

## Prevention of vibriosis in sea bass, *Dicentrarchus labrax* using ginger nanoparticles and *Saccharomyces cerevisiae*

Fatma M. M. Korni<sup>1†</sup>, Al Shimaa A. Sleim<sup>2</sup>, Jehan I. Abdellatief<sup>3</sup>  
and Rehab A. Abd-elaziz<sup>4</sup>

<sup>1</sup>Department of Fish Diseases and Management, Faculty of Veterinary Medicine,  
Beni-Suef University, Beni-Suef, Egypt.

<sup>2</sup>Unit of Bacteriology, Alexandria Provincial Lab, Animal Health Research Institute, ARC, Egypt.

<sup>3</sup>Department of Fish diseases, Animal Health Research Institute, ARC, Dokki, Giza, Egypt.

<sup>4</sup>Fish Diseases Dpet. Provincial Alexandria Lab, Animal Health Research Institute, ARC, Egypt.

Vibriosis is an important septicemic bacterial disease that affects a variety of commercial fish species, including cultured *Dicentrarchus labrax*. Nanotechnology has become an important modern tool for fish diseases prevention. Furthermore, nanomaterials have the ability to prevent and treat fish diseases. The current study was aimed to identify the causative agent of massive mortality of *D. labrax* commercial farm in Alexandria, Egypt. Experimental infection and the median lethal dose (LD<sub>50</sub>) of pathogenic isolate were assessed. Also, the effect of ginger nanoparticles (GNPs) and *Saccharomyces cerevisiae* as feed additives for prevention of vibriosis in *D. labrax* was carried out. Similarly, the tissue immunostimulant genes, IL-1 $\beta$  and TLR2 were measured in the spleen of feeding groups. The clinical signs of naturally diseased *D. labrax* showed corneal opacity and paleness of gills with excessive mucous secretion. The post-mortem abnormalities were severe hemorrhage and adhesion of internal organs. After bacteriological isolation and identification, the causative agent of mortality in the current study was *Vibrio alginolyticus*. The LD<sub>50</sub> of *V. alginolyticus* was  $1.5 \times 10^{5.4}$  CFU/ml. The experimentally infected *D. labrax* showed ulceration, exophthalmia and skin hemorrhages. The post-mortem findings of the experimentally infected *D. labrax* revealed internal hemorrhage, spleen darkness and paleness of liver. There is no mortality and 100% RPS in groups fed GNPs then injected with *V. alginolyticus*, in those fed a combination of GNPs and *S. cerevisiae* and a group fed normal diet then injected with physiological saline (control negative), respectively. Contrarily, there was 10% mortality and 87.5 RPS in the group fed *S. cerevisiae* then injected with *V. alginolyticus*. On the other hand, the control positive group showed 79% mortality. The spleen IL-1 $\beta$  and TLR2 immunostimulant genes were significantly increased in groups of fish fed GNNP, *S. cerevisiae* and a combination of GNPs and *S. cerevisiae*, respectively compared to control group. The highest stimulation of those immunostimulant genes was found in the group fed a combination of GNPs and *S. cerevisiae*, while fish fed *S. cerevisiae* had the lowest level. Dietary combination of GNPs and *S. cerevisiae* was shown to be efficient in preventing of vibriosis, with greatest stimulation of spleen IL-1 $\beta$  and TLR2 immunostimulant genes.

**Key words:** Vibriosis, *V. alginolyticus*, *Dicentrarchus labrax*, prevention; ginger nanoparticles, *Saccharomyces cerevisiae*, immune-related genes

---

<sup>†</sup>Corresponding author: Fatma M. M. Korni  
Tel: +20 201100991799  
E-mail: naglaamostafal@yahoo.com

## Introduction

Vibriosis is a serious septicemic bacterial disease that affects a number of economically significant fish species, including farmed sea bass (*Dicentrarchus labrax*, *D. labrax*), which is the most valuable fish species in the Mediterranean (Varsamos et al., 2006; Korun and Timur 2008). Vibriosis presents itself as a hemorrhagic septicemia with extensive skin hemorrhages, pale gills and systemic visceral invasion (Toranzo et al., 2005).

*Vibrio alginolyticus* (*V. alginolyticus*) is one of the most dominant vibrio species caused massive mortality in cultured *D. labrax* along the Egyptian provinces (Abdelaziz, et al., 2017). Control of vibriosis in aquaculture feeding infected fish with antibiotic-medicated food (Pridgeon et al., 2012) is the most common practice (Defoirdt et al., 2007). However, owing to the emergence of resistance, this strategy may be unsuccessful (Sugita et al., 1990). An assessment of "functional alternatives" is necessary to reduce the use of antibiotics in farmed fish and their possible harmful effects on public health and the environment (Hayatgheib et al., 2020). Nowadays, several types of beneficial feed additives such as probiotics and natural substances as dietary supplements in fish diets are being used in aquaculture. These feed additives improve immune responses and diseases prevention as well as providing antibiotics-free alternative (Irianto and Austin, 2002; Hoseinifar et al., 2016; Korní and Khalil 2017; Sayes et al., 2018). *Saccharomyces cerevisiae* (*S. cerevisiae*) yeast improves immune responses (Ortuño et al., 2002) as well as prevents certain septicemic bacterial diseases in fish (Abdel-Tawwab et al., 2008 and Pinoargote and Ravishankar 2018). Probiotics are microbial or cultured products of feed additives, which beneficially affect the fish by producing inhibitory compounds, competing with pathogenic bacteria for adhesion sites, enhancing the intestinal microbial balance and stimulating and regulating the immune function (Kim

and Austin 2006).

Nanotechnology is a novel technology and many promising applications are starting to appear in the area of aquaculture, where the antimicrobial properties of certain nanomaterials are of particular interest (Li et al., 2008). Nanomaterials have the potential to play a significant role in prevention and treatment of fish diseases (Korní and Khalil 2017). The use of nanoparticles as potential antimicrobials, nanoparticle-based vaccines, and the development of a specific and sensitive tool for diagnosis of bacterial, fungal, and viral diseases in aquaculture had been reported (Shalan et al., 2016). Ginger nanoparticles (GNPs) are developed easily for a large-scale manufacture and are thought to be effective therapeutic natural products for the prevention of some bacterial septicemic diseases such as motile aeromonas septicemia and Edwardsielliosis (Korní and Khalil 2017 and Korní et al., 2021).

Interleukin 1 $\beta$  (IL-1 $\beta$ ) is a multifunctional pro-inflammatory cytokine that is largely responsible for the activation of innate and acquired immune responses in response to a variety of bacterial, parasite, and viral diseases (Bird et al., 2005). In addition, IL-1 $\beta$  is an attractive candidate for resistance to bacterial vibriosis (Chistiakov et al., 2010).

