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EFFECTS OF DIETARY SODIUM BUTYRATE ON THE BIOLOGICAL INDICES, GENE EXPRESSION, AND RESISTANCE OF OREOCHROMIS NILOTICUS TO MULTIDRUG-RESISTANT PSEUDOMONAS AERUGINOSA INFECTION

SAFAA M. SHABANA [1]; SHAIMAA ELBAZ [2]; MONA E. ABASS [2] AND NAGWA I. S. ABU-ZAHRA [3]

[1] Bacteriology Unit, Kafr Elsheikh Provincial Lab, Animal Health Research Institute, Agricultural Research Center (ARC), Giza, Egypt

[2] Biochemistry, Nutritional Deficiency Diseases and Toxicology Unit, Kafr Elsheikh Provincial Lab, Animal Health Research Institute, Agricultural Research Center (ARC), Giza, Egypt

[3] Fish Diseases Unit, Kafr Elsheikh Provincial Lab, Animal Health Research Institute, Agricultural Research Center (ARC), Giza, Egypt. ORCID: https://orcid.org/0000-0002-6683-1566

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ABSTRACT

Using immunostimulants and acidifiers and their salts as antibiotic alternatives and growth promoters has become important due to the emerging antimicrobial resistance and is currently employed in livestock and aquaculture feed. So, this study is designed to assess the ameliorative properties of sodium butyrate (SB) on *Oreochromis niloticus* growth indices, immune responses, antioxidant, growth-related gene expression, and its resistance against the *Pseudomonas* aeruginosa infection. Five groups of *O. niloticus* were distributed (30 fish/group); the first control (CTR) group was fed a basal diet without any additives, while the 2nd to 5th groups were fed on a SB supplemented diet with 0.25, 0.50, 0.75, and 1% concentrations, respectively. After 8 weeks, all groups were subdivided into; the diet-treated group with enrofloxacin and the untreated ones. Following the consumption of the medicated feed, all groups were infected with *P.* aeruginosa. The SB-fed fish showed an upregulated expression of growth-related genes (GH and *IGF-1*) and the antioxidant enzyme genes (catalase, and superoxide dismutase). The hematological parameters, immune indices, bactericidal activity, and phagocytosis were improved. After *P.* aeruginosa infection, a high mortality rate was recorded in the CTR-infected untreated group (50%), while it was markedly reduced (10%) in 0.75% and 1% SB-infected untreated groups. The enhanced potential synergistic effect of SB in 0.75% and 1% with ENRO was also observed. Therefore, diet supplementation with sodium butyrate, particularly at 0.75%, either alone as a growth enhancer or combined with ENRO, is recommended to protect fish completely from *P.* aeruginosa infection.

Keywords: *Oreochromis niloticus*, Sodium butyrate, Biological indices, *Pseudomonas aeruginosa,* Antibiotic resistance

INTRODUCTION

In Egypt and many other countries, Nile tilapia (*Oreochromis niloticus*) has become a top priority for culture. In 2018, the production of this species increased to approximately 4.5 metric tons (FAO, 2018). This rise could be attributed to the expansion of intensive farming practices, which may be a reason for overcrowding stress, increasing the risk of infection, and ultimately resulting in fish death (Assefa and Abunna, 2018). However, fish raised in

Corresponding author: Nagwa I. S. Abu-Zahra
E-mail address: nagwaabuzahra09@gmail.com
Present address: Fish Diseases Unit, Kafrelsheikh Provincial Lab, Animal Health Research Institute, Agricultural Research Center (ARC), Giza, Egypt

intensive systems have suppressed immune responses and general health, making them more susceptible to infectious diseases and having lower output (Dawood, 2020). In addition, fish kept in extreme conditions have poor feed intake and compromised digestive abilities. Recently, feed additives have been used to enhance the health and performance of fish. Previous studies showed that medicinal plants (Abu-Zahra *et al.,* 2024; Hassan *et al.,* 2021), immunostimulants (Abu-Zahra *et al.,* 2023 a, b), and probiotics (Truong *et al.,* 2017) have improved fish immunity, health status, and disease resistance.

The majority of diseases affecting cultured fish are caused by bacterial infections (Hasan *et al*., 2021). More than 30% of global production losses are thought to be caused by fish diseases, and bacterial infections represent a significant threat to tilapia farming globally (Haenen, *et al*., 2023). In cultured fish and shrimp, Gram-negative bacteria belonging to the genera *Pseudomonas*, *Aeromonas*, *Flavobacterium*, and *Vibrio* cause high mortalities and significant financial losses (Aboyadak *et al*., 2017). *Pseudomonas aeruginosa* is a highly pathogenic Gram-ve bacterium that can infect a variety of farmed freshwater and marine fish species, such as *O. niloticus* and *Sparus aurata* (El-Bahar *et al.,* 2019; Bikouli *et al*., 2021). One of the major problems facing fish farms is controlling fish disease, which is often accomplished by using antibiotics, which were outlawed by the European Union. Furthermore, this method is usually highly expensive, impairs the immune system (Abu-Zahra *et al.,* 2023 a, b), contaminates the environment, leaves residues in the tissues of fish that might be detrimental to humans, and helps the emergence of bacteria resistant to antibiotics (Polianciuc *et al.,* 2020). Consequently, it is essential to search for natural immunostimulant feed additives that can improve fish health, performance, and resistance to infections (Abu-Zahra *et al.,* 2024, 2023 a, b).

In aquaculture, organic acids are nonnutritive feed supplements that are well known for their ability to promote growth, antibacterial agents, and maintain the balance of the intestinal microbiota (Abdel-Latif *et al*., 2020), additionally facilitate the absorption of minerals, resulting in optimal feed utilization, as well as regulated metabolic processes and osmoregulation (Hoseinifar *et al.,* 2016), and also stimulate local intestinal immunity by decreasing hazardous bacteria and increasing intestinal acidity (Zhang *et al.,*2020). Earlier studies investigated the effects of acidifiers and their salts in aquaculture (Sheikhzadeh *et al.,* 2021; Yusefi *et al.,* 2022; El-Sharkawy *et al.,* 2023), and the results showed enhanced feed digestion and growth, antibacterial activity, local gut immunity, and antioxidative capability. Also, Zhang *et al.* (2020) reported that the feed digestibility, growth, and immunological responses of *Carassius auratus gibelio* were improved by citric acid. The addition of propionic acid in *O. niloticus* diets had increased the immune capacity and resistance of the fish against *Aeromonas hydrophila* infection (El-Adawy, 2018). Since it is more stable than other organic salts, sodium butyrate (SB) is a well-known commonly used organic acid salt in aquaculture (Hoseinifar *et al.,* 2016), and due to its beneficial effects on intestinal regeneration and protection from bacterial toxins (El-Sharkawy *et al.,* 2023). The positive effects of SB have been demonstrated in many fish species (Abdel-Latif *et al.,* 2020). Notably, dietary SB promoted feed digestion, immunological activation, and growth.

Enrofloxacin (ENRO) is a synthetic antibacterial medication relevant to fluoroquinolones and is still widely used in veterinary medicine. Because of its low minimum inhibitory concentration (MIC) against several Gram-negative bacteria, ENRO has strong broad-spectrum

bactericidal effects at comparatively low concentrations (Zhou *et al.,* 2021). *Renibacterium salmoninarum*, *Aeromonas*, *Pseudomonas*, and *Vibrio* are only a few of the several bacterial fish diseases that are effectively combated by enrofloxacin (Vesna *et al.,*2009).

Since few previous studies have examined the impact of dietary SB on the performance of *O. niloticus*, this study is intended to investigate the effects of dietary SB on growth performance, antioxidant and immune capacity, biochemistry, and disease resistance to antimicrobial-resistant *P. aeruginosa* infection, and to investigate whether SB enhances the effectiveness of ENRO. To the best of our knowledge, no earlier studies have evaluated the combined effect of SB and ENRO on the disease resistance of fish, particularly *O. niloticus*. Also, SB has antioxidative and growth-promoting properties and has not been thoroughly investigated in *O. niloticus* at the transcriptome level. This will be the first report on the effects of SB dietary inclusion on the transcriptomic profile of antioxidant (CAT and SOD) and growth-related genes (GH and *IGF-1*).

MATERIALS AND METHODS

Samples of naturally infected fish

From various farms in the Kafr Elsheikh governorate, 110 samples of *O. niloticus* were collected. The fish showed fin rot, hemorrhages all over the body, skin darkness and ulceration, scale detachment, exophthalmia, ascites, hepatomegaly and splenomegaly, and distended gall bladder. Fish were taken directly to the laboratory and kept in aerated tanks that were partially filled with the pond's water.

Isolation and identification of Pseudomonas aeruginosa

Tissues from the kidney, liver, gills, heart, and spleen of the investigated fish were sampled under aseptic conditions, inoculated in tryptic soya broth (TSB), and incubated at 37°C for 24 h. A loopful of the broth was streaked onto nutrient agar and cetrimide agar supplemented with 10% glycerol and incubated at 37°C for 24-48 h. The obtained colonies were purified on cetrimide agar and morphologically and biochemically identified, according to Austin and Austin (2016).

