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Improving fish health and their resistance to diseases through balanced feeding programs

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ABSTRACT

This study was designed to evaluate the effect of ***Spirulina & Moringa*** supplementation on mixed bacteria (***Pseudomonas*spp & *E.coli***), mixed parasite (one species for each trematode ***Allocredium* sp.** & nematode ***Camallanus* sp)** and challenged in ***Clarias gariepinus***. From naturally infected 100 *Clarias gariepinus* fish, (55%) helminthes parasites were be recorded while by bacteriological examination (17%) *E.coli* and (23%)*Pseudomonas* spp. were be isolated. Studying genotypic attributes of bacterial isolates by PCR revealed that, *16sr*RNAgene was detected in (100%) of studied ***Pseudomonas***spp., mean while, *tox*A virulence gene was detected in (62.5%). On otherwise, (100%) of strains were contained *pho*A gene which consider as general marker of **E. *coli*** while only (62.5%) of *E.coli* strains were positive for *tsh* virulence gene. We observed that the dietary inclusion of ***Spirulina, Moringa*** significant improved the level of (ALT), (AST). Moreover, lower the cholesterol & triglycerides level compared with control group. Also, there is increase in Hemoglobin level, lymphocytes, total protein & albumin, immunity evidenced by increase phagocytic percent and phagocytic index. The present study showed that ***Spirulina, Moringa*** have a good potential for use as fish meal.

INTRODUCTION

Through the past decades, the world fishery production decreased and the human consumption for aquatic product increased **(FAO, 2020)**. The need for disease resistance, feed efficiency and growth of cultured organism is substantial for various sectors of this industry **(EL-Haroun, 2007)**. Fish is considered as an important and valuable source of protein, omega-3, and vitamins so, the demand for fish is on arise **(Karrari *et al.*, 2012 and Gopalakrishnan *et al.*, 2016)**.

The moringa plant (*Moringa oleifera*) has the object of research due to its multiple uses and well-known bactericidal and parasitic potential **(Suarez *et al.*, 2005).** The dietary inclusion of moringa leaf powder at 5 % significantly improved growth performance, an increase in final body weight and improved feed conversion ratio. Moreover, lower cholesterol and triglycerides were reported in fish fed **(El-Kassas *et al.*, 2020).** Moringa leaf causes increase the immunity of the fish to fight bacterial infections and parasitic diseases **(Bbole *et al.*, 2016).**

Antibiotic resistance of various bacterial pathogens in fish aquaculture attracted more attention in last years and imposes detection of cautious alternatives, including immunostimulants, medicinal plants, natural extracts and vaccination protocols (**Hoseinifar *et al.*, 2020)** from those were ***Spirulina*** which represents one of the most promising micro-organisms that produce a potential therapeutic agent, owing to its extracellular and intracellular metabolites that have anti-fungal (**MacMillan *et al.*, 2002)** and antibacterial activities **(Kumar *et al.*, 2011).** For these reasons, *spirulina* and ***Moringa*** considered as a good source of natural compounds to treat, prevent and/or control fish diseases better than using antibiotics in aquatic feed.

The effect of *Spirulina* inclusion in diets for ***Clarias gariepinus***, increased antioxidant protective capacities. It also affected humoral immunity parameters as well as don't have negative effect on fish growth **(Amer, 2016).** ***Spirulina*** has been speculated with modulation of the host immune system **(Hironobu *et al.*, 2006)**. The fluctuation in the number of bacteriain ***Spirulina*** treated fish after an artificial challenge with ***Pseudomonas*** and ***E.coli.*** They found that the bacteria numbers were lower in the liver and kidneys of carp treated with ***Spirulina*** than the control group the increased resistance against bacteria infection **(Watanuki *et al.* 2006).**

PCR is the most sensitive of the existing rapid methods to detect virulence genes of pathogenic bacteria in clinical specimens, which dependent on target genes, primer sequences, PCR techniques, DNA extraction procedures and PCR product detection methods **(Yamamoto, 2002).**

This work aimed to study parasitological, bacteriological and hematological effect of addition ***Moringa*** and ***Spirulina*** to fish meal on experimental fish infected with parasites and bacteria.

Material and Methods

I. Collecting samples:

One hundred of ***Clarias gariepinus*** fish we recollected from different localities of sharkia governorate during the period of 15 June 2021 to 15 July 2021. Samples we retransferred to Animal Health Research Institute-Zagazig laboratories, in Aquaculture Diseases Unit. Samples examined for presence of helminth parasites (***Allocredium* sp.**, ***Camallanus* sp. and *Polyonchobothrium*)** and bacteria **(*Pseudomonas*spp.** and **E. *coli***).

II. Experimental design:

Forty fish were selected from the naturally infected fish (15 fish single infected with parasites + 15 fish single infected with bacteria+10 fish mixed infected with both bacteria and parasite), and distributed equally into 4 groups and treated as following in **Table (1).**

Table (1): Experimental design of different groups

Group	No. of infected fish	Type of infection	Treatment/Ratio
G 1	5	Bacterial infection	Spirulina and Fish meal/ (1:3)
	5	Parasitic infection	
G 2	5	Bacterial infection	Moringa and Fish meal/ (1:3)
	5	Parasitic infection	
G 3	5	Bacterial infection	Spirulina & Moringa and Fish meal/ (1:1:2)
	5	Parasitic infection	
G 4	10	Mixed infection	Control

III. Parasitological Examination:

Using a moderate squeezing motion with the thumb and forefinger pressure was applied to the abdomen, starting in line at the front of the pelvic fins and finishing at the anus. Feces collected in a petri dish according to **(Scott and Pamela, 2015 and Hoffman, 2019)** andWe collected the most infected fish with helminth parasites to its specific groups in the experiments.