Toll-like receptors (TLRs) are one of the most essential components of the innate immune system, since they play a role in pathogen detection and disease resistance (Cebeci et al., 2019). There are no previous studies that reported the prevention of vibriosis by using GNPs and *S. cerevisiae* as feed additives in *D. labrax*. Therefore, the current study was aimed to identify the cause of mortality of *D. labrax* in a commercial fish farm in Alexandria, Egypt. Also, the experimental infection and median lethal dose (LD50) of pathogenic isolate were assessed. Then, the prevention of vibriosis by using GNPs and *S. cerevisiae* as feed additives in *D. labrax* was carried out. Furthermore, the transcription levels of the immune-related genes encoding IL-1 $\beta$  and TLR2 in the

spleen of feeding groups were determined.

## Material and methods

### Fish collection and management

#### Naturally diseased fish for clinical examination, bacteriological isolation and identification

A total of 20 *D. labrax* had  $55 \pm 2$  g an average body weight with severe skin hemorrhages were collected alive from a commercial fish farm in Alexandria, Egypt. The collected fish were transported to laboratory of Fish Health and Diseases Unit of Animal Health Research Institute, Agriculture Research Centre, Alexandria, Egypt.

#### Experimental fish

One hundred and fifty apparently healthy *D. larax* had  $50 \pm 3$  g an average body weight was collected alive for assessment of pathogenicity and median lethal dose ( $LD_{50}$ ) of *V. alginolyticus*. On the other hand, 240 apparently healthy *D. labrax* ( $50 \pm 3$  g) were collected alive for running the experiments of vibriosis prevention. Also, a total of 120 apparently healthy *D. labrax* ( $50 \pm 3$  g) were collected alive for spleen collection to measure IL-1 $\beta$  and TLR2 immune-related genes (Table 1).

#### Management of experimental fish

All experimental fish were collected a live from

a commercial fish farm in Alexandria, Egypt and transported in polyethylene plastic bags containing water enriched by oxygen (2/3) to Fish Health and Diseases Unit in Alexandria provincial laboratory of Animal Health Research Institute, Agriculture Research Centre, Egypt. Fish were acclimation for 14 days prior to the experiments.

During the experiments, fish were distributed in glass aquaria ( $80 \times 40 \times 60$  cm) with a water volume of 30 liters. Each aquarium was supported with continuous artificial aeration (1 air stone) through air blower and another one as spare in case of emergency. The water exchange rate in the experimental aquaria was 10% per day. Fish were fed 3% of their body weight and the water temperature was  $26 \pm 2$  °C, Dissolved Oxygen D.O was  $6 \pm 2$  mg/l, pH was 7-8 and the salinity was  $12 \pm 3$ ‰.

#### Synthesis and characterization of GNPs

Ginger nanoparticles were produced by reduction of dried ginger size in ball milling as a top down nanoparticle synthesis approach (Takacs 2002). Ginger was added in container of stainless steel ball mill (the balls: ginger = 10:1 by weight). The vessel size equal 7.5 cm and the ceramic ball diameters were 1.11-1.75 cm. The container was rotated at 900 rpm for 15 hrs using ball mill machine (photon, Egypt).

Ginger nanoparticles were characterized by scanning electron microscopy (SEM), hydrodynamic par-

Table 1. Number of apparently healthy *D. labrax* in each experiment

Number of healthy <i>D. labrax</i>	Experiment	Observation and sampling at the end of experiment
150 (10 fish/5 groups/ 3 replicates)	Experimental infection and evaluation of $LD_{50}$ of <i>V. alginolyticus</i>	<ul style="list-style-type: none"> <li>• <math>LD_{50}</math> was calculated.</li> <li>• Fish were observed for 2 weeks.</li> <li>• Mortality was recorded daily.</li> </ul>
240 (10 fish/8 groups/ 3 replicates)	For <i>V. alginolyticus</i> prevention	<ul style="list-style-type: none"> <li>• For <i>V. alginolyticus</i> challenge.</li> <li>• Fish were observed for 2 weeks after experimental infection.</li> <li>• Mortality was recorded daily.</li> <li>• RPS was calculated (Amend, 1981).</li> </ul>
120 (10 fish/4 groups/ 3 replicates)	Spleen collection	<ul style="list-style-type: none"> <li>• Spleen collection for IL-1<math>\beta</math> and TLR2 measurement.</li> </ul>

ticle size and zeta potential at the Faculty of Post-graduate Studies of Advanced Science, Beni-Suef University.

#### *Saccharomyces cerevisiae*

(S.I. Lesaf-fre-Marcq-France). Each one gram of yeast contains  $6.5 \times 10^9$  CFU.

#### Diet Preparation

The commercial pelleted fish diet [(Brsiek factory, Egypt), (Table 2)] was ground to obtain fine powder using a mortar. The GNPs and *S. cerevisiae* were mixed with the previously prepared powder to form four diets. Diet 1 had no additives (control), diet 2 had 1 g of GNPs/kg of feed, diet 3 had 1 g of *S. cerevisiae*/ kg of feed and diet 4 containing mixture of GNPs and *S. cerevisiae*, respectively (each of 1 g). The mixture was passed through a manual hand-minced meat processing machine (Italy) to produce extruded strings (Rattanachaikunsopon and Phumkhachorn, 2010).

#### Experimental Design

##### Clinical examination

External and internal examination of fish samples were carried out for detection of any clinical abnormalities according to Austin and Austin (2012).

##### Bacterial isolation and identification

The collected samples from lesions of organs of naturally diseased *D. labrax* were inoculated on TSA supplemented with 3% NaCl and TCBS. The plates were incubated at 25°C for 24 hrs. The selected colonies were subjected to identification by Gram staining technique, biochemical tests (Holt et al., 1994

and Liu, et al., 2004) and API\*20NE (BIO-Merieux).

#### Detection of thermostable related hemolysin (trh) and thermostable direct hemolysin (tdh) virulence genes by PCR

Thermostable related hemolysin (trh) and thermostable direct hemolysin (tdh) virulence genes were used for confirming the pathogenicity of the isolates. DNA extraction was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit. The primer sets and the cycling conditions used in this study were described in Table 3.

#### Assessment of pathogenicity and LD<sub>50</sub> of *V. alginolyticus*

One hundred and fifty apparently healthy *D. labrax* were divided into five groups (10 /group) with three replicates. The isolate culture was adjusted to  $1.5 \times 10^8$ ,  $1.5 \times 10^7$ ,  $1.5 \times 10^6$  and  $1.5 \times 10^5$ . Each dilution was injected intraperitoneally into a fish group at 300 µL /fish and the fish of the 5<sup>th</sup> group was injected with 300 µL of physiological saline (control). The groups were noticed for two weeks for recording of mortality.

