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

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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 (LD50) of pathogenic isolate were assessed. Also, the effect of ginger nanoparticles (GNPs) and *Sacchromyces cerevisiae* as feed additives for prevention of vibriosis in *D. labrax* was carried out. Similarly, the tissue immunstimulant genes, IL-1β 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_{50} of *V. alginolyticus* was $1.5\times10^{5.4}$ 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. cerevisae* then injected with *V. alginolyticus*. On the other hand, the control positive group showed 79% mortality. The spleen IL-1β 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-1β and TLR2 immunostimulant genes.

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

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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; Korni 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 (Korni 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 (Shaalan 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 Edwardsiellosis (Korni and Khalil 2017 and Korni et al., 2021).

Interleukin 1β (IL-1β) 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-1β 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-1β 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 ± 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 ± 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 ± 3 g) were collected alive for running the experiments of vibriosis prevention. Also, a total of 120 apparently healthy *D.* labrax (50 ± 3 g) were collected alive for spleen collection to measure IL-1β 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 × 40 × 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 ± 2 ºC, Dissolved Oxygen D.O was 6 ± 2 mg/l, pH was 7-8 and the salinity was 12 ± 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 *D. labrax*	Experiment	Observation and sampling at the end of experiment
150 (10 fish/5 groups/ 3 replicates)	Experimental infection and evalution of LD_{50} of *V. alginolyticus*	● LD50 was calculated. ● Fish were observed for 2 weeks. ● Mortality was recorded daily.
240 (10 fish/8 groups/ 3 replicates)	For *V. alginolyticus* prevention	● For *V. alginolyticus* challenge. ● Fish were observed for 2 weeks after experimental infection. ● Mortality was recorded daily. ● RPS was calculated (Amend, 1981).
120 (10 fish/4 groups/ 3 replicates)	Spleen collection	● Spleen collection for IL-1β and TLR2 measurement.

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 6.5×10^9 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 56ºC 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_{50} 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 1.5×10^8, 1.5×10^7, 1.5×10^6 and 1.5×10^5. Each dilution was injected intraperitoneally into a fish group at 300 µL /fish and the fish of the 5th 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 CATTTCCGCTCTCATATGC	250	94˚C 5 min.	94˚C 30 sec.	54˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Mustapha *et al*., 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 1.5×10^8 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:

RPS = 1 − (% of mortality in treated groups / % of mortality in control group) × 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,

target genes, amplicon sizes and cycling conditions for SYBR green rt-PCR were listed in Table 4 (Gröner et al., 2015 and Castro et al., 2011).

(3) SYBR green rt-PCR

Primers were utilized in 25 µl reaction containing 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl of Revert Aid Reverse Transcriptase (200 U/µl) (Thermo Fisher), 0.5 µl of each primer of 20 pmol concentration, 8.25 µl of water and 3 µl of RNA template. The reaction was performed in a Strata gene MX 3005P real time PCR machine.

(4) Analysis of the SYBR green rt-PCR results

Amplification curves and CT values were determined by the strata gene MX3005P software. To estimate the variation of gene expression on the RNA of the different samples, the CT of each sample was compared with that of the control group according to the "ΔΔCt" method stated by Yuan *et al*., (2006) using the following ratio: (2^{-DDct}).

Whereas ΔΔCt = ΔCt reference – ΔCt target

Δ*Ct target* = *Ct control – Ct treatment* and Δ*Ct reference* = *Ct control- Ct treatment*

Statistical Analyses

Statistical analyses were performed using all data one-way ANOVA (post hoc test; Dunnet's test) Advanced Models 16.0 software (SPSS, Tokyo, Japan). A P-value < 0.05 was considered statistically significant. Effective concentration causing 50% mortality (LD50), with 95% confidence limits (95% CL) was calculated with SPSS v.22.

Results

Ginger nanoparticles characterization

Ginger particles had large size in the range of 12-27 µm. Very small particles were obtained after milling (1.8-2.6 µm, Fig. 1). The sample before milling had a hydrodynamic size range of 100 to 1000 nm with larger particles (4-6 µm). After milling, all particles had size distribution less than 2 µm (Fig. 2). The zeta potential of ginger was -23.1 before milling and it was -17.2 mV after milling.

Clinical signs of naturally diseased *D. labrax*

The naturally diseased *D. labrax* showed corneal opacity and paleness of gills with excessive mucous secretion (Fig. 3, a & b). On the other hand, the post-mortem abnormalities were hemorrhage and adhesion of internal organs (Fig. 3, c).