After two weeks from experimental we repeated the previous technique to examine the fish for helminthes parasites. After four weeks, an internal examination was carried to each fish for helminths infestations. The examination involved the skin, fins, gills, buccal cavity and alimentary tract of fish, which made in a petri dish containing saline solution then opened, scraped and examined under dissecting micro-scope (**Lucky,1977)**, to examine the effect of ***Spirulina* & *Moringa*.** The parasites were identified by light-microscope by using the standard keys in the literature according to **(Bray *et al.*, 2005)** for trematodes, and **(Vernon, 2006)** for nematodes.

IV. Bacteriological examination:

Under aseptic conditions, swabs from skin and gills were taken and inoculated into TSB (**Difco**) then incubated at 37°C for 24 hours, then, a loop full of broth was streaked onto MacConkey's agar plates for ***E.coli*** isolation and *Pseudomonas* (citrate) agarplates for isolation of ***Pseudomonas*** spp., and incubated at 37° C for 24-48 hours. Identification of the isolates based on colonial morphology, biochemical characters and conformation of isolates by conventional PCR technique **(Austin and Austin, 2016).** Same procedures were applied to the experimental fish in different groups after 2 weeks. After 4 weeks, internal organs of experimental fish were dissected and examined for bacterial isolation.

V. In vitro antibacterial activity:

The antibacterial activity of ***Spirulina*** and ***Moringa*** extracts was determined against (4) ***E.coli*** and (4) ***Pseudomonas*** spp. randomly selected isolates, using agar disk Diffusion method described by **(Bauer *et al*., 1966).** Nutrient agar was inoculated with the ***E.coli*** and ***Pseudomonas***, and wells of 6mm were punched in the agar and filled with the extracts and control wells with no extract in the same plate. The plates were incubated at 37°C for 24 hours and the antimicrobial activity was assessed by measuring the diameter of the zone of inhibition. Strains surrounded by≥13 mm halo zone were considered to be susceptible **(Vieira *et al*., 2010).**

VI. Conventional PCR technique:

Extraction of DNA:

DNA extraction was extracted using commercially available kit, QIAamp DNA Mini kit (Qiagen, Germany, GmbH). Briefly, 200 µl of sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's instructions. Nucleic acid was eluted with 100 µl of elution buffer.

Amplification of DNA:

Pseudomonase *genes (16sr*RNA and *tox*A), were amplified according to **(Matar *et al*., 2002 and Spilker *et al*., 2004),** while ***E.coli*** genes (*pho*A and *tsh* gene) were amplified according to **(Delicato *et al*., 2003 and Hu *et al.,* 2011).**

Primers were supplied from Metabion **(Germany)** as listed in **Table (2),** which utilized in 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

Electrophoresis:

Using PCR for separating the product by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/ cm. For gel analysis, 20 µl of the uniplex PCR products and 40 µl of the duplex PCR products were loaded in each gel slot. gel pilot 100 bp and 100 bp plus DNA ladders (Qiagen, Germany, GmbH) and gene ruler 100 bp ladder (Fermentas, Germany) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) **Table (3).**

Table (2): Primers' sequences, target genes and amplicon sizes:

Test Target	Target gene	Primers' sequences (5´-3´)	Amplified segment (bp)
Pseudomonas	**16*S r*RNA**	GACGGGTGAGTAATGCCTA CACTGGTGTTCCTTCCTATA	**618**
	***tox*A**	GACAACGCCCTCAGCATCACCAGC CGCTGGCCCATTCGCTCCAGCGCT	**396**
E.coli	***tsh***	GGT GGT GCA CTG GAG TGG AGT CCA GCG TGA TAG TGG	**620**
	***pho*A**	CGATTCTGGAAATGGCAAAAG CGTGATCAGCGGTGACTATGAC	**720**

Table (3): PCR protocol for amplification conditions of PCR:

Bacteria	Target gene	Prim. Den. C˚/min	Amplification (35 cycles) Sec. den. C˚/sec	Ann. C˚/sec	Ext. C˚/sec	Final extension C˚/min
E. coli	*tsh*	94/ 5	94/30	54/40	72/45	72/10
	phoA	94/5	94/30	55/40	72/45	72/10
Pseudomonas	*16S rRNA*	95/5	94/30	50/40	72/45	72/10
	toxA	95/5	94/30	55/40	72/40	72/10

VII. Determination of haematoogical parameters:

Blood samples were collected at the end of the experiment, following the procedures by **(Wedemeyer and Yasutake, 1977)** at termination of the experiment, 2ml of blood was collected using a syringe and needle by piercing the vein located on the caudal peduncle. Each blood sample was placed separately in each sterile vacuum tube, containing Ethylene Diamine Tetra acetic acid (EDTA) as an anticoagulant and sent to the laboratory for hematological analysis. The following hematological parameters were analyzed; Red Blood Cell count (RBCs), Hemoglobin Concentration (Hb), Hematocrit (Hct), White Blood Cell count (WBCs), Mean Cell hemoglobin concentration (MCHC), Mean cell Volume (MCV), and Mean Cell hemoglobin (MCH).Blood film was prepared according to the method described by (**Lucky, 1977)**. Differential leukocytic count was calculated according to **Schalm (1986)**. Hemoglobin concentration **Hb** was calculated according to the formula mentioned by (**Dacie and lewis, 1975).** Red blood cell (**RBCs**) and White blood cell (**WBCs**) counts were counted by hemocytometer according to **Stoskopf (1993).**