#### Prevention of vibriosis in *D. labrax*

After acclimatization of 240 apparently healthy *D.*

Table 2. Composition of the commercial fish diet

Ingredients composition*	Percentage (%)	Ingredients composition*	Percentage (%)
Fish meal	6	Soya bean meal	36
Yellow corn	34	Rice polish	22
Common salt	0.5	Mono-calcium phosphate	1
Premix	0.5	*Crude protein	25

Table 3. The primers sequences, target genes, amplicon sizes and cycling conditions

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Trh	GGCTCAAAATGGTTAAGCG CATTTCGCTCTCATATGC	250	94°C 5 min.	94°C 30 sec.	54°C 30 sec.	72°C 30 sec.	72°C 7 min.	Mustapha <i>et al.</i> , 2013
Tdh	CCATCTGTCCCTTTTCCTGC CCAATACATTTTACTTGG	373	94°C 5 min.	94°C 30 sec.	54°C 30 sec.	72°C 40 sec.	72°C 7 min.	

*labrax*, they were divided into eight groups (10 fish/each) with three replicates. Fish in the first and second groups were fed diet 1 while fish in the third and fourth groups were fed diet 2. On the other hand, fish in the fifth and sixth groups were fed diet 3. The seventh and eighth groups were fed diet 4. Throughout the experimental period, fish of all groups were fed 3% of body weight with its specific diet once a day at 10 a.m. for 30 days.

At the end of dietary experimental period (30 days), the first (control positive), third, fifth and seventh groups with their replicates were challenged intraperitoneally with *V. alginolyticus* strain at a dose of 300 µl of  $1.5 \times 10^8$  concentration. On the other hand, the second (control negative), fourth, sixth and eighth groups were injected intraperitoneally with 300 µl physiological saline. The injected fish were maintained in separate glass aquaria for 2 weeks. The mortality was recorded and the Relative Percent Survival (RPS) was calculated according to Amend (1981) using the following formula:

$$\text{RPS} = 1 - (\% \text{ of mortality in treated groups} / \% \text{ of mortality in control group}) \times 100.$$

### Expression of IL-1β and TLR2 immune-related genes

#### 1) Tissue collection

After acclimatization of 120 apparently healthy *D. labrax*, they were divided into four groups (10 fish/each) with three replicates. Fish in the first group was fed diet 1 and fish in the second group was fed diet 2. On the other hand, fish in the third

group was fed diet 3. The fish groups were fed as in previous section. After feeding period, three fish from each group were rapidly netted and euthanized by an overdose (150 mg/l) of tricaine methane sulfonate (MS222, Sigma-Aldrich Chemical Co. Egypt) and a piece of spleen was collected and stored in 2 ml tubes containing RNAlater solution (Merc, Egypt) then the samples were kept overnight in the refrigerator at 4°C and frozen at -80°C for RNA extraction.

#### 2) Protocol of expression of IL-1β and TLR2 immune-related genes

##### (1) Extraction of RNA

RNA extraction from spleen samples was applied using QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH) when 30 mg of the tissue sample was added to 600 µl RLT buffer containing 10 µl β-mercaptoethanol per 1 ml. the tubes were placed into the adaptor sets, which were fixed into the clamps of the Qiagen tissue Lyser. Disruption was performed in a high-speed (30 Hz) shaking step for two minutes. One volume of 70% ethanol was added to the cleared lysate, and these steps were completed according to the Purification of Total RNA from Animal Tissues protocol of the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH).

N.B. On column DNase digestion was done to remove residual DNA.

##### (2) Oligonucleotide Primers

The primers of immune-related genes were supplied from Metabion (Germany). Primers sequences,



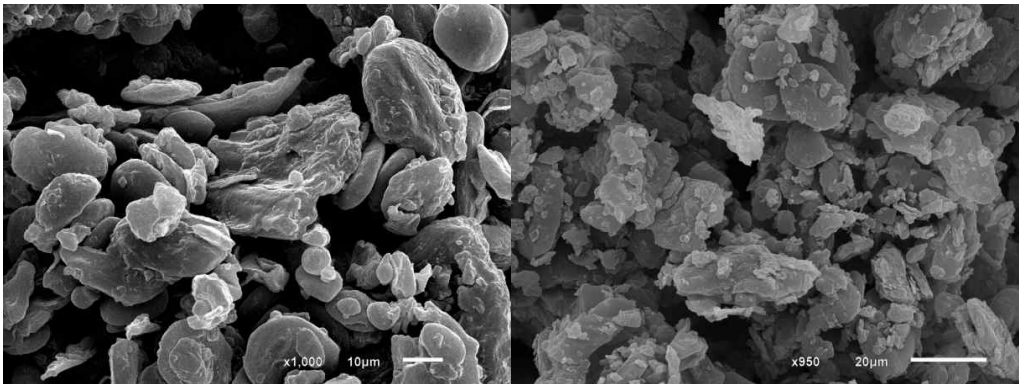


Fig. 1. shows SEM images of ginger before and after milling.

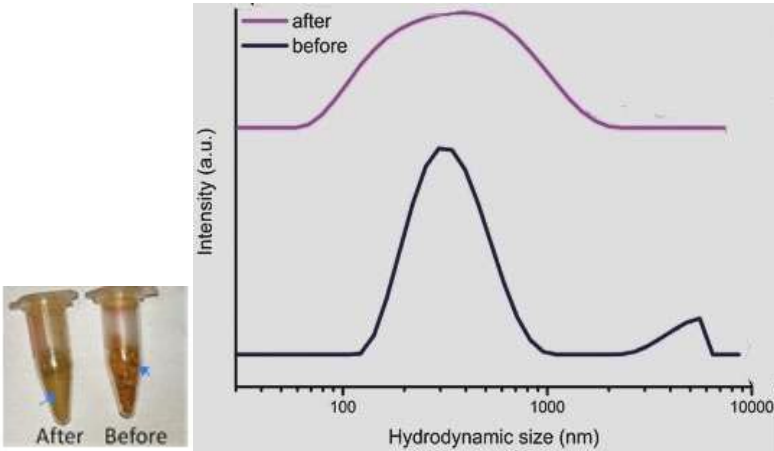


Fig. 2. Hydrodynamic size distribution of ginger samples before and after milling.



Fig. 3. clinically diseased *D. labrax* with *V. alginolyticus* showed corneal opacity and excessive mucous secretion on the gills (a). b. congested gills with excessive mucous secretion. c. internal hemorrhages and adhesion of internal organs.

rods, motile, oxidase positive, Indole production, gelatin hydrolysis, catalase positive and negative to urease and Simmons citrate. Also, all the isolates showed Swarmed large whitish colonies on TSA supplied with 3% NaCl and they showed yellow colonies on

TCBS agar. They were sensitive to O/129 (150 µg) Vibriostate (Table 5).