Antibiogram sensitivity test

All samples were tested against 8 antimicrobial discs (Oxoids, UK) of amoxicillin + clavulanic acid (AMC, 30µg), gentamycin (CN, 10µg), tetracycline (TE, 30µg); doxycycline (DO, 30µg); trimethoprim-sulfamethoxazole (SXT, 25µg); norfloxacin (NOR, 10µg); ciprofloxacin (CIP, 5µg); and azithromycin (AZM, 15µg), and classified as resistant, moderately susceptible, or susceptible according to Gaur *et al.* (2023).

Molecular characterization of *P. aeruginosa* and recognition of several virulence genes

Molecular identification was conducted in the accredited laboratories of the Animal Health Research Institute, Egypt. The oligonucleotide primers (Metabion, Germany) used are listed in Table 1. Using primers targeting 956 bp of the 16S rDNA gene specific for *P. aeruginosa*, seven isolates were randomly selected for molecular identification (n=7).

The seven molecularly identified *P. aeruginosa* isolates were examined for the presence of three virulence genes, namely, *tox* A (exotoxin A), *psl* A (exopolysaccharide synthesis locus), and *opr* L (outer membrane lipoprotein L), using primers targeting 396 bp, 656 bp, and 504 bp, respectively.

Table 1: Target genes, primer sequences, cycling conditions, and amplicon sizes

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Psl A	F: TCCC TACCT CAGCAGCAAGC R: TGTT GTAGCC GTAGCGTTTCTG	656	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec	72˚C 45 sec	72˚C 10 min.	Gha daksaz *et al.* (2015)
Opr L	F: ATG GAA ATG CTG AAA TTC GGC R: CTTCTT CAG CTC GAC GCG ACG	504	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec	72˚C 45 sec	72˚C 10 min.	Xu *et al.* (2004)
Tox A	F: GACAACGCCCT CAGCATCACCAGC R: CGCT GGCCCAT TCGCTCCAGCGCT	396	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec	72˚C 45 sec	72˚C 7 min.	Matar *et al.* (2002)
P. aeruginosa 16S rDNA	F: GGGGG ATCT TCGGACCTCA R: TCCTTA GAGT GCCCACCCG	956	94˚C 5 min.	94˚C 30 sec.	52˚C 40 sec	72˚C 1 min	72˚C 10 min	Spilker et al. (2004)

Experimental diets

The items used in the experimental diets were purchased from a commercial market, and SB was obtained from AVITASA, Spain. The same basic components were used to prepare five nitrogenous diets (Table 2). The various SB concentrations were used as follows: 0, 0.25, 0.5, 0.75, and 1.0 g/kg. A survey conducted by Ng and Koh (2016), who examined the potent dosage of SB for several fish species, served as the basis for determining the optimal dose. All ingredients were carefully measured, ground into a fine powder, mixed, and pelletized using a pelletizer to produce a consistent dough. The pellets were then sun-dried for 72 h. After being sealed in polythene bags, the pellets were kept at -20°C. Using techniques from AOAC (2005), the proximate and chemical components of the experimental diets were determined (Table 2).

Experimental design and setup

The tested substance (SB) was the only source of variation among all the homogeneous experimental units. *O. niloticus* (n=150, 40.81±0.16 g) was acquired from a nearby fish farm in Kafr Elsheikh governorate, Egypt. Before the feeding trials began, the fish were fed their corresponding control diet (*ad-libitum*) and allowed to adapt to laboratory conditions for 14 days. This was done to ensure homogeneity and uniformity. Figure 1 shows the experimental design for the *O. niloticus* groups. Fish (n=30/group; 10/replicate) were haphazardly distributed to fifteen glass aquariums with a 50 L water capacity. The treatment groups were fed on an SB-supplemented diet (SB diet) with 0.25, 0.50, 0.75, or 1% conc. of feed from groups 2–5, respectively. A base diet, devoid of additives, was given to the control (CTR) group. Fish were fed at a rate of 2% of their body weight, twice a day, at 8 a.m. and 1 p.m.; in two equal parts for the duration of the eight-week feeding trial. To ensure good water quality, the culture water was partially (50%) emptied and replaced daily, and the remaining feed particles and debris were syphoned daily from the tanks. On a weekly basis (from the start of the feeding trial and throughout the experiment till the end), the three main indicators of water quality (pH, temperature, and dissolved oxygen) were recorded. Using the Standard Polarographic DO Probe-HI76407-Hanna Instruments Inc., RI, USA, the dissolved oxygen was measured. The pH was estimated using a portable pH meter. and the water temperature was determined using a mercury thermometer.

Table 2: Proximate and chemical composition of the CTR diet

Proximate composition		Chemical composition	
Components	%	Item	%
Soya bean meal	40	Moisture	10.09
Fish meal	9	Dry matter	89.91
Corn	34.1	Crude protein (CP)	31.53
Corn gluten	10	Ether extract	6.8
Wheat bran	2	Ash	5.7
Soya oil	4	Crude fiber (CF)	4.3
Vitamin and Mineral mixture [a]	0.3	NDF (Non digestible fiber)	41.58
Carboxy Meth. Cellulose	0.2	Calcium [b]	0.74
Salt	0.25	Phosphorus [b]	0.65
DL. Methionine	0.150	Lysine [b]	1.71
Total	100	Methionine [b]	0.78
		Digestible energy (DE) [c]	3363 (Kcal/kg diet)

[a] Vitamin mixture (IU or mg/kg diet); Vit D3 1000 IU, Vit A 5000 IU, menadione (k3) 2g, α−tocopherol acetate 20.1g, riboflavin (B2) 5g, thiamine (B1) 2g, cyanocobalamin (B12) 0.02g, pyridoxine (B6) 1.4, Pantothenic acid (B5) 10g, Biotin 0.2g, Folic acid 0,75g, nicotinic acid 30g

[a] Minerals mixture (mg/kg diet); ZnCO3 50; Cu (OAc) 2.H2O 4; CoCl3.6H2O 0.2; CaIO3.6H2O 0.5 Na2SeO3 0.2; MnCl2.4H2O 80; and FeC6H5O7.3H2O 40

[b] The levels of methionine, lysine, calcium, and phosphorus were estimated using a formula based on the chemical composition of feedstuff components (Jobling, 2011).

[c] Digestible energy was computed using a formula based on the chemical composition of the nutrients in feedstuffs (Jobling 2011).

The experimental setups were inspected for mortality; if any were found, they were eliminated, and their number was noted daily. Every two weeks, the fish were weighed, and an electronic scale (with a maximum capacity of 5 kg) was used to determine any weight changes. Growth variables were computed with the appropriate methods (Abu-Zahra *et al.,* 2024):

$$\text{WG (g)} = \text{FW} - \text{IW}$$

$$\text{SGR} = \frac{\ln(\text{FW}) - \ln(\text{IW})}{\text{P}} \times 100$$

$$\text{TFI (g)} = \text{Quantity of the consumed feed} \times \text{P}$$

$$\text{G \%} = \frac{\text{FW}}{\text{IW}} \times 100$$

$$\text{FCR} = \frac{\text{TFI}}{\text{WG}}$$

$$\text{FE} = \frac{\text{WG}}{\text{TFI}}$$

$$\text{PER} = \frac{\text{WG}}{\text{PI (g)}}$$

Where IW is the initial weight, FW is the final weight, WG is the weight gain, SGR is the specific growth rate, P is the number of experimental days, G% is the gain %, TFI is the total feed intake, FE is the feed efficiency, FCR is the feed conversion ratio, PER is the protein efficiency, and PI is the protein intake.

Sampling

After the eight-week feeding trial, sterile syringes coated or not coated with a saturated EDTA solution (Abu-Zahra *et al.,* 2023a, 2024) were used to draw blood samples (n=9/group) from the caudal vein of the fish that had been gently anesthetized (50 mg clove oil/L), and then the samples were divided into two groups; one consisted of EDTA tubes and used for the hematological assay and phagocytosis, and the second one underwent a 10-min room temperature centrifuged at 5000 rpm, for sera which, preserved at -18°C for subsequent immunological and biochemical tests.

Once the blood samples were collected, 3 fish per replicate (n = 9/group) were collected, rinsed with deionized water, and dissected. To estimate the expression of the genes, portions of the head, kidney and liver tissues were extracted and stored in liquid nitrogen (-80°C).

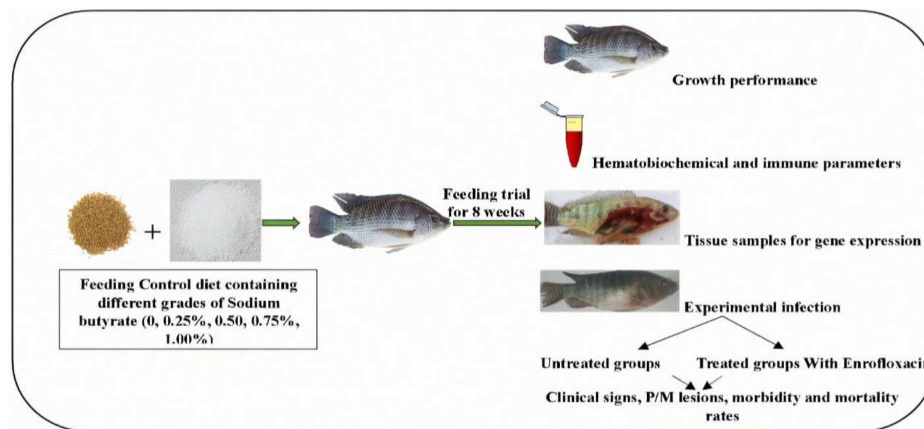

Fig. 1 Experimental design of *O. niloticus* groups fed various levels of sodium butyrate for 8 weeks

Hematobiochemical analysis

Hematological assays were carried out using Dacie and Lewis' techniques (Dacie and Lewis, 2006). Red blood cells (RBCs) were diluted with phosphate-buffered saline (pH 7.2) and counted using a Neubauer hemocytometer under a light microscope. Following blood collection, fresh blood was centrifuged in glass capillary tubes for 10 min, using a microhematocrit centrifuge to determine the hematocrit (Ht) levels. Along with Katsumata *et al.* (1982), colorimetric measurement of hemoglobin (Hb) levels was performed by monitoring the synthesis of cyanmethemoglobin. Following Dacie and Lewis (2006), the resulting blood indices of mean corpuscular hemoglobin (MCH), the mean corpuscular volume (MCV), and MCH concentration (MCHC) were computed.