Serum biochemical samples collection and immune response assay:-

Blood samples were used for serum separation by centrifugation after clotting (at 4 °C for 60 min). The serum was kept at –20 °C until further biochemical analysis. Serum samples were collected for determination of the concentration of total protein (**TP**) and albumin (**Alb**) were measured by colorimetric methods according to **(Weichsellbaum, 1946** and **Doumas *et al.*, 1971)**, respectively. While, globulin (**Glo**) concentrations were determined by subtracting the concentration of total protein from albumin concentration. The activity of the liver enzymes, Aspartate Amino Transaminase (**AST**) and Alanine Amino Transaminase (**ALT**) was determined according to **(Reitman and Frankel 1957)**. Serum total cholesterol, triglycerides, high density lipoprotein (HDL-c) and low-density lipoprotein (LDL-c) were measured using commercial kits (Bio diagnostic Co., Egypt) according to **(Allain *et al.* 1974).**

Cellular immune response:

Phagocytic activity and phagocytic index:

Peripheral blood mononuclear cells (PBMC) were isolated according to the method described by **(Goddeeris *et al*., 1986)**.In brief,2 ml heparin zed blood was harvested from each calf and then diluting the blood 1:2 in heparinized (10 I.U. heparin/ml) PBS. The diluted blood was overlaid on the surface of lymphocyte separation medium Ficoll-Hypaque (1:1) in sterile centrifuge tubes, and centrifuged for 15 min at 80 xg. The PBMC was aspirated by 5 ml pipette from the interphase layer, diluted with an equal volume of heparinized PBS and pelleted by centrifugation at 800 xg for 15 min. The PBMC was washed three times (3x) in heparinized PBS by repeated pipetting with 10 ml heparinized PBS, resuspension and centrifugation. The cells were resuspended in RPMI- 1640 of pH 6.8 and containing 20% heat-inactivated AS, penicillin (100 IU/ml), streptomycin (100µg/ml) and fungizone (250 µg /ml) at a concentration of 4-5×10^6 cells/ml.

Phagocytic Assay:

To assess the cell phagocytic activity, 0.25 ml of adjusted viable leukocytes suspension was added to 0.25 ml heat inactivated *C. albicans* in a sterile plastic tubes. The tubes were incubated at 37ºC for 30 minutes in a humidified CO_2 incubator. Subsequently, the tubes were centrifuged at 2500 rpm for 5 minutes and the supernatant was removed with Pasteur pipette leaving a drop into which the sediment was re-suspended. Smears were prepared from the deposit, dried in air and stained with Leishman`s stain.

Evaluation of phagocytic activity:

Under a light microscope using oil immersion lens, a total number of 100 phagocytic cells were counted randomly in about ten microscopic fields. The number of ingested yeast cells in each individual phagocytes were determined to calculate the phagocytic cell activity in each of the tested group. The phagocytic-activity is considered as the percentage of phagocytic cells by microscope field. The phagocytic-index is the mean number of *C.albicans*, ingested by one phagocytic cell.

Statistical analysis:

Statistical analysis was performed using the statistical software package SPSS (Version 18.0; SPSS Inc., Chicago, IL). The significance of differences between the experimental groups were evaluated by one-way analysis of variance (ANOVA). If one-way ANOVA indicated a significant difference, the differences between individual groups were estimated using post hoc Fisher's least significant difference (LSD) test. Results were expressed as the mean ± standard error of mean. A *P*- value of less than 0.05 was considered significant **(Kinnear and Gray, 2006).**

Results

Parasitological finding:

In the present study, 55 ***Clarias gariepinus*** fish were highly infected by helminthes parasites (55%)(40 single infected with parasite only+15 mixed infection with parasite and bacteria), one species of trematode (*Allocredium* sp.) was isolated as shown in **Figure (1),** one species nematode (*Camallanus* sp.) was isolated as shown in **Figure (2)** but the examination failed to detect cestode species (*Polyonchobothrium*) **Table (4).**

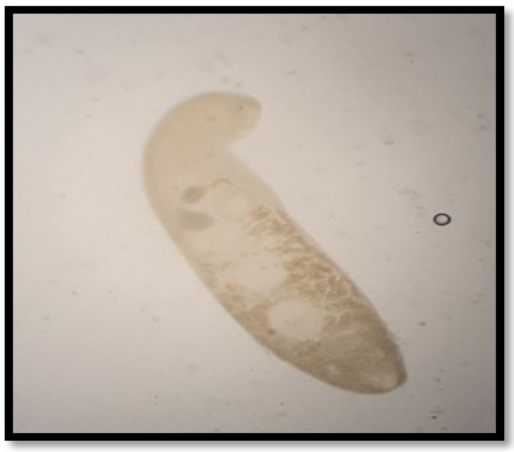

Figure (1) *Allocredium* sp

Figure (2) *Camallanus* sp.