The virulence genes  
The agarose gel electrophoresis of PCR products

Table 5. Biochemical identification of isolated *V. alginolyticus* by API\*20 NE

Biochemical tests			Results	Biochemical tests			Results
NO3	Potassium nitrate		+	GLU	Glucose assimilation		+
TRP	Tryptophane production		+	ARA	Arabinose assimilation		-
GLU	Glucose fermentation		+	MNE	Mannose assimilation		-
ADH	Arginine Dihydrolase		-	MAN	Mannitol assimilation		+
URE	Urease		-	NAG	N acetyl Glucosamine assimilation		-
ESC	Esculin		+	MAL	Maltose assimilation		+
GEL	Gelatin		+	GNT	Potassium GlucoNate aasimilation		+
PNG	Para Nitrophenyl D Galactopyranosidase B Glucosidase		-	CAP	Capric acid assimilation		-
LDI	Adipic acid assimilation		-	PAC	Phenyle acetic acid assimilation		-
MLT	Malate assimilation		+	OX	Oxidase		+
CIT	Tri sodium Citrate assimilation		-				

determined the presence of *trh* and *tdh* virulence genes at bands 250 bp and 373 bp respectively (Fig. 4). The results clearly indicate the presence of virulence toxins (*trh* and *tdh*).

#### Pathogenicity and the LD<sub>50</sub>

The experimentally infected *D. labrax* showed skin hemorrhages, ulceration and exophthalmia (Fig.

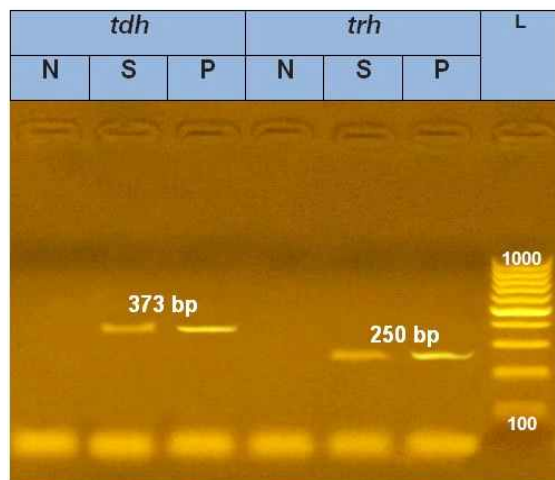


Fig. 4. Ethidium bromide stained agarose gel of PCR products representing (L) the ladder, (N) control negative, (P) control positive (S) amplification of 250 pb amplicons of *trh* and 373 bp amplicons of *tdh* gene of *V. alginolyticus*.

5, d & e). The post-mortem findings of the experimentally infected *D. labrax* revealed internal hemorrhage and darkness of spleen (Fig. 5, f) and paleness of liver (Fig. 5, g). The fish death occurred during the 1<sup>st</sup> week of the experimental infection (Fig. 6), and the LD<sub>50</sub> was  $1.5 \times 10^{5.4}$  CFU/ml.

#### Prevention of vibriosis in *D. labrax*

There were no mortality and 100% RPS in the groups fed GNPs and injected by *V. alginolyticus*,

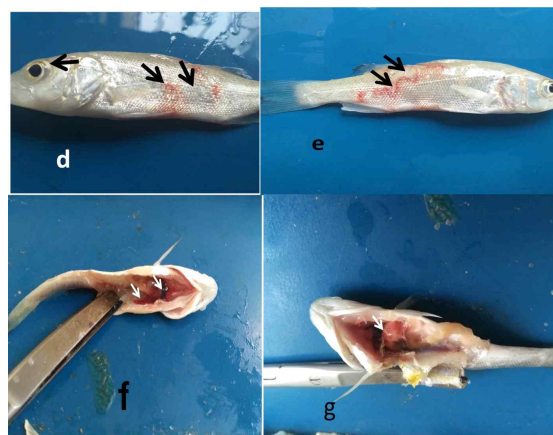


Fig. 5. Experimentally infected *D. labrax* with *V. alginolyticus* showed skin hemorrhage, ulceration and exophthalmia ((d & e). f. Internal hemorrhage and darkness of spleen. g. paleness of liver.



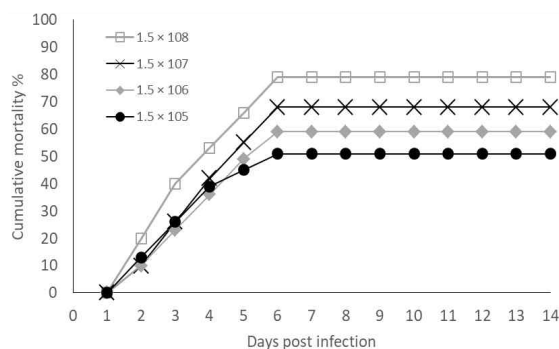


Fig. 6. Cumulative mortality after infection of *D. labrax* by *V. alginolyticus*. Fish were intraperitoneally challenged with pathogenic *V. alginolyticus*. The isolate culture was adjusted to  $1.5 \times 10^8$ ,  $1.5 \times 10^7$ ,  $1.5 \times 10^6$  and  $1.5 \times 10^5$  with 300  $\mu$ L per fish. Graph represents cumulative mortality in each group with SD 0.57.

group fed combination of GNPs and *S. cerevisiae* and group fed normal diet then injected with physiological saline (control negative), respectively. Contrarily, there were 10% mortality and 87.5 RPS in group fed *S. cerevisiae* then injected with *V. alginolyticus*. On the other hand, the control positive group showed 79% mortality (SD 0.57), (Fig. 7).

The effect of dietary GNPs and *S. cerevisiae* on the tissue immune-related genes of *D. labrax*

Compared to the control group, groups of fish fed

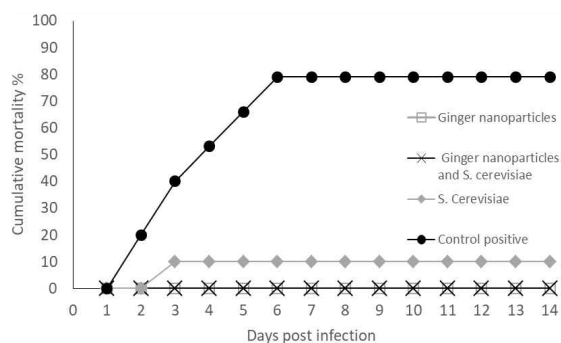


Fig. 7. Cumulative mortality of experimental feeding groups of dietary GNPs, combination of GNPs and *S. cerevisiae*, *S. cerevisiae* (SD 0.57) and the control positive group.

GNPs, *S. cerevisiae* and the combination of GNPs and *S. cerevisiae*, respectively, significantly ( $P < 0.05$ ) up-regulated the expression of IL-1 $\beta$  by 1.6, 1.3 and 2 fold change, respectively and TLR2 by 1.7, 1.5 and 2.4, respectively (Table 6).

## Discussion

In aquaculture, the application of nanotechnology is still at an infant stage but it has the potential to solve most problems in fish diseases and management, disease diagnosis, nutrition and fish production and reproduction (Govindaraju et al 2020). Nano-

Table 6. Expression of immune-related genes in the spleen of *D. labrax*

Sample Groups	EF-1 $\alpha$	IL-1 $\beta$		TLR-2	
	CT (cycle threshold)	CT	Fold change	CT	Fold change
1	19.42	22.84	-	23.78	-
2- ginger nanoparticles (GNPs)	24.58	25.19	1.6 $\pm$ 0.15	26.21	1.7 $\pm$ 0.15
3- <i>Saccharomyces cerevisiae</i>	20.04	21.07	1.3 $\pm$ 0.10	22.59	1.5 $\pm$ 0.1
4- combination of GNPs and <i>S. cerevisiae</i>	18.62	18.67	2 $\pm$ 0.15	19.73	2.4 $\pm$ 0.2

For each gene, the mRNA level of the control fish was set as 1. Data were the means for triplicate experiments and presented as the mean  $\pm$  SD.