Differential leukocytic counts were carried out according to Noga (2010). In brief, one drop of blood was smeared onto glass slides (n=3/replicate, 9/group). After air drying, the slides were fixed for one minute in pure methanol and for three minutes in May-Grünwald solution. To precisely detect the cell type, the slides were first incubated for one minute in PBS solution, and then stained for 10 minutes in 5% Giemsa solution. The subsequent technique was used to compute the percentage of leukocytes:

$$\text{Leukocytes \%} = \frac{\text{Absolute no of specific leukocyte in the blood}}{\text{Total leukocyte count}} \times 100$$

Glucose, total protein, albumin, liver function (ALT, AST, and alkaline phosphatase) and renal function (urea and creatinine) were estimated colorimetrically following the manufacturer's instructions (Spinreact Co., Spain). The triglyceride (TG) and cholesterol (CHO) activities were determined according to the methods of Sullivan *et al.* (1985) and Amundson and Zhou (1999), respectively.

Immune parameters

Turbidimetric methods based on the lysis of *Micrococcus lysodeikticus* (Sigma, USA), as described by Abu-Elala (2013), were used to determine the activity of serum lysozyme (LYZ). In summary, a standard suspension of 0.75 mg/mL *M. lysodeikticus* (pH 6.0) was produced using 66 mM phosphate buffer. Twenty-five microliters of serum and 1 mL of the bacterial culture were mixed, and the absorbance decrease at 450 nm was measured using a spectrophotometer. Lysozyme activity was quantified by measuring the 0.001 units/min decrease in absorbance.

Total serum immunoglobulin (TIg) and phagocytic activity and indices (PA and PI) were assessed using the procedures detailed by Siwicki and Anderson (1993) and Kawahara *et al.* (1991), respectively.

$$\text{PA} = \frac{\text{Macrophage containing yeast}}{\text{Total No. of macrophages}} \times 100$$

$$\text{PI} = \frac{\text{No. of cells phagocytised}}{\text{No. of phagocytic cells}}$$

Serum bactericidal activity

The methods of Biller-Takahashi *et al.* (2013) were used to calculate the serum bactericidal activity. At -20°C, the bacterial strain was preserved in a glycerol solution. A spectrophotometer set to 600 nm was used to adjust the bacterial density to 1×10^7 CFU/mL. By inoculating the serial dilutions in TSA (Sigma–Aldrich), viability was evaluated. Equal parts of the bacterial suspension were combined with the serum samples, and the blend was then incubated at 30°C for 24 h. The CFUs were manually counted after a 24-h growth period, and the serum bactericidal activity was defined as the ratio of CFUs in the test groups (two plates per sample) to that in the positive control group. A decreased bacterial count will correlate with improved serum bactericidal activity.

RNA extraction, cDNA synthesis, and qRT-PCR

The head, kidney and liver tissues were treated with TRIzol reagent to extract total RNA. Spectrophotometric analysis was used to measure the total RNA amount and purity at 260/280 nm. cDNA synthesis was carried out using an RT-PCR kit (Takara, Japan) as directed by the manufacturer. The primer sequences that were designed for *O. niloticus* and obtained from the NCBI gene bank for qRT-PCR of the identified genes are listed in Table (3). The expression levels of the genes were normalized against those of *β-actin*, a non regulatory reference gene. The results were standardized by eliminating variances in mRNA and cDNA quantity and quality using ΔΔ 2 Ct method (Livak and Schmittgen, 2001).

Table 3 :Primers used for the expression of antioxidant enzymes-encoding and growth-related genes

Genes	Primer sequences (5′ -3′)	Amplification size (bp)	Accession no.
β-actin	*F: AGCAAGCAGGAGTACG ATGAG *R: TGTGTGGTGTGTGGTTG TTTTG	135	XM_003443127.5
SOD	*F: GGTGCCCTGGAGCCCTA *R: ATGCGAAGTCTTCCACT GTC	377	JF801727.1
CAT	*F: TCCTGAATGAGGAGGA GCGA *R: ATCTTAGATGAGGCGGT GATG	232	JF801726.1
IGF-1	*F: TTCTCCAAAAACGAGCC TGCG *R: TCTGCTACTAACCTTGGG TGC	233	AF033796.1
GH	*F: CTGGTTGAGTCCTGGGA GTT *R: AGGTGGTTAGTCGCAT TGG	177	KT387598.1

*F: forward primer; R: reverse primer; *β-actin*: beta actin; SOD: superoxide dismutase; CAT: catalase; GH: growth hormone; *IGF-1*: insulin-like growth factor-1 precursor

Therapeutic efficacy of SB and ENRO

Following the eight-week feeding trial, each group (n=20 fish/group) was divided into two subgroups (n=10/subgroup). The fish received medicated feed containing 10 mg/kg body weight enrofloxacin. Ten milliliters of vegetable oil were mixed with enrofloxacin powder (Xi'an SENYI New Material Technology Co., China) with a purity of 99%, and the resulting mixture was uniformly sprayed onto half of the feed of both CTR and SB after being left at room temperature for one day to allow the drug to be absorbed, and the medicated feed was stored at 4°C. Following the consumption of the medicated feed by each treated group, the experimental infection was conducted. The selection of Enro was based on the results of an antibiotic sensitivity test, which revealed the sensitivity of the *P. aeruginosa* isolate used for the challenge (sample no. 6) to norfloxacin (norfloxacin and ENRO are fluoroquinolone antibiotics). When the feeding trial was over (8 weeks), all the fish were experimentally infected through intraperitoneal injection of a virulent *P. aeruginosa* strain, that was previously isolated and molecularly identified from *O. niloticus*. The chosen virulent bacterial isolate was sub-cultured in TSB and incubated for 24 h at 37°C. Using McFarland standard tubes, bacterial suspensions were adjusted after being produced (Hardi *et al.,* 2015). Each fish was injected with 0.2 ml of bacterial suspension containing $3x10^7$ CFU/ml (Ezzat *et al.,* 2018). Treatment continued for 7 consecutive days, and the mortality rate was recorded daily.

$$\text{MR\%} = \frac{\text{No. of fish mortalities}}{\text{No. of total population}} \times 100$$

$$\text{RPS\% (relative percent of survival)} = 1 - \frac{\text{\% Mortality in the treated group}}{\text{\% Mortality in the control group}} \times 100$$

Freshly dead and morbid fish were subjected to clinical and bacteriological examination. *P. aeruginosa* re-isolated and identified bacteriologically on the 5th day after challenge from the gills, liver, kidney, and spleen under complete aseptic conditions.

Statistical analysis

The means ± standard errors (SE) were used to present the results of the experiment. The Shapiro-Wilk and Bartlett tests were used to confirm the normality and homogeneity of variance prior to one-way analysis of variance (ANOVA). The differences in the treatments were evaluated employing SPSS 22.0 (SPSS® version 22, SPSS Inc.; IL, USA) using Tukey's test and a post hoc test. Significant statistical differences were considered at $P < 0.05$.

RESULTS

Bacteriological isolation and phenotypic identification of *P. aeruginosa*

Out of 120 examined fish samples, 14 isolates of *P. aeruginosa* were isolated, with a prevalence rate of 11.7%. The isolates were identified based on microbiological examination (morphological, conventional, and biochemical analysis).

Antibiotic resistance patterns of *P. aeruginosa*

The results revealed that 50% of the isolates (Table 4) were highly resistant to amoxicillin clavulanic acid and azithromycin (50%), doxycycline and trimethoprim-sulfamethoxazole (42.9%). Conversely, they were sensitive to ciprofloxacin and norfloxacin (92.9%), followed by gentamicin and tetracycline (71.4% and 57.1%, respectively).

Table 4: Incidence of phenotypic antimicrobial resistance in *P. aeruginosa*

Antimicrobial drugs	*P. aeruginosa*					
	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Amoxicillin clavulanic acid	4	28.6%	3	21.4%	7	50%
Doxycycline	8	57.1%	0	0%	6	42.9%
Tetracycline	8	57.1%	4	28.6%	2	14.3%
Gentamycin	10	71.4%	1	7.1%	3	21.4%
Azithromycin	7	50%	0	0%	7	50%
Norfloxacin	13	92.9%	1	7.1%	0	0%
Ciprofloxacin	13	92.9%	1	7.1%	0	0%
Trimethoprim-Sulfamethoxazole	8	57.1%	0	0%	6	42.9%

The results showed that *P. aeruginosa* strains (Table 5) were multidrug resistant (MDR) to azithromycin, doxycycline amoxicillin clavulanic acid and trimethoprim-sulfamethoxazole (42.85%), where the multiple antibiotic resistance (MAR) index ranged from 0.12 to 0.75.