Bacteriological identification

A total of fourteen *V. alginolyticus* strains were isolated from diseased *D. labrax* with a prevalence of 70%. All strains were Gram negative, Short-curved

Table 4. Primers sequences, target genes, amplicon sizes and cycling conditions for SYBR green rt-PCR

Target gene	Primers sequences	Reverse transcription	Primary Denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)		
				Secondary denaturation	Annealing (Optics on)	Extension	Secondary denaturation	Annealing	Final denaturation
EF-1α	CCTTCAACGCTCAGGTCATC	50°C 30 min.	94°C 15 min.	94°C 15 sec.	62°C 30 sec.	72°C 30 sec.	94°C 1 min.	62°C 1 min.	94°C 1 min.
	TGTGGGCAGTGTGGCAATC								
TLR-2	CCCACAATGGATTCACCAG								
	AAAGATCAAGACTCAAGG CACTG								
IL-1ß	GCTGGAGAGTGCTGTGGA AGAACATATAG								
	CCTGGAGCATCATGGCGTG								

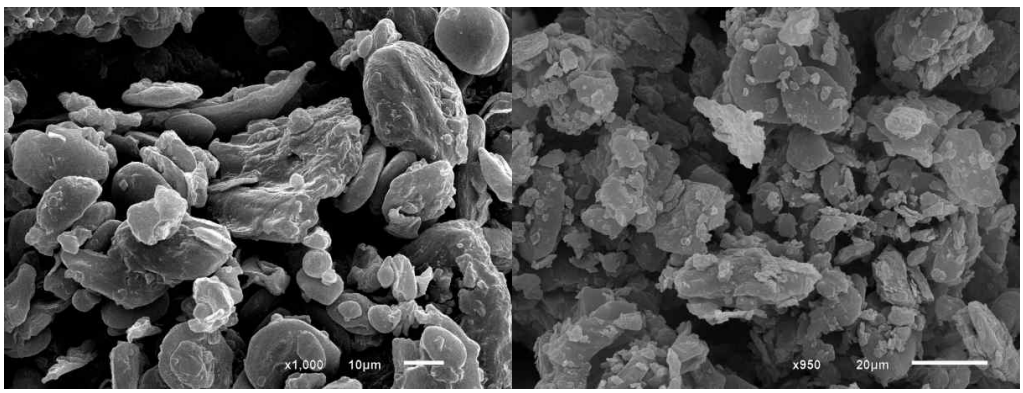

Fig. 1. shows SEM images of ginger before and after milling.

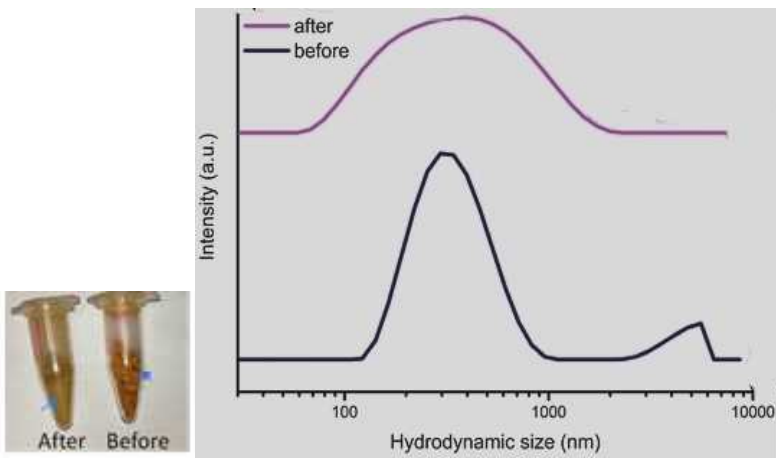

Fig. 2. Hydrodynamic size distribution of ginger samples before and after milling.

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	Trytophane 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 assimlation	-
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_{50}

The experimentally infected *D. labrax* showed skin hemorrhages, ulceration and exophthalmia (Fig. 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 1st week of the experimental infection (Fig. 6), and the LD_{50} was $1.5 \times 10^{5.4}$ CFU/ml.

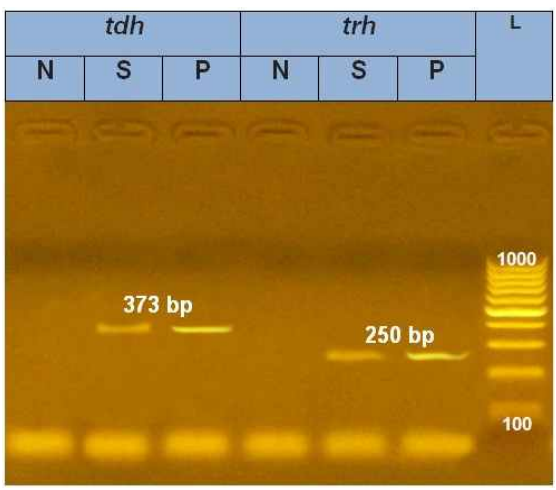

Fig. 4. Ethidium bromide stained agarose gel of PCR products representing (L) the ladder, (N) control negative, (P) control positive (S) amplification of 250 pb amplicons of trh and 373 bp amplicons of tdh gene of *V. alginolyticus*.