## Highlights

- The causative agent of mortality in *D. labrax* was *V. alginolyticus*.
- GNPs were synthesized by ball milling and characterized by SEM, hydrodynamic and zeta.
- Combination of GNPs and *S. cerevisiae* succeeded in preventing vibriosis in *D. labrax*.

technology provides the ability to engineer the properties of materials by controlling their size that has driven many researches towards a multitude of potential uses for nanomaterials (Saifuddin et al., 2009).

The mariculture development is restrained by disease, technical and economic problems [General Authority for Fish Resources Development (GAFRD), 2009]. Bacterial diseases are thought to be the most important type of disease problems that had a direct economic impact on Egyptian mariculture (Grisez et al 1995). Vibriosis is at the top of bacterial diseases that induce high economic losses in mariculture development due to high mortality associated with its invasion to fish (Austin and Austin 2012). In the current study, there was mortality in *D. labrax* farm in a commercial fish farm in Alexandria, Egypt. The samples were collected from that farm to identify the cause of mortality and to find the most effective preventive action against the causative agent.

The naturally diseased *D. labrax* samples showed hemorrhages on the skin with severe congestion of internal organs. The same clinical abnormalities were recorded by Toranzo et al., (2004). Hemorrhages and congestion could be related to severe tissue damage caused by the invasiveness of the organism. It was reported that the severe tissue damage caused by vibriosis was mainly due to the release of proteinases and other extra-cellular enzymes secreted by the bacteria (Sharma et al., 2013). The mortality and the hemorrhagic septicemia picture could be related to *trh* and *tdh* virulence genes which were detected in the *V. alginolyticus* strain in this study. These findings were supported by Mustapha et al. (2013) and Gargouti et al. (2015) who mentioned that the two pathogenic genes (*trh* and *tdh*) of *V. alginolyticus* exert variety of biological activities such as hemolytic activity, cytotoxicity, and enterotoxigenicity causing damage to cells of aquatic organisms.

After bacteriological isolation and identification, the causative agent of mortality in the current study was *V. alginolyticus*. These findings were supported

by Abdelaziz et al. (2017) who reported that *V. alginolyticus* is one of the most dominant vibrio species caused massive mortality in cultured sea bass, sea bream and crustaceans in the Egyptian provinces. Furthermore, these findings were matched with the results of Zorrilla et al. (2003) and Abdelaziz et al. (2013) who recorded that *V. alginolyticus* causes many epizootic outbreaks in Gilthead sea bream and European sea bass aquaculture, which have high economic value in Mediterranean communities and Egyptian provinces.

The LD<sub>50</sub> of *V. alginolyticus* in sea bass was  $1.5 \times 10^{5.4}$  CFU/ml. Some studies reported that the LD<sub>50</sub> in Asian sea bass was  $10^{3.2}$  CFU g<sup>-1</sup> (Sharma et al., 2013). The challenge tests in the present study have established that the present isolate of *V. alginolyticus* was virulent to sea bass. To the best of our knowledge, this is the first study use a dietary combination of GNPs and *S. cerevisiae* to prevent vibriosis in *D. labrax* followed by analysis of transcription levels of the immune-related genes encoding interleukin (IL-1  $\beta$ ) and Toll-like receptors (TLR2) in the spleen of feeding groups.

Our decision to use GNPs instead of ginger was based on their better antibacterial and immuno stimulating properties in prior research (Korni and Khalil 2017; Korni et al 2021).

In the current study, GNPs, as well as a combination of GNPs and *S. cerevisiae*, were shown to be more effective at preventing vibriosis than *S. cerevisiae* alone. There was 10% mortality in groups of fish fed *S. cerevisiae* for 30 days then injected with pathogenic *V. alginolyticus*. On the other hand, there was 79% mortality in the control positive. These results were supported by Korni and Khalil (2017) and Fatma et al. (2021) who proved that dietary incorporation of GNPs succeeded in preventing MAS and Edwardsiellosis in *C. carpio* and *Clarias gariepinus*, respectively. Also, the present findings were supported by Norhidayah et al. (2015) who reported that the in-vitro application of GNPs rhizome water

extract inhibited the growth of *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Salmonella typhimurium*, *Streptococcus pyogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Rhizopus* sp, *Candida albicans* and *Aspergillus nige*.

In the current study, the superior effect of GNPs for prevention of vibriosis could be related to the changing of ginger properties after reduction of size to nanoscale. Ginger nanoparticles might cause damage to the bacterial cell membrane, cellular contents leakage and cell death (Basniwal et al., 2011). Nanoparticles have unique physico-chemical properties which differ from their bulk materials such as a greater surface area to volume ratio, resulting in a larger reactivity (Khosravi-Katuli et al., 2017). Also, Li and Gatlin (2004) recorded that feeding of hybrid striped bass diets containing *S. cerevisiae* significantly increase survival rates after *Streptococcus iniae* infection. Similarly, Abass et al. (2018) demonstrated that *S. cerevisiae* succeeded in protecting of *Oreochromis niloticus* against *A. hydrophila* with higher survival rate. Feeding yeast-supplemented diets improved resistance to other pathogenic infections and survival rates in other aquaculture fish species such as the rainbow trout (Tukmechi and Bandboni 2014), the grouper, *Epinephelus coioides* (Chiu et al. 2010); and the Indian white shrimp, *Fenneropenaeus indicus* (Sajeevan et al., 2009). The enhanced survival rates of yeast-fed fish challenged with pathogen infections are generally related to the presence of immune-stimulatory compounds in yeasts such as  $\beta$ -glucans, nucleic acids, and/or mannan oligosaccharides (Li and Gatlin 2006; Lokesh et al., 2012). These compounds have the potential to stimulate the non-specific immune responses (Harikrishnan et al., 2011). Also, dietary feeding of *S. cerevisiae* improved cellular immunity (Ortuño et al. 2002), serum immunoglobulin M (Cuesta et al. 2004), lysozyme activity (Rodríguez et al. 2003), and phagocytosis (Esteban et al., 2004).