Table 5 : Antimicrobial resistance patterns of *P. aeruginosa* strains

P. aeruginosa strains	Resistance pattern								Resistance pattern	*MAR Index	**MDR isolates	
	AMC	DO	TE	CN	AZM	NOR	CIP	COT			NO.	(%)
1	I	S	S	S	S	S	S	S	-	-	-	(6 out of 14) (42.85%)
2	R	R	I	S	R	S	S	R	AMC, DO, AZM, COT	0.5	1	
3	S	R	R	R	R	S	S	R	DO, TE, CN, AZM, COT	0.6	1	
4	R	S	S	S	R	S	S	S	AMC, AZM	0.25	-	
5	R	S	S	S	S	S	S	S	AMC	0.12	-	
6	R	R	R	I	R	I	S	R	AMC, DO, TE, CN, AZM, COT	0.75	1	
7	R	R	I	R	R	S	I	R	AMC, DO, CN, AZM, COT	0.6	1	
8	R	R	I	S	R	S	S	R	AMC, DO, AZM, COT	0.5	1	
9	R	R	I	R	R	S	S	R	AMC, DO, CN, AZM, COT	0.6	1	
10	S	S	S	S	S	S	S	S	-	-	-	
11	I	S	S	S	S	S	S	S	-	-	-	
12	S	S	S	S	S	S	S	S	-	-	-	
13	S	S	S	S	S	S	S	S	-	-	-	
14	I	S	S	S	S	S	S	S	-	-	-	

*MAR index (multiple antibiotic resistance index) = the number of antibiotics to which the isolates were resistant/the total number of antibiotics tested

**MDR: multidrug resistance to at least three antimicrobial classes

Molecular identification and detection of virulence genes of *P. aeruginosa*

The results of PCR for the specific 16S rDNA gene of *P. aeruginosa* were positive for all seven isolates (100%) with amplicon weighted 956 bp (Figure 2a). Also, the virulence genes *psl* A, *opr* L, and *tox* A, were detected at 656 bp, 504 bp, and 396 bp bands, with percentages of 57.1%, 85.7%, and 100%, respectively, in the seven examined isolates.

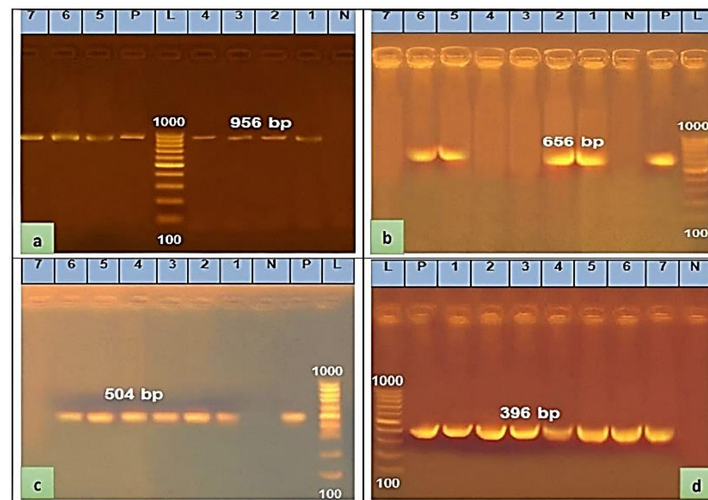

Fig. 2. Agarose gel electrophoresis (n=7 samples) of a) 16S rDNA gene amplification for the molecular identification of *P. aeruginosa* isolates with an amplicon size of 956 bp; lanes 1-7: positive 16SrDNA gene (100%). **b)** PCR amplification of the *psl* A gene (656 bp) of *P. aeruginosa*; lanes 1, 2, 5, and 6: positive *psl* A gene (57.1%). **c)** PCR amplification of the *opr*L gene (504 bp) of *P. aeruginosa*; lanes 1, 2, 3, 4, 5, and 6: positive *opr*L gene (85.7%); **d)** PCR amplification of the *tox* A gene (396 bp) of *P. aeruginosa*; lanes 1–7: positive *tox* A gene (100%). Lane (L): DNA ladder; P: positive control; N: negative control

Water quality parameters

Figure 3 displays the water quality indices that were measured during the experiment. The temperature, dissolved oxygen, and pH did not significantly vary (P >0.05) between the treatments.

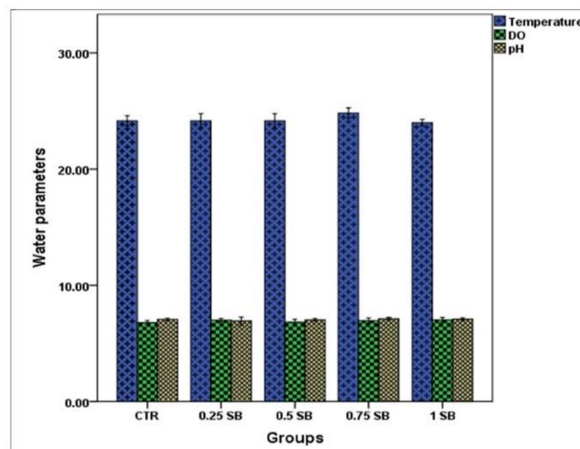

Fig. 3: Water quality indices (dissolved oxygen (DO, mg/L), temperature (°C), pH) of *O. niloticus* groups fed varying levels of sodium butyrate (SB, 0.25-1%) for 8 weeks. CTR: control fed 0% SB. The values (n= 6) are the means ± SEs

Growth performance

The growth indices of *O. niloticus* fed SB-supplemented diets for 8 weeks are shown in Table 6. Dietary SB significantly (P < 0.05) improved fish growth, as the highest FW, WG, G%, TFI, SGR, and PER were reported in groups fed 7.5-10 g SB/kg feed compared to the CTR. The fish groups fed 7.5 g SB/kg feed had the highest growth indices. The FE and survival of *O. niloticus* fed the SB-containing diets were greater than those of *O. niloticus* fed the CTR diet (Table 6). Compared with the CTR, all groups fed meals containing SB exhibited a significant reduction in FCR.

Table 6 : Growth indices of *O. niloticus* after 8 weeks of feeding with varying SB concentrations

Parameters	CTR	0.25% SB	0. 5% SB	0.75% SB	1% SB	*P*-value
IW (g)	41.09±1.10	40.74±2.31	40.72±1.74	40.70±2.90	40.80±2.08	1.000
FW (g)	54.70±1.27^{c}	60.90±1.07^{ab}	59.10±0.12^{b}	65.80±0.70^{a}	65.40±2.00^{a}	0.000
WG (g)	13.61±0.17^{c}	20.16±1.24^{ab}	18.38±1.86^{b}	25.10±2.21^{a}	24.60±0.12^{a}	0.001
G%	33.15±0.48^{c}	50.15±5.91^{b}	45.70±6.54^{b}	63.10±10.01^{a}	60.64±3.39^{a}	0.038
TFI (g)	44.86±0.16^{d}	49.33±0.10^{a}	48.94±0.21^{a}	47.28±0.24^{b}	46.00±0.10^{c}	0.000
FCR	3.30±0.03^{a}	2.47±0.16^{b}	2.72±0.27^{ab}	1.91±0.16^{bc}	1.87±0.00^{bc}	0.000
FE	0.30±0.00^{b}	0.41±0.03^{ab}	0.38±0.04^{b}	0.53±0.04^{a}	0.53±0.00^{a}	0.001
SGR	0.22±0.00^{b}	0.31±0.03^{ab}	0.29±0.03^{ab}	0.38±0.05^{a}	0.37±0.03^{a}	0.030
PER	0.43±0.01^{b}	0.64±0.04^{ab}	0.58±0.06^{b}	0.80±0.07^{a}	0.78±0.00^{a}	0.001
MR%	3.33	-	-	-	-	-

Means (n=30 fish/group) followed by different letters in the same row are significantly different at *P*<0.05. FW: final weight; IW: initial weight; G%: gain %; WG: weight gain; FCR: feed conversion ratio; TFI: total feed intake; SGR: specific growth rate; FE: feed efficiency; PER: protein efficiency ratio; MR%: mortality rate

Hematological parameters

The hematological indices of O. niloticus fed SB diets for 8 weeks are described in Table 7. The 0.75 SB group showed substantial (P < 0.05) increases in Hb values and RBC counts compared to those of the CTR group, as well as significant decreases in MCV and MCH along with substantial increases in MCHC (Table 7). However, other inclusion levels of SB did not significantly affect hematological parameters (0.25%, 0.5%, and 1%). Also, all groups showed non significant increases in the PLT and RDW. Moreover, there was a significant increase in the WBC count, especially in the 0.75% SB and 1% SB groups, compared to the CTR group. The differential leukocyte count did not significantly change among the SB-fed groups.