After two weeks of experimental fish, **Group 1**, which was fed by **Spirulina** and fish meal, a little decrease in infection rate by which (4/5) of experimental fish only was infected by gastrointestinal helminth parasites. While in **Group 2,** which was fed by **Moringa** and fish meal, there was no change in infection rate. In **Group 3,** which was fed by **Spirulina & Moringa** and fish meal, (2/5) of experimental fish only infected by gastrointestinal helminth parasites. In **Control group,** there was no change in number of infected fish, **Table (4).**

After four weeks in **Group 1**, which was fed by **Spirulina** and fish meal, a decrease in infection rate by which (2/5)of experimental fish only was infected by gastrointestinal helminth parasites. While in **Group 2,** which was fed by **Moringa** and fish meal, a decrease in infection rate by which (2/5)of experimental fish was infected by gastrointestinal helminth parasites. In **Group 3,** which was fed by **Spirulina & Moringa** and fish meal, there was no infection by gastrointestinal helminth parasites. In **Control group,** there was no change in number of infected fish, **Table (4).**

Bacteriological examination :

The bacteriological examination revealed that 40 ***Clarias gariepinus*** were highly infected with bacteria (25 single infection with bacteria only +15 mixed infection with parasite and bacteria), (17%) of the isolates were positive for ***E.coli*** while (23%) were positive for ***Pseudomonas*** *spp*., **Table (4).**

After 2 weeks of the experimental fish , the number of isolated ***E.coli*** were (3, 4, 2 and 7) isolates while ***pseudomonas*****spp**. was isolated as (2, 3, 1 and 5) isolates from gr1, gr2, gr3 and gr4, respectively, while After 4 weeks the lowest number of isolated bacteria was in gr3 in which only one ***E.coli*** isolate was identified, **Table(6).**

Table (4): Distribution of parasitic and bacteria in natural fish:

Type of infection		No. of isolates	Prevalence rate
Single infection	Trematode and cestode Parasite	40/100	40%
	Bacteria	25/100	25%
Mixed infection	Parasite+ Bacteria	15/100	15%

Table (5): Distribution of helminth parasites during the experiment:

Groups	Total no. of infected fish	
	After 2 weeks	After 4 weeks
(Gr1)Spirulina + Fish meal (n=5)	4/5	2/5
(Gr2) Moringa + Fish meal (n=5)	5/5	2/5
(Gr3) Spirulina + Moringa + Fish meal (n=5)	2/5	0/5
(Gr4) Control (n=10)	10/10	10/10

Table (6): Distribution of bacteria during the experiment:

Groups	Total no. of infected fish			
	After 2 weeks (Skin and gills)		After 4 weeks (Liver and kidney)	
	E.coli	*Pseudomonas*	*E.coli*	*Pseudomonas*
(Gr1) Spirulina + Fish meal (n=5)	3/5	2/5	2/5	1/5
(Gr2) Moringa + Fish meal (n=5)	4/5	3/5	3/5	3/5
(Gr3) Spirulina + Moringa + Fish meal (n=5)	2/5	1/5	1/5	0/5
(Gr4) Control (n=10)	7/10	5/10	6/10	5/10

In vitro antibacterial activity:

Zones of inhibition caused by spirulina, moringa and the blind of both extracts against ***E.coli*** and ***Pseudomonas*** isolates were illustrated in **Table (7)** and the results showed that zones of inhibition ranged from 15mm and 19mm with ***Spirulina*** extract, and from12mm to 16mm with ***Moringa*** extract, while it reaches 20mm with the blend of both extracts.

Table (7): In vitro antibacterial activity:

Type of treatment	**Zone of inhibition (mm)**							
	E.coli				***Pseudomonas spp.***			
Bacterial isolate no.	**E1**	**E2**	**E3**	**E4**	**P1**	**P2**	**P3**	**P4**
Spirulina	17	15	17	19	18	18	19	17
Moringa	12	14	16	17	14	16	15	15
Spirulina + Moringa	19	19	21	20	18	17	19	18

Investigation of genotypic virulence attributes of isolates by PCR:

Eight isolates with mixed *E.coli* and *pseudomonase* infection were selected from (random and experimental groups isolates) and tested by PCR to confirm the isolated strains, as shown in, **Table (8)** PCR targeted the relevant species conserved genes (*16sr*RNA, *pho*A) as demonstrated in all isolates (100%),as shown in **Figures (3and 4).** The results revealed that, (62.5%) of the tested *pseudomonase* and *E.coli* isolates were positive for *tox*A and *tsh* virulence genes, respectively**,** as shown in **Figures (5and 6).**

Table (8): PCR amplifications results of different virulence genes of isolates*:*

Isolates no.	**Source of samples**	**Conserved genes**		**Virulence genes**	
		***16sr*RNA**	***pho*A**	***tox*A**	***tsh***
1	Random samples	+	+	-	-
2	Random samples	+	+	+	-
3	Random samples	+	+	-	-
4	Random samples	+	+	-	+
5	Exp.G1	+	+	+	+
6	Exp.G2	+	+	+	+
7	Exp.G3	+	+	+	+
8	Exp.G4	+	+	+	+
Total (%)		8/8(100%)	8/8(100%)	5/8(62.5%)	5/8(100%)

16SrRNA*: conserved genus-specific gene, *tox*A gene:** pseudomonas exotoxin A gene, ***pho*A*: Alkaline phosphatase gene **,** ***tsh* gene** :Temperature-sensitive hemagglutinin, **Exp.G**: experimental group.