The fold changes of spleen IL-1 $\beta$  and TLR2 genes were significantly increased in groups of fish fed GNPs, *S. cerevisiae* or a combination of GNPs and *S. cerevisiae* compared to the control group. The highest level was found in fish fed a combination of GNPs and *S. cerevisiae*. IL-1 $\beta$  is produced by activated macrophages, monocytes and dendritic cells that affects almost every cell type. It plays a major role in the generation of local and systemic responses to injury, infection and immunological challenges (Sims and Smith 2010). Chistiakov et al., (2010) mentioned that IL-1 $\beta$  is an attractive candidate for resistance to bacterial vibriosis. IL-1 $\beta$  is a multifunctional pro-inflammatory cytokine and it is involved in the induction of innate and acquired immune responses against multiple bacterial, parasitic and viral diseases (Bird et al., 2005). Also some studies showed that polymorphism on TLR gene region can be responsible for disease resistance (Heng et al., 2011). TLRs are very important since they recognize various recognizes pathogen associated molecular patterns (PAMPs) of bacterial, protozoal, fungal and viral pathogens and activate signaling for stimulation of innate immunity to protect the host against diseases (Arancibia et al., 2007).

## Conclusions

Dietary GNPs, *S. cerevisiae* and the combination of GNPs and *S. cerevisiae* succeeded in preventing vibriosis in *D. labrax*. The dietary combination of GNPs and *S. cerevisiae* had the superior effect on prevention of vibriosis with highest up-regulation of IL-1 $\beta$  and TLR2 expression.

## Recommendations

Researches should be discussed the application of nanomaterials in management of aquaculture, prevention and treatment of fish diseases.

## Acknowledgement

We would like to thank Biotechnology unit Reference lab of veterinary quality control on poultry production Animal health research institute, Dokki Giza Egypt. Agricultural Research Centre for determination of virulence genes and transcription levels of the immune-related genes.

### Declarations:

#### - Funding

No funding was received.

#### - Conflict of Interest

The authors declare that they have no conflict of interest.

#### - Ethics approval

All applicable guide lines for the care and use of experimental fish were followed by authors.

#### - Availability of data and material:- n/a

#### - Code availability (software application or custom code):- not applicable

## References

- Abass, DA., Obirikorang, KA., Campion, BB. et al.: Dietary supplementation of yeast (*Saccharomyces cerevisiae*) improves growth, stress tolerance, and disease resistance in juvenile Nile tilapia (*Oreochromis niloticus*). *Aquacult Int* 26: 843-855, 2018.
- Abdelaziz M., Mai D. IbrahimMarwa A. IbrahimNermeen M. Abu-ElalaDalia A. and Abdel-moneam: Monitoring of different vibrio species affecting marine fishes in Lake Qarun and Gulf of Suez: Phenotypic and molecular characterization. *Egyptian Journal of Aquatic Research* 43: 141-146, 2017. <http://dx.doi.org/10.1016/j.ejar.2017.06.002>
- Abdel-Aziz, M.; Eissa, A.; Hanna, M. and Abou Okada, M.: Identifying some pathogenic *Vibrio* /Photobacterium species during mass mortality of cultured Gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) from some Egyptian coastal provinces. *Int. J. Vet. Sci. Med.*, 1, 87-95, 2013. <https://doi.org/10.1016/j.ijvsm.2013.10.004>
- Abdel-Tawwab, M.; Abdel-Rahman, A. and Ismael, N. E.: Evaluation of commercial live bakers' yeast, *Saccharomyces cerevisiae*, as a growth and immunity promoter for fry of Nile tilapia, *Oreochromis niloticus* (L.) challenged in situ with *Aeromonas hydrophila*. *Aquaculture* 280: 185-189, 2008. <https://doi.org/10.1016/j.aquaculture.2008.03.055>
- Amend, D. F.: Potency testing of fish vaccines. International symposium on fish Biologics: Serodiagnostics and Vaccines. *Developmental biology*. 49: 447-454, 1981.
- Arancibia, S. A.; Caroll jbeltran; Isabel M Aguirre; Paulinasilva; Alexis L Peralta; Frano Malinarich and Marcela A H.: Toll-like Receptors are Key Participants in Innate-Immune Responses. *Biol Res* 40: 97-112, 2007. [http// doi: 10.4067/s0716-97602007000200001](http://doi:10.4067/s0716-97602007000200001).
- Austin, B. and Austin, A. D.: Bacterial fish pathogens: diseases of farmed and wild fish (5thed.), Springer/ Prazis Publishing, Chichester, UK, 2012. <https://www.springer.com/gp/book/9781402060687>
- Basniwal, R. K., Buttar, H. S., Jain, V. K. and Jain, N.: Curcumin nanoparticles: preparation, characterization and antimicrobial studies. *Journal of Agricultural Food Chemistry*. 59: 2056-2061, 2011. <https://doi.org/10.1021/jf104402t>
- Bird, S.; Zou, J., Kono, T., Sakai, M., Dijkstra, J. M., Secombes, C.: Characterisation and expression analysis of interleukin 2 (IL-2) and IL-21 homologues in the Japanese pufferfish, *Fugu rubripes*, following their discovery by synteny. – *Immunogenetics* 56 (12): 909-923, 2005.
- Castro, R.; Zou, J.; Secombes, C.J. and Martin, S.A.M.: Cortisol modulates the induction of inflammatory gene expression in a rainbow trout macrophage cell line. *Fish & Shellfish Immunology* 30: 215-223, 2011. [http//DOI: 10.1007/s00251-004-0741-7](http://DOI:10.1007/s00251-004-0741-7)
- Cebeci, A.; Saltan, A. N. and Sahayaraju K.: Prediction of Toll-Like Receptor Gene Family in Asian Sea Bass. *Genetics of Aquatic Organisms* 3(2): 79-91, 2019. [http// doi: 10.1016/j.fsi.2019.07.010](http://doi:10.1016/j.fsi.2019.07.010). Epub 2019 Jul 6
- Chiu C-H, Cheng C-H, Gua W-R, Guu Y-K, Cheng W.: Dietary administration of the probiotic, *Saccharomyces cerevisiae* P13, enhanced the growth, innate immune responses, and disease resistance of the grouper, *Epinephelus coioides*. *Fish Shellfish Immunol* 29: 1053-1059, 2010. [http// doi: 10.1016/j.fsi.2009.02.018](http://doi:10.1016/j.fsi.2009.02.018)
- Chistiakov, D. A.; Kabanov, F. V.; Troepolskaya O. D. and Tischenko, M. M. A.: A variant of the interleukin-1beta gene in European sea bass, *Dicent-*