Prevention of vibriosis in *D. labrax*

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

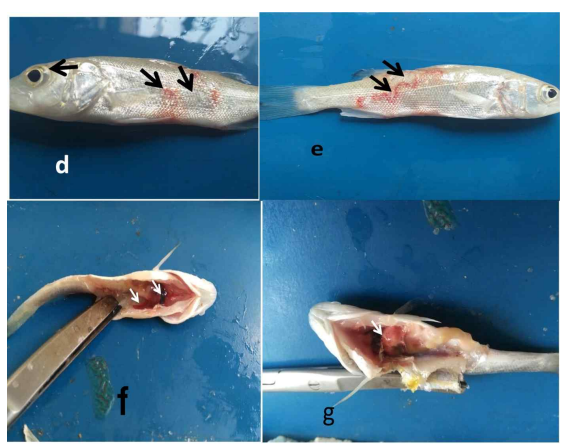

Fig. 5. Experimentally infected *D. labrax* with *V. alginolyticus* showed skin hemorrhage, ulceration and exophthalmia ((d & e). f. Internal hemorrhage and darkness of spleen. g. paleness of liver.

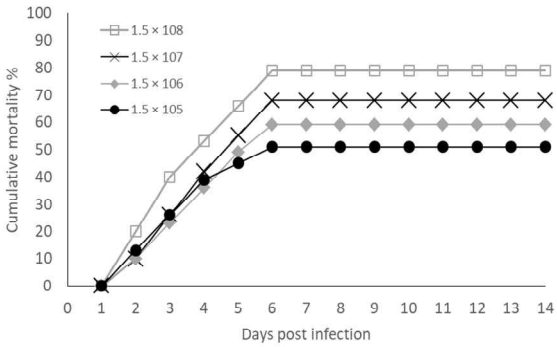

Fig. 6. Cumulative mortality after infection of *D. labrax* by *V. alginolyticus*. Fish were intraperitoneally challenged with pathogenic *V. alginolyticus*. The isolate culture was adjusted to 1.5×10^8, 1.5×10^7, 1.5×10^6 and 1.5×10^5 with 300 µL per fish. Graph represents cumulative mortality in each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

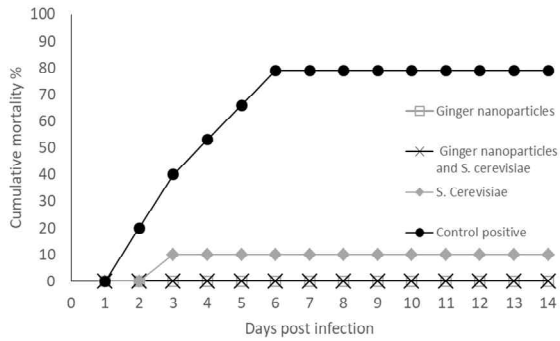

Fig. 7. Cumulative mortality of experimental feeding groups of dietary GNPs, combination of GNPs and *S. cerevisiae*, *S. cerevisiae* (SD 0.57) and the control positive group.

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. cerevisae* 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 GNPs, *S. cerevisiae* and the combination of GNPs and *S. cerevisiae*, respectively, significantly ($P < 0.05$) up-regulated the expression of IL-1β 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	EF-1α	IL-1ß		TLR-2	
Groups	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±0.0.15	26.21	1.7±0.15
3- *Sacchromyces cerevisae*	20.04	21.07	1.3±0.10	22.59	1.5±0.1
4- combination of GNPs and *S. cerevisae*	18.62	18.67	2 ± 0.15	19.73	2.4±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 ± SD.

Highlights

- The causative agent of mortality in *D. labrax* was *V. alginolyticus*.
- GNPs were synthetized 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 enterotoxicity 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_{50} of *V. alginolyticus* in sea bass was $1.5 \times 10^{5.4}$ CFU/ml. Some studies reported that the LD_{50} in Asian sea bass was $10^{3.2}$ CFU g^{-1} (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 β) 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. cerevisae*, were shown to be more effective at preventing vibriosis than *S. cerevisae* alone. There was 10% mortality in groups of fish fed *S. cerevisae* 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 *Clariras 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. cerevisae* 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 β-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-1β 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-1β 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 thatIL-1β is an attractive candidate for resistance to bacterial vibriosis. IL-1β 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-1β and TLR2 expression.

Recommendations

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

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- **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

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