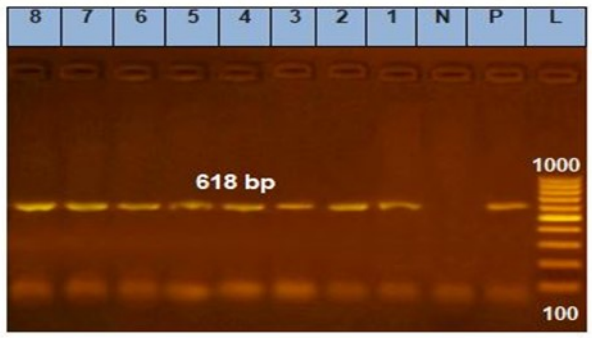

Figure (3):16S r*RNA* gene of *pseudomonase spp.*
Lane L: DNA molecular size marker 100-1000 bp
Lane (P): Positive control
Lane (N): Negative control
Lane 1-8: Positive for 16S r*RNA* gene at 618bp

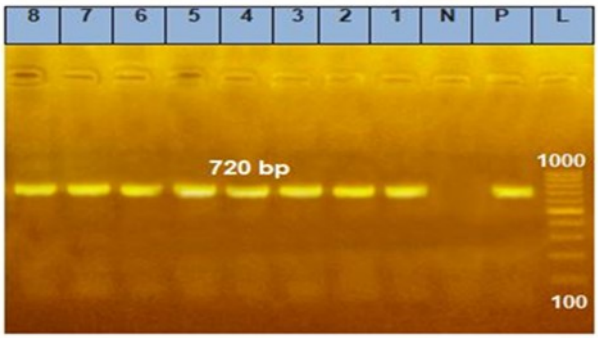

Figure (4): *pho*A gene of *E.coli spp.*
Lane L: DNA molecular size marker 100-1000 bp
Lane (P): Positive control
Lane (N): Negative control
Lane 1-8: Positive for *pho*A gene at 720 bp

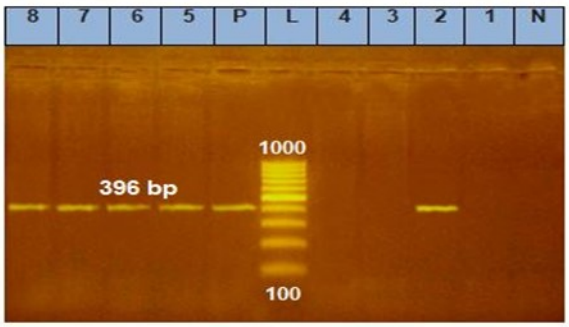

Figure (5): *tox*A gene of *pseudomonase spp.*
Lane L: DNA molecular size marker 100-1000 bp
Lane (P): Positive control
Lane (N): Negative control
Lane 5-8,2: Positive for *tox*A gene at 396 bp

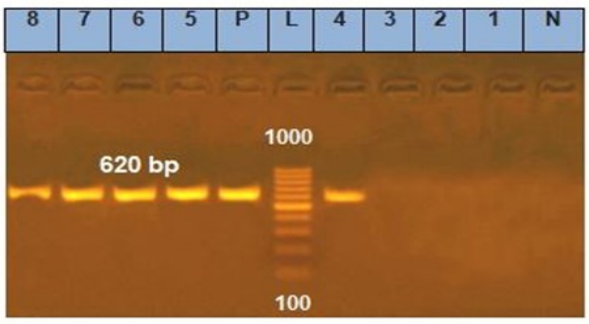

Figure (6): *tox*A gene of *pseudomonase spp.*
Lane L: DNA molecular size marker 100-1000 bp
Lane (P): Positive control
Lane (N): Negative control
Lane 5-8, 4: Positive for *tox*A gene at 620 bp

Heamatological analysis:

Results of our experiment showed that, feeding fish on both of ***Spirulina*** and ***Moringa*** diet had significantly enhancement of RBC, Hb, PCV, MCV, MCH and MCHC in different treatments represented in **Table (9)**. Meanwhile WBCs count and differential leukocyte count revealed that lymphocyte exhibited the higher count in all treatments and groups fed on diet compared with infected groups when compared with control group, **Table(10).**

Serum biochemical analysis:

Serum Total protein, albumin, globulin, (AST) and (ALT) represented in **Table (11)** recorded were significantly ($P<0.05$) higher than control whereas albumin showed respectively compared with infected groups when compared with control group. Serum cholesterol and triglycerides revealed show hypocholesterolemia activities and serum lipid profilein treated groups when compared with infected group compared when compared with control group.

There were improve in the cellular immunity due to inclusion of moringa & spirulina in dietary fish ,this evidenced by significant increase in the phagocytic index & phagocytic % compared with control groups, **Table (12).**

Table (9): Mean values of hematological parameters in fish before and after treatment by Spirulina and Moringa through out the experimental period

Groups/ Parameters		Hb(g/dl)	RBCs * 10^6/ mm^3	PVC %	MCV (fl)	MCH(pg)	MCHC (g/dl)
Control		8.11 b ±0.96	1.42 c ±0.01	2.21 a ±0.51	8.1a ±1.11	30.13 a ±4.47	39.25 a ±11.18
Spirulina	**with bacterial infection**	9.16 a ±0.16	1.83 b ±0.04	2.19 b ±0.50	7.9 b ±1.14	29.1 b ±4.32	39.04 a ±11.74
	with Parasitic infection	9.56 a ±0.13	2.11 a ±0.11	2.15 b ±0.49	8.4 a ±1.07	29.8 b ±4.42	39.01 a ±11.11
Moringa	**with bacterial infection**	9.77 a ±0.55	1.99 b ±0.014	2.24 b ±0.52	8.6 a ±1.09	29.6 b ±4.39	38.7 b ±11.02
	with Parasitic infection	9.91 a ±0.56	2.20 a ±0.02	2.25 b ±0.519	8.9 a ±1.13	29.4 b ±4.36	38.4 b ±10.94
Moringa + Spirulina	**with bacterial infection**	8.71 b ±0.49	1.74 b ±0.012	2.28 b ±0.53	8.4 a ±1.07	29.7 b ±4.40	38.01 b ±10.82
	with Parasitic infection	8.61 b ±0.48	1.52 b ±0.01	2.27 b ±0.52	8.7 a ±1.10	29.5 b ±4.38	38.0 b ±10.82