- rarchus labrax* L., is associated with increased resistance against *Vibrio anguillarum*. Journal of Fish Diseases. 33(9): 759-767, 2010. <https://doi.org/10.1111/j.1365-2761.2010.01182.x>
- Cuesta A., Meseguer J., Esteban M. A.: Total serum immunoglobulin M levels are affected by immunomodulators in seabream (*Sparus aurata* L.) Vet Immunol Immunopathol 101: 203-210, 2004. <https://doi.org/10.1016/j.vetimm.2004.04.021>
- Defoirdt, T.; Boon, N.; Sorgeloos, Verstraete, W. and Bossier, P.: Alternatives to antibiotics to control bacterial infections: luminescent vibriosis in aquaculture as an example. Trends in Biotechnology. 25 (10): 472-479, 2007. <https://doi.org/10.1016/j.tibtech.2007.08.001>
- Esteban MA, Rodríguez A, Meseguer J.: Glucan receptor but not mannose receptor is involved in the phagocytosis of *Saccharomyces cerevisiae* by seabream (*Sparus aurata* L.) blood leucocytes. Fish Shellfish Immunol 16: 447-451, 2004. <http://doi.org/10.1016/j.fsi.2003.07.004>
- Gargouti A.S., Ab-Rashid M.N.K., Ghazali MF, Mitsuki N., Haresh K. K., et al.: Detection of tdh and trh Toxic Genes in *Vibrio Alginolyticus* Strain from Mantis Shrimp (Oratosquilla Oratoria). J Nutr Food Sci 5: 405, 2015. <http://doi.org/10.4172/2155-9600.1000405>
- General Authority for Fish Resources Development (GAFRD), Statistics of fish production Govindaraju, K., Dilip Itroutwar, P., Veeramani, V. et al.: Application of Nanotechnology in Diagnosis and Disease Management of White Spot Syndrome Virus (WSSV) in Aquaculture. J. Clust. Sci. 31: 1163-1171, 2020.
- Grisez, L. and Ollevieribrio, F.: (Listonella) anguillarum infections in marine fish larviculture P. Lavens, E. Jaspers, I. Roelands (Eds.), Fish and crustacean larviculture symposium, European Aquaculture Society, Gent (1995).
- Gröner, F.; Ziková, A. and Kloas, W.: Effects of the pharmaceuticals diclofenac and metoprolol on gene expression levels of enzymes of biotransformation, excretion pathways and estrogenicity in primary hepatocytes of Nile tilapia (*Oreochromis niloticus*). Comparative Biochemistry and Physiology, Part C 167 51-57, 2015. <http://doi.org/10.1016/j.cbpc.2014.09.003>
- Harikrishnan R, Kim M. C., Kim J. S., Balasundaram C., Heo M. S.: Immunomodulatory effect of probiotics enriched diets on Uronema marinum infected olive flounder. Fish Shellfish Immunol 30: 964-971, 2011. <https://doi.org/10.1016/j.fsi.2011.01.030>
- Hayatgheib, N., Moreau, E., Calvez, S. et al.: A review of functional feeds and the control of Aeromonas infections in freshwater fish. Aquacult Int 28: 1083-1123, 2020. <https://doi.org/10.1007/s10499-020-00514-3>
- Heng, J.; Su, J.; Huang, T. and Chen, L.: The polymorphism and haplotype of TLR3 gene in grass carp (*Ctenopharyngodon idella*) and their associations with susceptibility/resistance to grass carp reovirus. Fish & Shellfish Immunology, 30(1): 45-50, 2011. <https://doi.org/10.1016/j.fsi.2010.09.004>
- Holt, J. G.; Krieg, N. R.; Sneath, P.H.A.; Staley, J. T. and Williams, S.T.: Bergey's Manual of Determinative Bacteriology, 9<sup>th</sup> ed. Baltimore: Williams & Wilkins, 1994.
- Hoseinifar, S. H.; Ringø, E.; Shenavar Masouleh, A. and Esteban, M. Á.: Probiotic, prebiotic and synbiotic supplements in sturgeon aquaculture: a review. Rev. Aquacult. 8: 89-102, 2016. <https://doi.org/10.1111/raq.12082>
- Irianto, A., and Austin, B.: Probiotics in aquaculture. Fish Shellfish Immun. 25: 633-642, 2002. <https://doi.org/10.1046/j.1365-2761.2002.00422.x>
- Khosravi-Katuli, K., Prato, E., Lofrano, G., Guida, M., Vale, G. and Libralato, G.: Effects of nanoparticles in species of aquaculture interest. Environ Sci Pollut Res. 24: 17326-17346, 2017. <https://doi.org/10.1007/s11356-017-9360-3>
- Kim D. H., Austin B.: Innate immune responses in rainbow trout (*Oncorhynchus mykiss*, Walbaum) induced by probiotics. Fish Shellfish Immunol 21: 513-524, 2006. <http://doi.org/10.1016/j.fsi.2006.02.007>
- Korni, F. and Khalil, F.: Effect of ginger and its nanoparticles on growth performance, cognition capability, immunity and prevention of motile Aeromonas septicaemia in *Cyprinus carpio* fingerlings. Aquaculture Nutrition. 23. 10.1111/anu.12526, 2017. <https://doi.org/10.1111/anu.12526>
- Fatma M.M. Korni, Fatma I. Abo El-Ela, Usama K. Moawad, Rehab K. Mahmoud, Yasser M. G.: Prevention of Edwardsielliosis in *Clarias gariepinus* using ginger and its nanoparticles with a reference to histopathological alterations, Aquaculture, Volume 539, 2021, 736603, ISSN 0044-8486, <https://doi.org/10.1016/j.aquaculture.2021.736603>
- Korun J. and Timur G.: Marine Vibrios associated with diseased Sea bass (*Dicentrarchus labrax*) in Turkey. Journal of Fisheries and Science. 2(1): 66-76, 2008.

