, A., Clauzet, M., May, P.H., 2018. Envolvendo os pescadores artesanais no desenvolvimento sustentável urbano e periurbano no Brasil. *Revibec: revista de la Red Iberoamericana de Economía Ecológica* 28, 1–20.
- Udayangani, R.M.C., Dananjaya, S.H.S., Fronte, B., Kim, C.H., Lee, J., De Zoysa, M., 2017. Feeding of nano scale oats  $\beta$ -glucan enhances the host resistance against *Edwardsiella tarda* and protective immune modulation in zebrafish larvae. *Fish Shellfish Immunol.* 60, 72–77.
- Vaezifar, S., Razavi, S., Golozar, M.A., Karbasi, S., Morshed, M., Kamali, M., 2013. Effects of some parameters on particle size distribution of chitosan nanoparticles prepared by ionic gelation method. *J. Cluster Sci.* 24, 891–903.
- Wang, X., Chi, N., Tang, X., 2008. Preparation of estradiol chitosan nanoparticles for improving nasal absorption and brain targeting. *Eur. J. Pharm. Biopharm.* 70, 735–740.
- Wu, Y., Yang, W., Wang, C., Hu, J., Fu, S., 2005. Chitosan nanoparticles as a novel delivery system for ammonium glycyrrhizinate. *Int. J. Pharm.* 295, 235–245.
- Xu, Y., Du, Y., 2003. Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles. *Int. J. Pharm.* 250, 215–226.
- Yadav, M., Goswami, P., Paritosh, K., Kumar, M., Pareek, N., Vivekanand, V., 2019. Seafood waste: a source for preparation of commercially employable chitin/chitosan materials. *Bioresour. Bioprocess.* 6, 8.
- Yoksan, R., Jirawutthiwongchai, J., Arpo, K., 2010. Encapsulation of ascorbyl palmitate in chitosan nanoparticles by oil-in-water emulsion and ionic gelation processes. *Colloids Surf. B Biointerfaces* 76, 292–297.
- Yun, Y.S., Kim, K.S., Lee, Y.N., 1999. Antibacterial and antifungal effect of chitosan. *J. Chitin Chitosan.* 4, 8–14.
- Zaki, M.A., Shatby, E., Shatby, E., 2015. Effect of chitosan supplemented diet on survival, growth, feed utilization, body composition, and histology of sea bass (*Dicentrarchus labrax*). *World J. Eng. Technol.* 3, 38.