Table 7: Hematological parameter variations in *O. niloticus* after 8 weeks of feeding with varying SB concentrations

Parameters	CTR	0.25% SB	0. 5% SB	0.75% SB	1% SB	*P*-value
Erythrocyte indices						
RBCs (x10^6/mm^3)	2.18±0.21	2.31±0.21	2.18±0.20	3.64±0.30*	2.26±0.03	0.002
Hb (g/dl)	10.57±0.93	10.77±0.42	10.50±0.92	13.47±0.23*	10.77±0.22	0.033
Ht (%)	35.33±3.19	35.87±1.23	37.00±4.26	39.03±0.52	36.90±0.76	0.853
MCV (F1)	162.10±2.15	157.23±8.47	168.90±4.19	108.87±10.37*	163.23±3.58	0.000
MCH (pg)	48.73±1.97	47.20±2.45	48.23±0.26	37.53±3.29*	47.70±1.04	0.017
MCHC (%)	30.07±0.83	30.07±0.15	28.57±0.84	34.50±0.29*	29.27±1.22	0.002
RDW (F1)	14.90±0.50	15.13±0.97	15.90±0.87	15.80±0.15	14.00±0.76	0.383
PLT (x10^3/mm^3)	5.67±0.33	6.67±0.33	6.67±0.33	7.33±0.88	7.00±1.00	0.489
Leukocyte indices						
WBCs (x10^3/mm^3)	66.83±3.17^{b}	63.07±1.73^{b}	68.99±3.26^{ab}	79.49±2.50^{a}	78.67±0.88^{a}	0. 003
Lymphocytes %	92.17±0.15	92.20±0.15	92.17±0.28	92.63±0.22	91.97±0.57	0.668
Granulocyte %	1.13±0.07	1.30±0.10	1.17±0.15	1.17±0.03	1.50±0.23	0.339
MID %	6.70±0.10	6.50±0.15	6.67±0.20	6.20±0.21	6.53±0.37	0.570

Means (n=9 fish/group) followed by asterisks or different letters in the same row are substantially different at *P*<0.05. RBCs: red blood cell; Ht: hematocrit; Hb: hemoglobin; MCH: mean corpuscular hemoglobin; MCV: mean corpuscular volume; MCHC: MCH concentration; PLT: platelet count; RDW: red blood cell distribution width; WBCs, white blood cell; granulocytes: mostly refers to neutrophil, which are the most prevalent type and the other types (eosinophils, basophils, and mast cells); MID (mid-range): total value of the other types of WBCs that are not categorized as granulocytes or lymphocytes

Biochemical parameters

Interestingly, the uppermost total protein (TP) and globulin levels were detected in fish fed 0.75-1% SB/kg feed (0.75% SB and 1% SB); consequently, the albumin/globulin (A/G) ratio significantly decreased in the same groups. Remarkably, feeding *O. niloticus* SB-supplemented diets insignificantly affected the levels of liver enzymes (ALT, AST and AKP), kidney function (urea and creatinine), ALB, and CHO. Conversely, insignificant decreases in blood glucose and triglycerides (TG) levels were observed in fish fed 7.5-10 g SB/kg feed compared to the CTR (Table 8).

Table 8 : Variations in the biochemical parameters of *O. niloticus* after 8 weeks of feeding with varying SB concentrations

Parameters	CTR	0.25% SB	0.5% SB	0.75% SB	1% SB	*P*- value
TP (g/dl)	4.11±0.09^{c}	4.76±0.42^{bc}	5.23±0.54^{ab}	6.80±0.22^{a}	6.09±0.44^{a}	0.004
ALB (g/dl)	2.89±0.16	2.65±0.18	2.35±0.34	2.89±0.07	2.48±0.03	0.246
Globulin(g/dl)	1.22±0.08^{c}	2.11±0.47^{b}	2.89±0.54^{ab}	3.91±0.16^{a}	3.61±0.43^{a}	0.003
A/G ratio	2.40±0.29^{a}	1.44±0.45^{ab}	0.89±0.25^{b}	0.74±0.02^{b}	0.71±0.09^{b}	0.005
Glucose (mg/dl)	96.33±12.55	92.67±8.65	95.33±10.33	72.33±11.10	68.00±5.29	0.188
CHO (mg/dl)	109.00±10.97	121.67±17.32	95.00±9.07	100.33±13.54	106.67±14.26	0.690
TG (mg/dl)	326.00±19.08	215.67±29.81	299.33±25.18	246.33±24.92	260.00±28.94	0.054
Creatinine (mg/dl)	0.47±0.03	0.55±0.08	0.52±0.03	0.42±0.01	0.53±0.003	0.188
Urea (mg/dl)	13.67±1.20	15.00±0.58	12.33±0.88	12.33±0.33	14.67±0.33	0.082
ALT (IU/L)	55.00±10.69	62.33±7.80	73.00±5.51	66.33±6.69	63.00±8.66	0.635
AST (IU/L)	84.00±1.15	72.67±4.19	79.00±1.15	90.00±2.57	90.33±3.05	0.064
ALT/AST ratio	0.65±0.12	0.42±0.15	0.35±0.01	0.37±0.07	0.23±0.06	0.093
AKP (IU/L)	50.44±4.93	55.82±2.35	57.20±0.95	47.79±1.66	46.64±0.92	0.059

Means (n=9 fish/group) followed by different letters in the same row are significantly different at *P*<0.05. ALB: albumin; TP: total protein; A/G ratio: albumin/globulin ratio; TG: triglyceride; CHO: cholesterol; AST: aspartate aminotransferase: ALT: alanine aminotransferase; AKP: alkaline phosphatase

Immune parameters

In terms of immunological indices, *O. niloticus* fed SB-enriched diets exhibited dose dependent, statistically significant (P < 0.05) increases in total Ig, PA, and PI values compared to those in the CTR group (Fig. 4). The fish groups fed 7.5–10 g SB/kg feed had the highest values of these indices. Lysozyme activity did not significantly differ among the experimental groups and the CTR group (P > 0.05).

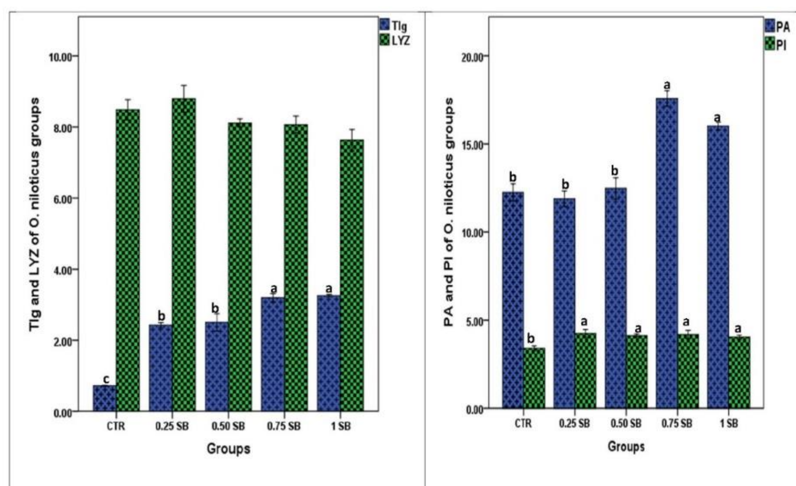

Fig. 4: Immune responses of *O. niloticus* fed varying concentrations of SB for 8 weeks. The values are the means; the error bars represent the SEMs (n=9/group). Bars with different letters are significantly different at *P*<0.05. TIg: total immunoglobulin (mg/ml); LYZ: lysozyme activity (µg/ml); PA: phagocytic activity (%); PI: phagocytic index (No)

Serum bactericidal activity

When SB was added to the fish diet, the *O. niloticus* serum bactericidal activity against *P. aeruginosa* increased significantly (P < 0.05). It reached its maximum levels in the fish groups that received 7.5 g SB/kg feed, with insignificant (P>0.05) differences among the SB-supplemented groups (Fig. 5). Following incubation with the serum of *O. niloticus* fed the CTR diet, the bacterial counts were significantly (P < 0.05) higher.

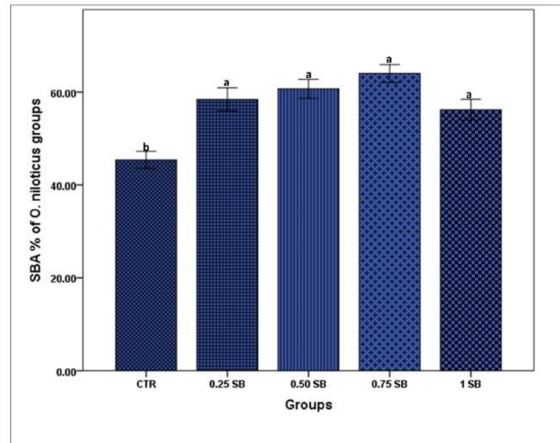

Fig. 5: Serum bactericidal activity (SBA %) of *O. niloticus* after 8 weeks of feeding with varying SB concentrations against *P. aeruginosa*. The values are the means; the error bars represent the SEMs (n=9 fish/group). Bars with different letters are significantly different at *P*<0.05

Gene expression of growth-related and antioxidant enzyme-encoding genes

Significant increases in *IGF-1* and GH gene mRNA expression verified the accelerated growth of fish fed SB-supplemented diets (Fig. 6), especially in the 0.75% SB and 1% SB groups. The fish fed the CTR diet had the lowest expression of these genes.