Group with different letters within the same column are significantly different at $P < 0.05$

Table (10): Mean values of Leucocytic count parameters in fish before and after treatment by Spirulina and Moringa through out the experimental period

Groups/ Parameters		WBCs * 10^3	Lympho-cyte %	Heterophil %	Monocyte	Eosinophil	Basophil
Control		7.26 b ±0.04	50.3 b ±0.3	39.0 a ±2.6	3.1 a ±0.1	2.67 b ±0.30	1.0 a ±0.01
Spirulina	**with bacterial infection**	8.51 a ±0.047	56.26 b ±0.33	29.21 d ±1.95	2.71 b ±0.090	3.31 a ±0.37	1.0 a ±0.01
	with Parasitic infection	8.47 a ±0.047	56.6 b ±0.34	34.01 c ±2.27	2.64 b ±0.088	3.28 a ± 0.36	1.0 a ±0.01
Moringa	**with bacterial infection**	8.62 a ±0.047	58.1 a ±0.35	28.01 d ± 1.87	2.75 b ±0.092	3.38 a ±0.38	1.0 a ±0.01
	with Parasitic infection	8.54 a ±0.047	57.90 a ±0.345	26.53 d ±1.77	2.66 b ±0.089	3.34 a ±0.375	1.0 a ±0.01
Moringa + Spirulina	**with bacterial infection**	7.72 b ±0.042	55.2 b ±0.33	30.27 c ±2.02	2.94 b ±0.098	2.87 b ±0.32	1.0 a ±0.01
	with Parasitic infection	7.54 b ± 0.041	54.9 b ±0.327	30.85 c ±2.06	2.97 b ±0.099	2.74 b ±0.31	1.0 a ±0.01

Group with different letters within the same column are significantly different at $P < 0.05$

Table (11):Mean values of biochemical parameters in fish before and after treatment by Spirulina and Moringa through out the experimental period:

Groups		TP(g/dl)	Alb (g/dl)	Glob (g/dl)	Alt (u/l)	ASt (u/l)	Cholesterol (mg/dl)	Tri glycerides (mg/dl)
Control		4.40 c ±0.80	1.88 b ±0.14	2.12 b ±0.66	18.21 b ±1.0	24.01 c ±0.10	180.4 c ±6.43	145.3 c ±9.79
Spirulina	**with bacterial infection**	5.72 b ±1.14	2.10 a ±0.16	3.62 a ±0.98	17.31 b±0.95	23.8 b ±0.39	177.9 c ±6.34	160.4 b ±10.81
	with Parasitic infection	5.88 b ±1.17	2.04 a ±0.15	3.84 a ±1.02	17.11 b ±0.94	23.6 a ±0.66	180.4 c ±6.43	155.2 b ±10.45
Moringa	**with bacterial infection**	5.92 b ±1.18	2.21 a ±0.16	3.71 a ±1.02	19.16 a ±1.05	25.9 c ±0.11	198.40 b ±7.07	165.3 a ±11.14
	with Parasitic infection	5.80 b ±1.16	2.26 a ±0.17	3.54 a ±0.99	19.19 a ±1.054	25.37 c ±0.10	197.1 a ±8.56	157.6 b ±10.62
Moringa + Spirulina	**with bacterial infection**	4.51 c ±0.90	1.98 b ±0.15	2.53 b ±0.75	18.02 b ±0.99	25.0 c ±0.10	164.6 d ±4.22	142.2 d ±5.41
	with Parasitic infection	4.48 c ±0.89	1.92 b ±0.14	2.56 b ±0.75	18.17 b ±0.99	25.07 c ±0.11	162.4 d ±5.71	143.8 d ±5.64

Group with different letters within the same column are significantly different at $P < 0.05$

Table (12) Mean values of Phagocytic index and Phagocytic % in fish before and after treatment by Spirulina and Moringa through out the experimental period:

Groups		Phagocytic index	Phagocytic %
Control		4.96c±0.08	44.0 c ±2.18
Spirulina	**with bacterial infection**	5.76 b ±0.092	45.35 b ±2.25
	with Parasitic infection	5.36 b ±0.086	45.84 b ±2.27
Moringa	**with bacterial infection**	5.16 b ±0.083	44.71 c ±2.21
	with Parasitic infection	5.20b ±0.083	44.32 c ±2.19
Moringa + Spirulina	**with bacterial infection**	7.71 a ±0.12	46.41 a ±2.28
	with Parasitic infection	7.78 a ±0.13	46.52 a ±2.30

Group with different letters within the same column are significantly different at $P < 0.05$

DISCUSSION

In the present study, prevalence rate of infection by helminth parasites was 55%, while **Mgbemena *et al.* (2020)** informed that 2 classes of parasites in *Clarias gariepinus* was infected by two species one trematode *Allocreadium* and one nematode *Camallanus* were higher as their percentage infection was 88.2 % and 91.2, respectively. **Amos *et al.* (2018)** reported that, a total number of 60 adult *C. gariepinus* were infected out of which 31 parasites were recovered, a total of 41% fish were infected by *Allocreadium*, while 80% infected by *Camallanus*. Parasitic infestations were significantly higher than our study. **Kawe *et al.* (2016),** reported that 56 of the examined fishes were infected with various species of helminth parasites giving a prevalence of 57.5% nearly as our study, parasites were identified as *camallanus* (32.5%), *Allocraedium* species. (3.6%)