- <http://DOI: 10.3153/jfscm.2008007>
- Li P, Gatlin, D.: Nucleotide nutrition in fish: current knowledge and future applications. *Aquacult* 251: 141-152, 2006. <https://doi.org/10.1016/j.aquaculture.2005.01.009>
- Li Q, Mahendra S, Lyon DY, Brunet L, Liga MV, Li D, Alvarez P.J.J.: Antimicrobial nanomaterials for water disinfection and microbial control: potential applications and implications. *Water Res* 42: 4591-4602, 2008. <https://doi.org/10.1016/j.watres.2008.08.015>
- Liu P.C.; Lin, J.Y.; Hsiao, P.T. and Lee, K.K.: Isolation and characterization of pathogenic *Vibrio alginolyticus* from diseased cobia *Rachycentron canadum*. *Journal of Basic Microbiology*. 44: 23-28, 2004. <https://doi.org/10.1002/jobm.200310316>
- Lokesh J, Fernandes JMO, Korsnes K, Bergh Ø, Brinchmann MF, Kiron V.: Transcriptional regulation of cytokines in the intestine of Atlantic cod fed yeast derived mannan oligosaccharide or  $\beta$ -glucan and challenged with *Vibrio anguillarum*. *Fish Shellfish Immunol* 33:626–631, 2012. <http://doi: 10.1016/j.fsi.2012.06.017>. Epub 2012 Jul 5
- López-Castejón G., Sepulcre M. P., Roca F. J., Castellana B., Planas J. V., Meseguer J. and Mulero V.: The type II interleukin-1 receptor (IL-1RII) of the bony fish gilthead seabream *Sparus aurata* is strongly induced after infection and tightly regulated at transcriptional and post-transcriptional levels. *Mol Immunol* 44, 2772-2780, 2007. <http://doi: 10.1016/j.molimm.2006.10.027>
- Mustapha, S.; Mustapha, E.M.; Nozha, C.: *Vibrio Alginolyticus*: An Emerging Pathogen of Foodborne Diseases. *International Journal of Science and Technology* Volume 2 No. 4, 2013. <http:// Maejo International Journal of Science and Technology>
- Norhidayah, A., Noriham, A. and; Rusop, M. D.: Assessment of antimicrobial activity of nanoparticle ginger rhizome water extract. *International Journal of Scientific and Research Publications*, (5),11, 656-669, 2015. <http://ijsrp.org/>
- Ortuño J, Cuesta A, Rodríguez A, Esteban MA, Meseguer J.: Oral administration of yeast, *Saccharomyces cerevisiae*, enhances the cellular innate immune response of gilthead seabream (*Sparus aurata*). *Vet Immunol Immunopathol* 85: 41-50, 2002. [http://doi: 10.1016/s0165-2427\(01\)00406-8](http://doi: 10.1016/s0165-2427(01)00406-8)
- Pinoargote, G. and Ravishankar, S.: Evaluation of the Efficacy of Probiotics in vitro Against *Vibrio parahaemolyticus*, Causative Agent of Acute Hepato-pancreatic Necrosis Disease in Shrimp. *J Prob Health* 6: 193, 49-89, 2018. <http://doi:10.4172/2329-8901.1000193>
- Pridgeon, J. W. and Klesius, P. H.: Major bacterial diseases in aquaculture and their vaccine development. *CAB Reviews* 7, 1 -16, 2012. <http://doi:10.1079/PAVSNNR20127048>.
- Rattanachaikunsopon, P. and Phumkhachorn, P.: Assessment of synergistic efficacy of carvacrol and cymene against *Edwardsiella tarda* in vitro and in Tilapia (*Oreochromis niloticus*). *African Journal of Microbiology Research*. 4: 420-425, 2010. <http://www.academicjournals.org/ajmr>
- Sajeevan T.P., Philip R., Singh I.S.B.: Dose/frequency: a critical factor in the administration of glucan as immunostimulant to Indian white shrimp *Fenneropenaeus indicus*. *Aquaculture*, 287: 248-252, 2009. <https://doi.org/10.1016/j.aquaculture.2008.10.045>
- Rodríguez A., Cuesta A., Ortuño J, Esteban M. A., Meseguer J.: Immunostimulant properties of a cell wall modified whole *Saccharomyces cerevisiae* strain administered by diet to seabream (*Sparus aurata* L.) *Vet Immunol Immunopathol* 96: 183-192, 2003. <http://doi: 10.1016/j.vetimm.2003.07.001>.
- Saifuddin, N., C. W. W. and YASUMIRA, A.A.N.: Rapid Biosynthesis of Silver Nanoparticles Using Culture Supernatant of Bacteria with Microwave Irradiation. *E-Journal of Chemistry*, 2009, 6(1): 61-70, 2009. [http:// DOI: 10.1155/2009/734264](http://DOI: 10.1155/2009/734264)
- Sayes, C.; Leyton, Y. and Riquelme, C.: Probiotic bacteria as a healthy alternative for fish aquaculture, in *Antibiotic Use in Animals*, ed S. Savic (London: Intech Open) 115-132, 2018. [http/ DOI:10.5772/intechopen.71206](http://DOI:10.5772/intechopen.71206)
- Shaalán M, Saleh M, El-Mahdy M, El-Matbouli M.: Recent progress in applications of nanoparticles in fish medicine: a review. *Nanomedicine: NBM* 12: 701-710, 2015. [http/ doi: 10.1016/j.nano.2015.11.005](http://doi: 10.1016/j.nano.2015.11.005)
- Sharma S. R. K., Gaurav, R. , Dev K. V., Narasimhulu S. and Kuttickal K. P.: *Vibrio alginolyticus* infection in Asian seabass (*Lates calcarifer*, Bloch) reared in open sea floating cages in India. *Aquaculture Research*, 44: 86-92, 2013. <https://doi.org/10.1111/j.1365-2109.2011.03013.x>
- Sims, J. E. and Smith D. E.: The IL-1 family: regulators of immunity. *Nat Rev Immunol* 10: 89-102, 2010. [http/ DOI: 10.1038/nri2691](http://DOI: 10.1038/nri2691)
- Sugita H, Miyajima C, Deguchi Y.: The vitamin B12-producing ability of intestinal bacteria isolated from tilapia and channel catfish. *Nippon Suisan Gakkaishi*

- 56, 701, 1990.
- Takacs, L.: Self-sustaining reactions induced by ball milling. *Progress in Materials Science*. 47(4): 355-414, 2002. [http://doi.org/10.1016/S0079-6425\(01\)00002-0](http://doi.org/10.1016/S0079-6425(01)00002-0)
- Thune R.L., Stanely L.A. and Cooper R. K.: Pathogenesis of gram-negative bacterial infections in warm water fish. *Annual Review of Fish Diseases* 3: 37-68, 1993. [http://DOI: 10.1016/0959-8030\(93\)90028-A](http://DOI: 10.1016/0959-8030(93)90028-A)
- Toranzo, A.E. : Report about fish bacterial diseases P. Alvarez-ellitero, J.L. Barja, B. Basurco, F. Berthe, A.E. Toranzo (Eds.) (2004). Mediterranean Aquaculture Diagnostic Laboratories, CIHEAM, Zaragoza. <http://www.ciheam.org/>
- Toranzo, A. E., Magariños, B. and Romalde, J. L.: A review of the main bacterial fish diseases in mariculture systems. *Aquaculture*, 246: 37-61, 2005. <http://doi.org/ 10.1016/j.aquaculture.2005.01.002>
- Tukmechi A., Bandboni M.: Effects of *Saccharomyces cerevisiae* supplementation on immune response, hematological parameters, body composition and disease resistance in rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792). *Journal of Applied Ichthyology* 30: 55-61, 2014. <https://doi.org/10.1111/jai.12314>
- Varsamos, S., Flik, G., Pepin, J. F., Wendelaar B., S. E. and Brevil, G.: Husbandry stress during early life stages affects the stress response and health status of juvenile sea bass, *Dicentrarchus labrax*. *Fish & Shellfish Immunology*, 20: 82-96, 2006. <http://doi.org/ 10.1016/j.fsi.2005.04.005>
- Yuan, J.S.; Reed, A.; Chen, F. and Stewart, C.N.: Statistical analysis of real-time PCR data. *BMC Bioinformatics*, 7:85, 2006. <https://doi.org/10.1186/1471-2105-7-85>
- Zorrilla,I.; Arijo,S.; Chabrillon,M.; Diaz,P.; Martinez-Manzanares,E.; Balebona, M.C.and and Morínigo, M.A.: *Vibrio* species isolated from diseased farmed sole, *Solea senegalensis* (Kaup) and evaluation of the potential virulence role of their extracellular products. *Journal of Fish Diseases*, 26, pp. 103-108, 2003. <https://doi.org/10.1046/j.1365-2761.2003.00437.x>

---

Manuscript Received : Jul 09, 2021

Revised : Aug 01, 2021

Accepted : Sep 06, 2021