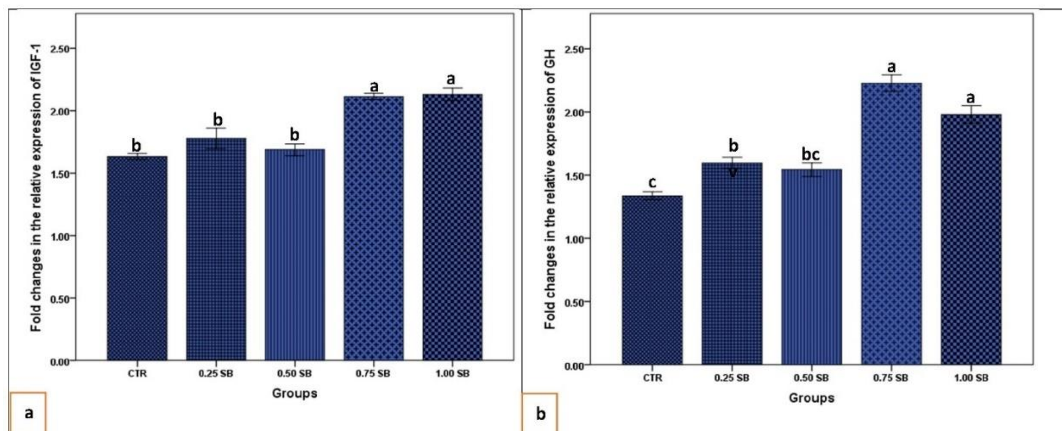

Fig. 6: Fold changes in the relative expression of growth-related genes (a: IGF-1; b: GH) in head kidney tissues of *O. niloticus* fed varying levels of SB for 8 weeks. The bars for each gene with different letters are substantially different at *P*<0.05. IGF-1: insulin-like growth factor-1; GH: growth hormone

The dietary addition of SB significantly (P <0.05) upregulated the expression of the hepatic CAT and SOD genes, mostly in the 0.75% SB group (Fig. 7).

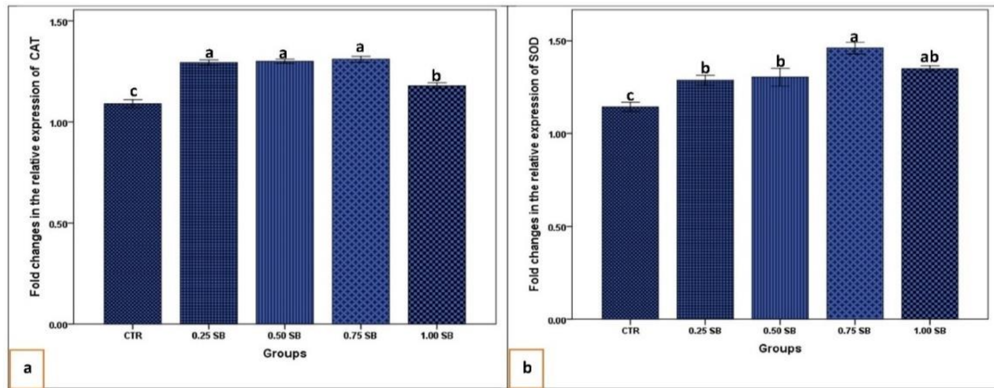

Fig. 7: Fold changes in the relative expression of antioxidant genes (a: SOD; b:CAT) in the liver tissues of *O. niloticus* fed different levels of SB for 8 weeks. The bars for each gene with different letters are significantly different at *P*<0.05. CAT: catalase; SOD: superoxide dismutase

Protective effect of SB and ENRO on *O. niloticus* infected with *P. aeruginosa*

When *O. niloticus* was subjected to ENRO treatment, there was a strong protective effect against *P. aeruginosa* infection (Table 9). By reducing the mortality rate from 50% in the CTR-infected untreated group to 30%. Ten mg/kg ENRO had protected the challenged fish against *P. aeruginosa* infection. On the other hand, SB supplementation markedly reduced the MR, which reached 10% in both conc. 0.75 SB and 1 SB groups. The combination of SB and ENRO completely protected the fish from *P. aeruginosa* infection.

Table 9: Morbidity, mortality rates, and RPS of *O. niloticus* after 8 weeks of feeding with varying SB concentrations and infected with *P*. aeruginosa for 7 days.

Groups	Fish No. (nontreated/ *Enro)	Morbidity (%)		Mortality (%)		RPS %	
		untreated	Enro	untreated	Enro	untreated	Enro
CTR	20 (10/10)	70	50	50	30	-	40
0.25 SB	20 (10/10)	50	30	30	10	40	80
0.5 SB	20 (10/10)	50	20	20	0	60	100
0.75 SB	20 (10/10)	30	10	10	0	80	100
1 SB	20 (10/10)	30	10	10	0	80	100

ENRO: fish treated with enrofloxacin, RPS%: relative percent of survival

Clinical Picture

The clinical and postmortem symptoms of the *P. aeruginosa*-infected fish are briefly represented in Table (10) as typical signs and clinical pictures were detected in the infected fish; including decreased feed intake, with disease progression, infected fish exhibited tail erosions, scale detachment, skin ulceration, and hemorrhaging (Fig. 8a–b). Some fish displayed exophthalmia and ascites. Fish swam close to or at the water surface and lost their ability to flee soon before they died.

The most notable gross internal finding noticed during the dissection of the infected fish was a darkly enlarged liver tinged with petechial hemorrhages and retention of bile in the liver. Other findings included a distended gall bladder with a greenish content, a congested posterior kidney, splenomegaly, and an empty intestine (Fig. 8c–f) *P. aeruginosa* was also reisolated in pure colonies from recently dead and moribund fish.

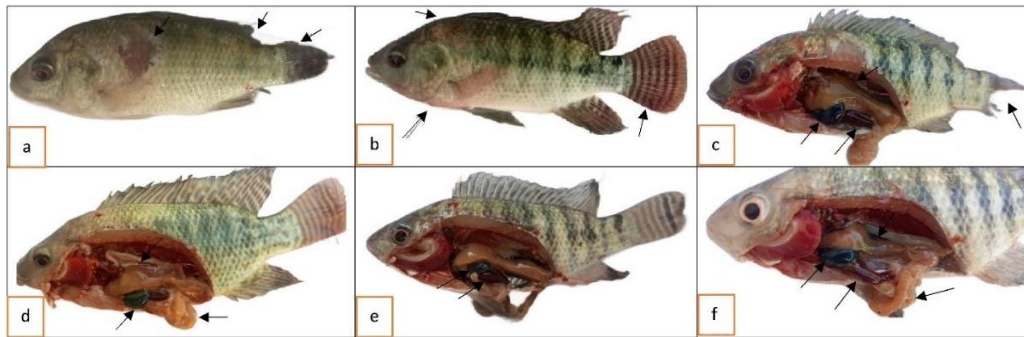

Fig. 8: Clinical and postmortem examination of *O. niloticus* in the experimental groups after *P. aeruginosa* infection. **a)** The CTR-untreated group showed slight exophthalmia, skin ulcers, and fin erosions. **b)** Fish fed 0.75% SB and treated with ENRO showed some skin and fin hemorrhages. **c)** The CTR-untreated group showed skin ulcers, tail erosions, dark enlarged hemorrhagic liver, distended gall bladder with greenish content, and splenomegaly. **d)** The CTR group treated with ENRO showed slight exophthalmia, a distended gall bladder with greenish content, an empty intestine, and retention of bile in the liver (greenish liver). **e)** Fish fed 0.75% SB and treated with ENRO showed slight splenomegaly and distension of the gallbladder. **f)** Fish fed 0.75% SB untreated group had partially empty intestines, distended gall bladders with greenish content, splenomegaly, and retention of bile in the liver.

Table 10: Clinical and postmortem symptoms of the *O. niloticus* in the experimental groups after *P. aeruginosa* infection

Item	CTR		0.25% SB		0.5% SB		0.75% SB		1% SB	
	Untreated	ENRO	Untreated	ENRO	Untreated	ENRO	Untreated	ENRO	Untreated	ENRO
Off food	+++	++	++	+	++	++	+	-	+	-
Loss of escape reflex	++	+	++	+	-	-	-	-	-	-
Tail erosions	+++	+	++	-	+	-	+	-	+	-
Scale detachment	++	+	-	-	-	-	-	-	-	-
Skin ulcers	+++	-	-	-	-	-	-	-	-	-
Skin hemorrhage	+++	++	++	+	++	+	+	-	+	-
Hemorrhagic liver	++	+	+	-	+	-	-	-	-	-
Retention of bile in the liver	+++	++	+++	++	++	+	+	+	++	+
Distended gall bladder	+++	++	++	++	+	+	+	+	++	+
Splenomegaly	+++	++	+++	++	+++	++	+	-	+	-
Congested posterior kidney	++	+	++	-	+	-	+	-	-	-
Empty intestine	+++	++	++	+	++	++	+	-	+	-

–: normal, +: weak (less than 10%), ++: moderate (10-50%), +++: severe (more than 50%)

DISCUSSION

Aquaculture is one of the global food-producing industries with the quickest rate of growth, and this trend is predicted to continue in the near future. Modern aquaculture is rapidly expanding due to a number of factors, such as the intensification of culture methods and the increasing use of prepared aquafeeds. One of the most restricting issues facing the aquaculture industry, as a result of increased farming activities, is disease outbreaks. The spread of infectious diseases caused by

different pathogens severely limits the sustainability and expansion of the worldwide aquaculture sectors, and results in considerable financial losses for farmers.