Moreover, we found that **Moringa** and **Spirulina** improve the fish health as they decrease the infection rate by helminth parasites. A study was designed to evaluate the effect of ***Spirulina*** algae supplementation at different levels on growth performance, feed utilization, and parasitic infection resistance, **Sherif *et al.* (2012).** Another study confirms that ***Moringa oleifera*** used as an anti-parasitic product indicating impacted immune status, **Sherif*et al.* (2014).*Moringa oleifera L. (moringa)*** is known as one of the most useful multipurpose plants. It can be effectively utilized as a natural biopesticide and inhibitor of several parasitic pathogens, **Abd El-Hack *et al.* (2018).** The use of blue green algae ***Spirulina***in aquaculture has many potential advantages over the culture of fish. Generally, ***Spirulina***is used in fish feed as a replacement of fish meal and a nutritional supplement as an anti-parasitic, **Islam *et al.* (2018).**

The bacteriological examination of 100 diseased ***Clarias gariepinus*** revealed that forty fish were highly infected with bacteria (25 single infection with bacteria only+15 mixed infection with parasite and bacteria), (17%) of the isolates were positive for ***E.coli*** while (23%) were positive for ***Pseudomonas*** *spp*. our results was in accordance with the finding that mentioned by **Masbouba, 2004; El-Hady and Samy, (2011)** and higher than what reported by **Akinobowale *et al.*, (2006)**.

The results showed inhibition activity of ***Spirulina*** extract against *and* ***E.coli*** and *P. aeruginosa,* that were appeared as inhibition zone diameters ranged from 17 and 22 mm, while the ability of moringa were inhibition of the bacterial isolates at 14 and 19mm. The mixture containing both spirulina and moringa was the most effective giving zones of inhibition ranged from 2 and 2.2mm. These results were agreed with **Murugan (2012)** and **Nayyef and Thalij (2020)** who found that Spirulina have antimicrobial activity against *P. aeruginosa* and *E.coli*. Antibacterial activity of ***Moringa*** was noticed against several bacteria namely *Escherichia Coli* and *Pseudomonas aeroginosa* as illustrated by **Napolean *et al.* (2009**). On the other hand, **Rajendrhan *et al.* (1998**) reported that *E. coli* isolates were resistant to Moringa extracts. Results of bacterial isolation along the experiment revealed that spirulina and moringa had antibacterial activity against *E.coli* and *pseudomonas* isolates. In our investigation spirulina was more effective as antibacterial agent than moringa and the blend of both extracts was the most effectual. Our results of spirulina antibacterial effect were supported by previousstudies as **Kumar *et al.* (2011)** who mentioned that *Spirulina* is one of the most important micro-alga showed antimicrobial activity against a variety of pathogenic bacteria and fungi.

Conventional PCR technique:

The diversity and composition of a microbial community are often determined by its 16S ribosomal RNA (rRNA) gene content, that by PCR using primers specific to a broad taxonomic range, which are a convenient tool for detecting bacterial genomic DNA contamination (**Acinas *et al.*, 2004**). The 16Sr*RNA* gene is used for phylogenetic studies as it is highly conserved between different species of bacteria (**Altaai *et al.*, 2014**). The PCR results for *16SrRNA* conserved gene of *pseudomonase isolates* was detected in all eight studied isolates (100%), the result was decided by **Ibrahim *et al.*, (2021)**.The results of PCR for amplification of exotoxin A (*toxA*) gene of *Pseu-*

domonase isolates revealed that the *toxA* gene was amplified in five out of 8 studied isolates (62.5%), this result was agreed with **Abd El Tawab *et al*. (2016).**

In our study, a general *E.coli pho*A gene was selected to confirm the presence of *E.coli* species (100%), this result agree with **(Hu *et al*., 2011)** who detected this gene in all fish *E.coli* isolates. Further, the pathogenic capability of *E.coli* is mainly due to virulence factors as adhesion genes (*tsh*) which was detected in 62.5% of *E.coli* isolates, other study showed that the *tsh*gene was detected in 85.3% of the *E. coli* strains **(Zhao *et al.*, 2005).**

Results of RBCS, WBCS Showed of infected fish showed significant decrease this results agree with **Jahn *et al*. (1986)** who identified the bactericidal substances in moringa seeds as pterygospermin, moringine and the glycosides 4-(α-L-rhamnosyloxy)- benzylisothiocyanate and 4-(α-L-rhamnosyloxy)-phenylacetonitrile. These substances have been shown to inhibit mainly *E. coli* and *Pseudomonas aeruginosa.* ALSO, the moringa plant (*Moringa oleifera*) has been the object of much research due to its multiple uses and well-known bactericidal potential **(Gallao *et al.*, 2006)**.

Results of our experiment showed that, feeding ***Spirulina*** diet had significantly higher RBCs respectively and higher WBCs count respectively. Differential leukocyte count revealed that lymphocyte exhibited the higher count in all treatments and groups fed on diet ***Spirulina and Moringa*** were the highest. Concerning hemoglobin in fish blood all groups fed on ***Spirluina*** had higher than control reflecting good nutritional status. Similar results obtained by **Abdel –Tawab *et al.* (2008)** who stated that 10 % ***Spirulina*** diet increased RBCs and WBCs counts.

Hemogram RBC, Hb, PCV, MCV, MCH and MCHC of ***O. niloticus*** in different treatments showed enhanced conditions indicating that incorporation of ***M. oleifera*** leaf in diet had enhanced health status. Declined results of hemogram with higher level of addition agreed with those obtained with **Ozovehe and Nzeh (2013)** who claimed that the decrease in RBC may be ascribed to the higher concentration of antimetabolite especially tannin in the diets containing more ***M. oleifera*** leaf meal. However, our results could be explained that low feed intake in higher addition levels had decreased the protein intake which cause reduction of RBCs and blood protein synthesis. **Robert *et al.* (2000)** supported our results as they stated that the reduction in the Hb concentration could imply that diets having higher substitutions contained low quality protein.

Results concerning WBCs and lymphocytes showed higher level in addition level 5, 10 and 15% and had returned to control level with higher addition. These findings could be explained by improvement of immune status along with improved feed utilization. However, decreased level after addition of higher levels of ***M. oleifera*** could be attributed to the fact that ***M. oleifera*** contained anti nutritional materials that cause sensitization of immune system but did not cause its destruction. High WBC count is usually associated with microbial infection or the presence of foreign body or antigen in the circulating system **(Oyawoye and Ogunkunle , 1998)**. Also, **Douglass and Janes (2010)** demonstrated that the amount of ***M. oleifera*** leaf meal incorporated in fish diet has implication in immune responses and the ability of the animal to fight infection. It was obvious that incorporation of ***M. oleifera*** leaf 5, 10 and 15 % had the lowest mortality rate comparing with other groups. This could be explained by the results of blood analyses which showed increased WBCs indicating enhancement of immune status in these groups.

Serum Total protein, albumin, globulin and albumin / globulin ratio represented in table () showed that treatments and had significant higher level respectively as compared with control group indicating improved immune status. Data concerning serum total proteins, albumin and globulin showed enhancement significantly ($p<0.05$) with lower addition levels as compared with higher incorporation levels. These findings could be due to impaction of high-level incorporation on hepatic tissues. As there was significant increase ($p<0.05$) in the activities of serum enzymes AST and ALT in all experimental groups in comparing with the control group. Elevated AST and ALT activi-

ties in fish fed higher levels of ***M. oleifera*** leaf meal diet and maybe due to hepatic cellular damage leading to their leakage into circulation **(Ozovehe and Nzeh, 2013)**. It was obvious that incorporation of ***M. oleifera*** leaf 5, 10 and 15 % had the lowest mortality rate comparing with other groups. This could be explained by the results of blood analyses which showed increased WBCs and serum protein indicating enhancement of immune status in these groups. In the same trend the immune status of ***Clarias gariepinus*** had improved with incorporation of ***M. oleifera*** leaf meal to level 10 % and increase the level of incorporation has implication in immune responses and the ability of the animal to fight infection **(Douglass and Janes , 2010).**

Serum total protein and globulin in all groups fed on different levels of ***Spirulina*** algae were significantly (P<0.05) higher than control whereas albumin showed no significant differences between different treatments indicating that ***Spirulina*** had enhanced immunity and improved health of ***O.niloticus*** table ().Moreover, the measurement of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma is of considerable diagnostic value in fish, as it relates to general nutritional status as well as liver function. There were no significant differences between treatments as ***Spirulina*** showed no adverse effects ***O.niloticus*** on liver function and good nutritional status in addition to integrity of vascular system **(Douglass and Janes , 2010)**

The moringa plant **(*Moringa oleifera*)** has been the object of much research due to its multiple uses and well-known bactericidal potential **Suarez *et al.* (2005)**. Leaf extracts show hypocholesterolemia activities and serum lipid profile **(El-Kassas *et al.*, 2020)**. Inducing a hypolipidemic effect confirmed by the reduced fat vacuolation observed in liver tissue. The reduced serum cholesterol levels seen in moringa-fed fish might be due to β-sitosterol **(Vergara-Jimenez *et al.*, 2017),** which lowers the absorption rate of endogenous cholesterol and increases its secretion into the intestinal tract and excretion in the form of neutral steroids**.** In addition, the reduced serum cholesterol, triglyceride, and lipoprotein levels might be attributed to the inhibition of cholesterol synthesis, resulting in a depletion of intracellular sterols in the liver **(Mehta *et al.*, 2003).**

In the present study, treatment with ***Spirulina*** in fish increased the bactericidal activities of serum these results may be attributed to the significant rise in antibody – mediated complement – killing ability of immune serum when compared with non–immune serum**.** Granulocytes and mononuclear phagocytes or macrophages play a central role in the cellular part of the nonspecific defense of fish **(Jian *et al.*, 2005).**

Phagocytic index was higher in all groups fed on ***Spirulina*** compared with control group showed increasing in phagocytic activities of monocytes. Blood cell count and Phagocytic index indicating that Spirulina had enhanced and improved fish immunity **(Duncan and Klesius , 1996)**

Feeding infected fish with spirulina showed increase in phagocytosis in blood, which have an important role for prevention of infectious disease. Phagocytosis by these cells is a process of internalization, killing and digestion of invading microorganisms, in phagocytosis; phagocytes produce oxygen free radicals during the respiratory burst, which is toxic to bacteria this result agree with **Abdel-Tawwab *et al.*(2008)** this results of bacteria challenge and bactericidal activity.

Conclusion

The present study was designed to evaluate the effect of ***Spirulina & Moringa*** supplementation on mixed bacteria (***Pseudomonas*spp & *E.coli***), mixed parasite (one species for each trematode ***Allocredium* sp.** & nematode ***Camallanus* sp)** and challenged in ***Clarias gariepinus***. The effect of ***Spirulina*** was more significant on fish health than ***Moringa***, the mixture of ***Moringa*** and ***Spirulina*** made the best result on fish health and resistance to bacterial and parasitic diseases.

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