Pseudomonas species, particularly *P. aeruginosa*, are thought to be one of the primary causes of ulcerative diseases in fish populations, which can result in significant mortality rates and financial losses (Shahrokhi *et al.,* 2022). The bacteriological examination revealed 14 *P. aeruginosa* isolates (11.7%) among the 120 examined samples, which was comparable to that obtained by El-Keredy and Naena (2020), who isolated *P. aeruginosa* at 14.2% from naturally infected *O. niloticus*. Our results disagreed with those of Ali *et al.* (2023) and Shahrokhi *et al.* (2022), who reported higher incidences (47.3%) and lower incidences (5.0%), respectively. Resistance to at least three antimicrobial drugs from various categories was deemed to be the definition of multidrug resistance (MDR) (Horcajada *et al.,* 2019). In the present study, multidrug-resistant *P. aeruginosa* were isolated with a resistance percentage (42.85%) to trimethoprim-sulfamethoxazole, oxycycline, azithromycin, and amoxicillin + clavulanic acid. Similarly, Algammal *et al.* (2020) identified MDR *P. aeruginosa* strains in Egypt. Additionally, the MAR index in the current study ranged from 0.12 to 0.75, which is inconsistent with the results of Mohamed *et al.* (2023), who demonstrated that the MAR index varied from 0.4 to 0.5.

The results of PCR for the specific 16S rDNA gene of *P. aeruginosa* were positive for all seven detected isolates (100%), and these results were in agreement with those of El-Keredy and Naena (2020). Also, the detection of the virulence genes *tox* A, *opr* L and *psl* A in the current study were 100%, 85.7% and 57.1%, respectively, in the seven examined isolates. The most extracellularly toxic protein of pathogenic *P. aeruginosa* in fish, humans, and animals is the exotoxin A gene (*tox* A). However, our results were similar to those of Algammal *et al.* (2020), who detected *Tox* A in all examined isolates (100%) of *P. aeruginosa,* but they were inconsistent with those of Ali *et al.* (2023), who detected *Tox* A in 3 out of 6 examined *P. aeruginosa* isolates. The innate antibacterial resistance of *P. aeruginosa* is largely attributed to the *Opr*L gene, which codes for outer membrane lipoprotein L. This gene is a strong necrotic factor for cells. The results for the *opr*L and *psl*A genes were similar to those of Ali *et al.* (2023).

The water quality parameters (pH, temperature, and dissolved oxygen) measured in all the experimental groups were within the acceptable limits for tropical fin fish culture, as stated by Jobling (2011).

The growth indices of *O. niloticus* were improved by dietary SB, with 7.5 g/kg diet being the ideal level. The higher growth performance was verified by Boroumand *et al.* (2024), who fed *Huso huso* different levels of butyric acid. The inclusion of organic acids or their salts to the diet has been stated to enhance the growth of several fish species (Reda *et al*., 2016; El-Adawy *et al*., 2018 and Boroumand *et al.,* 2024). Butyric acid increases villus height and epithelial cell proliferation, which increases the capacity of the intestine to absorb nutrients (Baruah *et al.,* 2005). These factors might be regarded the main causes of the improvement in fish growth performance and feed efficiency. Additionally, it was discovered that organic acid compounds improved the digestibility of crude protein and dry matter, as well as the absorption of minerals, leading to improved FCR and WG (Boroumand *et al.,* 2024). It is thought that butyric acid increases the absorption of nucleotide derivatives and several vital amino acids and minerals (such as phosphorus, calcium, and zinc) and alters the gut microbiome, which may improve intestinal health (Ng *et al.,* 2009). In general, organic acidifiers act by reducing the intestinal tract pH, and have the ability to dissociate acids and produce anions in bacterial cells that inhibit the growth of Gram-negative bacteria (Freitag and Lückstädt, 2007).

Enhanced growth has been associated with similar patterns of mRNA expression of the GH and *IGF-1* genes. These findings suggested that GH and *IGF-1* gene expression was positively impacted by the 7.5 g SB/kg diet. These genes contribute to hypertrophic muscle growth in fish. Fish growth is regulated by GH, which is mediated by *IGF-1*. Furthermore, the GH/*IGF-1* axis is a crucial signal for growth. The growth performance of white leg shrimp was positively correlated with the expression of the *IGF-II* gene in a study conducted by Sharawy *et al.* (2020). In a similar study, feeding *O. niloticus* sodium butyrate granules greatly increased the expression of *IGF-1* and GH mRNA (Abdel-Tawwab *et al.,* 2021).

It is well known that the first line of defense against oxidative damage is the production of SOD and CAT, which reduce or neutralize reactive oxygen species (ROS) produced when a host is subjected to a particular stressor (Abdel-Tawwab *et al.,* 2018; Hoseinifar *et al.,* 2020). Our findings suggested an improvement in the antioxidant activity of dietary SB. These findings may also be related to the increased expression of the CAT and SOD genes in the fish liver. Antioxidant-related genes are anticipated to employed as molecular indicators in fish inflammatory response to clarify the extent to which the fish are impacted by changes in their aquatic environment, either as a stressor or a stimulus (Yilmaz, 2019 and Li *et al.,* 2023).

Furthermore, by enhancing antioxidant capacity (such as upregulating SOD and GPx activities) in mucosa cells, sodium butyrate enhanced intestinal tight junctions and supported the recovery of intestinal wound healing, as demonstrated by Ma *et al.* (2013). This may subsequently improve nutrient absorption and growth rate. Furthermore, it has been demonstrated that fish fed organic acid diets have higher levels of genes encoding antioxidant enzymes (Safari *et al.,* 2017). Earlier studies have also demonstrated that organic acids and their salts, such as citric acid in *Larimichthys crocea* (Zhang *et al.,* 2016) and sodium butyrate in grass carp (Liu *et al.,* 2016), may protect fish from the damaging effects of oxidative stress. These findings are consistent with the results of the present study. Similarly, supplementing *O. niloticus* with SB improved the activity of antioxidant enzymes (GPx, CAT, and SOD) while lowering the MDA concentration, and hence enhancing the antioxidant capacity of the fish under heat stress (Dawood *et al.,* 2020). The results of this experiment and those obtained in yellow catfish showed that SB increased the expression levels of SOD and CAT mRNAs (Zhao *et al.,* 2021). Overall, these findings suggest that SB has beneficial effects on the antioxidant capacity of *O. niloticus* and increases the activity of antioxidant enzymes.

Fish hemato-biochemistry is a vital bioindicator of the nutritional status and general health of fish. Higher WBC and RBC counts, Hb levels, TP levels, and globulin levels were detected in this study, particularly in fish fed the 7.5 g SB/kg diet. These findings matched those of Boroumand *et al.* (2024), who showed notable increases in globulin and TP. Conversely, the TG and blood glucose levels were somewhat lower in the SB-fed *O. niloticus* group than in the CTR group. It has been suggested that acidic pH, which effectively increases the release of phosphorous, calcium, iron, and copper from feed constituents (El-Adawy *et al.,* 2018), may be responsible for the improvement of hematological parameters in fish fed diets supplemented with SB. In the current study, the inclusion of SB in fish diets augmented RBCs and WBCs count, indicating that SB has immunomodulatory effects. These elevated immunological and hematological parameters support the increased antibacterial impact of SB and suggest that SB is a safe dietary additive for *O. niloticus*.

Remarkably, feeding *O. niloticus* SB-supplemented diets did not significantly affect the levels of liver enzymes (ALT, ALT and AKP), kidney function (urea and creatinine), ALB, or CHO. According to these findings, dietary SB may have hepatic

and renal protective effects. Consistent with these results, a previous study demonstrated that feeding fish, such as *O. niloticus*, diets supplemented with organic acids or their salts can lower the serum levels of AST, ALT, and AKP, suggesting better liver function (Hoseini *et al.,* 2023). Boroumand *et al.* (2024) reported a significant reduction in liver enzymes (ALT, AST, AKP, LDH) in *Huso huso,* fed diets supplemented with butyric acid. In the present study, dietary SB insignificantly reduced serum glucose and TG. Oral intake of acidifiers such as butyrate can decrease lipid accumulation by increasing lipolysis and reducing lipogenesis in many tissues (Jiao *et al.,* 2018).

Immune responses are among the beneficial impacts of feeding fish SB on their physiology. In comparison with those in the CTR group, the serum levels of TP, globulin, PA, and PI; lysosomal activity; and total immunoglobulin increased significantly in the SB group. The serum protein and immunological parameter values were highest in the 0.75 SB group. Consistent with these findings, earlier studies have demonstrated that supplementing diets with organic acids, such as sodium propionate and butyric acid, may improve TP, ALB, and lysozyme activity in common carp and *Huso huso* (Safari *et al.,* 2017 and Boroumand *et al.,* 2024). Fish immune systems may be affected by acidifiers, which could change cellular and molecular signaling pathways (Safari *et al.,* 2017). According to earlier studies, adding acidifiers or their salts can increase immune gene expression while motivating the proliferation of immune cells (Safari *et al.,* 2017). The increase in serum total Ig concentrations and phagocytic activity in response to dietary SB inclusion can be associated with an increase in leucocyte counts, suggesting that these groups have better immunological competence. Similar results were reported by Abdel-Mohsen *et al.* (2018), who reported that feeding *Dicentrarchus labrax* dietary butyrate (1 to 3 g/kg) noticeably augmented the percentage of monocytes as well as the RBC and WBC counts. Additionally, feeding *O. niloticus* diets supplemented with blends of malic acid (5 and 10 g/kg) and 1.1 × 105 CFU/g *Bacillus subtilis* significantly enhanced Ht, Hb, RBC, and WBC (Hassaan *et al.,* 2017). The impact of acidifiers on fish health in general can be influenced by a number of factors, including fish species, health status, the conditions of the experimental trial, acidifiers type and level (Hoseinifar *et al.,* 2017).

Consistent with our results, earlier studies also reported that the dietary inclusion of organic acids and their salts, malic acid (Hassaan *et al.,* 2017), butyric acid (Boroumand *et al.,* 2024), sodium propionate (Safari *et al.,* 2017), and sodium butyrate (Abdel-Mohsen *et al.,* 2018), increased the serum total protein and total Ig in several cultured fish species, which resulted in significant enhancements in immune responses. Additionally, dietary acidifiers can modify the immune response by binding to GPR43, a G protein-coupled receptor primarily expressed on inflammatory and innate immune response cells (Maslowski and Mackay, 2010). The present study suggested a possible correlation between the improved immunological competence status of fish and the elevated serum protein, WBC count, phagocytic activity, and total Ig concentrations in fish fed a 7.5 or 10 g SB/kg diet.

The experimental challenge revealed that the mortality and morbidity rates reduced in the groups fed diets supplemented with SB compared with those in the CTR group, and the greatest survival was detected in the groups fed diets supplemented with 0.75% and 1% SB. These findings were consistent with those of Sikandar *et al.* (2017), who stated that butyrate contributes to enhancing immunity, decreasing the pH in the gastrointestinal tract, and suppressing the growth of pathogenic bacteria. Butyrate and its derivatives might be considered promising antibacterial and immunomodulatory agents for the treatment of bacterial infections without antibiotics (Du *et al.,* 2021). Furthermore, an earlier study has shown that

dietary inclusion of butyrate diminishes *Salmonella enteritidis* infection in chicken broilers by lowering SE adhesion and invasion of macrophages and enterocytes (Sikandar *et al.,* 2017). This decrease in bacterial adhesion and invasion was most likely caused by changes in the expression of the genes involved in attachment or invasion rather than a decrease in the number of bacteria (Sikandar *et al.,* 2017). The anti-inflammatory potential of butyrate has also been described by Bedford and Gong (2018). This reduction in the expression of proinflammatory cytokines, such as interleukin-1β (*Il-1β*), tumor necrosis factor-α (*TNF-α*), interferon-gamma (*IFN-g*), Il8, and Il6, may be the mode of action. Through the decrease in mortality and morbidity rates, our results demonstrated a significant increase in the antibacterial effects of dietary SB when it was administered either alone or in combination with ENRO. The combination of SB and ENRO had the greatest antibacterial effect on the fish, indicating a potential synergistic effect between the two compounds against infections. This can be explained by the fact that SB increases ENRO bioavailability and adds an antimicrobial effect to ENRO.

Earlier studies on acidifiers and their salts against *A. sobria* in *O. niloticus* (Reda *et al.,* 2016), *A. hydrophila* in *O. niloticus* (El-Adawy *et al.,* 2018), and total bacterial counts in the gut of red hybrid tilapia (Ng *et al.,* 2009) revealed comparable *in vitro* and *in vivo* antibacterial activities, which is consistent with our findings. The ability of acidifiers to reduce pH may be the cause of their antibacterial activity (Ng and Koh, 2016). Most intestinal pathogenic bacteria favor a pH of 7 or somewhat more. On the other hand, beneficial microorganisms survive in an acidic pH range of 5.8–6.2 and actively oppose pathogens. Using acidifiers to decrease the pH of the gastrointestinal tract in *O. niloticus* selectively reduces the population of hazardous microorganisms, particularly gram-negative microorganisms, while promoting the growth of beneficial microorganisms (Ng *et al.,* 2009). The lipophilic property of SB, which permits penetration of the cell membrane of Gram-negative bacteria and causes acidification of their cytoplasm, disruption of metabolism, and DNA damage, is another potential mechanism by which the bacterium is killed. Rather than reducing fish gut pH, the majority of researchers believe that this method is the primary mechanism of action of organic acids (Ng and Koh, 2016).

CONCLUSION

According to this study, organic acid salts (such as SB) can effectively increase fish growth and improve their health. As such, they can function as even more effective and ideal substitutes for antibiotics, and they also can work together synergistically to provide complete protection against infection. Therefore, it is recommended that fish producers include them in fish diets. Further studies should focus on obtaining certain active herbal compounds with organic acid properties so that fish farmers can easily obtain them. Given the commercial importance of *O. niloticus*, the addition of SB to their diet may improve their growth and fortify their antioxidant and immune capacity without any adverse effects on the environment or fish's health. The results of this research may aid in the development of more efficient and ecofriendly aquaculture practices for *O. niloticus* and other important fish species.

Authorship contribution statement

Nagwa I.S. Abu Zahra: Methodology, Formal analysis, writing – original draft, writing – review and editing, Resources, Supervision, Investigation, Visualization, **Safaa M. Shabana:** Ideas, writing– original draft, Formulation of overarching research goals and aims, Writing – review. **Shaimaa Elbaz:** Resources, Investigation, Visualization, Validation, Writing – review; **Mona E. Abas:** Ideas, Formulation of overarching research goals and aims, Project administration, Writing – review.

Data availability

The authors confirm that the data supporting the findings of this study are available within the manuscript, figures, and tables.

Declarations

Animal welfare and ethics statement

The study methodology, protocols, and animal care all followed the relevant guidelines and regulations of the Animal Health Research Institute, Agriculture Research Center, Giza, Egypt (Code No. 83429/2022) and European Union directive 2010/63UE.

The reporting of this study complies with the ARRIVE guidelines (https://arriveguidelines .org). None of the authors' investigations involving human subjects are included in this paper.

Consent to participate

Not applicable

Consent for publication

Not applicable

Conflict of interest disclosure

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

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آثار زبدات الصوديوم (Sodium butyrate) الغذائية على المؤشرات البيولوجية، والتعبير الجيني، ومقاومة أسماك البلطي النيلي لعدوى Pseudomonas aeruginosa المقاومة لمضادات الميكروبات

صفاء محمد شبانة ، شيماء الباز ، منى عليوة عباس ، نجوى إبراهيم سعد أبو زهرة

Email: nagwaabuzahra09@gmail.com Assiut University web-site: www.aun.edu.eg

أصبح استخدام المنشطات المناعية والأحماض العضوية وأملاحها كبدائل للمضادات الحيوية ومحفزات النمو أمرًا مهمًا بسبب ظهور مقاومة مضادات الميكروبات ويستخدم حاليًا في أعلاف الماشية وتربية الأحياء المائية. لذلك، تم تصميم هذه الدراسة لتقييم الخصائص التحسينية لزبدات الصوديوم (SB) على مؤشرات نمو سمك البلطي النيلي *Oreochromis niloticus*، والاستجابات المناعية، ومضادات الأكسدة، والتعبير الجيني المرتبط بالنمو، ومقاومتها ضد عدوى سيدموناس اريجنوزا *Pseudomonas aeruginosa*.

تم توزيع خمس مجموعات من أسماك البلطي *O. niloticus* (٣٠ سمكة لكل مجموعة)، وتم تغذية المجموعة الضابطة الأولى (CTR) على علف أساسي بدون أي إضافات، بينما تم تغذية المجموعات الثانية إلى الخامسة على علف مكمل SB بتركيزات ٠,٢٥، ٠,٥٠، ٠,٧٥، و١٪ على التوالي. وبعد ٨ أسابيع، تم تقسيم داخلي للمجموعات إلى مجموعتين؛ المجموعة المعالجة بالنظام الغذائي بالإنروفلوكساسين والمجموعة غير المعالجة به. بعد استهلاك العلف الدوائي، تم استحداث اصابة جميع المجموعات ببكتيريا *P. aeruginosa*. أظهرت الأسماك التي تتغذى على SB زيادة في التعبير الجيني المرتبط بالنمو (GH و*IGF-1*)، وجينات الكاتالايز، وفوق أكسيد ديسموتيز Superoxide dismutase المضادة للأكسدة. تم تحسين المعاملات الدموية، ومؤشرات المناعة، والتأثير المميت للبكتيريا، والبلعمة.

وبعد عدوى *P.* aeruginosa، تم تسجيل معدل نفوق مرتفع في المجموعة الضابطة غير المعالجة المصابة (٥٠٪)، في حين انخفض بشكل ملحوظ (١٠٪) في ٠,٧٥٪ و١٪ SB المجموعات المعالجة. وقد لوحظ التأثير التآزري المعزز لـ SB بنسبة ٠,٧٥% و١% مع ENRO. لذلك، يوصى باستخدام مكملات النظام الغذائي التي تحتوي على زبدات الصوديوم، خاصة بنسبة ٠,٧٥٪، إما بمفردها كمحسن للنمو أو بالاشتراك مع ENRO، لحماية الأسماك تمامًا من عدوى *P. aeruginosa*.