IMMUNOLOGICAL STUDIES ON AMMONIA CONTROL IN CULTURED SEABASS

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ABSTRACT

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The present study is to investigate the immunological effects of different sub-lethal concentrations of ammonia and trails to control these levels on immunological response by using different protexin probiotic concentrations (*Enterococcus faecium*). Differential leucocytes count, phagocytic activity, phagocytic index, cortisol level, serum total protein, albumin, globulin, Serum bactericidal activity, Serum lysozome activity and disease resistance against challenge with *Vibrio alginolyticus* were investigated. Three hundred Fish were divided in to ten equal experimental groups. 1st group (control) treated with 0.0 mg/l ammonium chloride and fed 0.0 g/kg feed protexin, The 2nd ,3rd ,4th group treated with 1 mg/l ammonium chloride (Unionized ammonia-Nitrogen 0.01 mg/l) and fed 0.1 g/kg feed, 0.2 g/kg feed, 0.3 g/kg feed of commercial probiotic protexin concentrate respectively. The 5th, 6th, 7th group treated with 2 mg/l ammonium chloride (Unionized ammonia-Nitrogen 0.02 mg/l) and fed 0.1 g/kg feed, 0.2 g/kg feed, 0.3 g/kg feed of commercial probiotic protexin concentrate respectively. The 8th, 9th, 10th group treated with 3 mg/l ammonium chloride (Unionized ammonia-Nitrogen 0.03 mg/l) and fed 0.1 g/kg feed, 0.2 g/kg feed, 0.3 g/kg feed of commercial probiotic protexin concentrate respectively. Fish fed at 3% body weight per day for ten weeks. Results showed that protexin supplementation adverse significantly the negative impacts of increasing ammonia concentrations on lymphocytes, phagocytic activity, phagocytic index, Serum total protein and globulin, serum bactericidal activity and serum lysozome activity in all treated groups compared to control. In contrast, neutrophils and albumin/globulin ratio significantly decreased in all treated groups comparing to the control as well as mortality rates after challenging with *Vibrio alginolyticus* were significantly lower in all treated groups comparing to the control. Results indicated that by increasing the concentration of the protexin probiotic (*Enterococcus faecium*) results getting better in all examined parameters comparing to control although increasing ammonia concentrations. Best results in the present study were by inclusion of 0.3gm / kg feed of protexin probiotic in seabass diet which improve the immune status of seabass to the favor of adverse the stress of increasing the ammonia concentrations.

Keywords: *Protexin - Probiotic- Enterococcus faecium- Ammonia control- immune status - European seabass (Dicentrarchus labrax).*

INTRODUCTION

Aquaculture has become an economic activity of great importance around the world. Aquaculture practices demand intensive production in shorter times. Under the conditions of intensive production, aquatic species are subjected to high-stress conditions, increasing the incidence of diseases and causing a decrease in productivity (Patricia *et al*., 2012). Sea bass is a commercially important euryhaline marine fish species which is intensively cultured in the Mediterranean area. The first stages of its culture take place in on-shore tanks and the last stages in sea cages. Lately, the potential of rearing sea bass only in tanks, in extremely high stocking densities, has been investigated (Sammouth *et al*., 2009). Ammonia and urea are the two main nitrogenous products excreted by teleost fish. Ammonia usually representing 75 to 90 % of nitrogenous excretion. The acute and chronic toxicities of ammonia have been extensively reviewed for freshwater species but ammonia toxicity data for marine fish species or salmonids in sea water are relatively scarce (Fivelstad *et al*., 1995). In seawater,

ammonia is measured as total ammonia nitrogen (TAN), which represents the sum of UIA-N and NH4+-N. (Lemariéa *et al*., 2004). Safe levels for growth, usually extrapolated from LC50 data, are reported to range from 0.05 to 0.2 mg l-1 UIA-N (Handy and Poxton, 1993), depending on species, age and environment (oxygen concentration, pH). Sub-lethal concentrations of ammonia can damage the gills and also impair immune function leading to increased susceptibility to infectious disease. The 0.26-mg l-1 UIA-N concentration can be considered as a safe long-term limit in seawater for sea bass juveniles (The EFSA Journal, 2008). Probiotics are usually live microorganisms which when administered in adequate amounts confer a health benefits on host. In last few years probiotics became an integral part of the culture practices for promoting growth and disease resistance to procure high production, certain probiotics used as water additives can also play a significant role in decomposition of organic matter, reduction of nitrogen and phosphorus level as well as control of ammonia, nitrite, and hydrogen sulfide (Sha.Ahmadvand *et al*., 2012). Protexin concentrate contains beneficial probiotics microorganisms (*Enterococcus faecium*). This product is designed for continuous use to promote efficient digestion and immunity or at times of stress.

The objective of the current study is conducted to investigate the effect of probiotic *Enterococcus faecium* "protexin" on immunity and survival of cultured sea bass against ammonia stress.

MATERIALS and METHODS

1. Fish and experimental design: A total of 300 apparently healthy sea bass with an average body weight of 40 ± 5 gram were obtained from private farm. Fish were kept in full glass aquaria measuring (90 X 45X 45 cm) and maintained in aerated water at 27^{o}c ± 1^{o}c, pH 8.3 ± 0.3 and salinity 32 for 7 days prior to use in experiments. The health status was examined throughout the acclimation period during the acclimation fish fed on the pelleted basic diet only contained 45% protein twice daily. Fish were randomly divided equally to ten experimental groups. Protexin probiotic were used and mixed thoroughly with the prepared basal fish diet during its preparation. Half of the water was changed daily.

Table 1: Outline of the experimental design:

Group	No.	Ammonium chloride	Diet	Protexin g/kg
1	30	0.0 mg/l	Basal diet	0.0
2	30	1mg/l	Basal diet	0.1
3	30	1mg/l	Basal diet	0.2
4	30	1mg/l	Basal diet	0.3
5	30	2 mg/l	Basal diet	0.1
6	30	2 mg/l	Basal diet	0.2
7	30	2 mg/l	Basal diet	0.3
8	30	3 mg/l	Basal diet	0.1
9	30	3 mg/l	Basal diet	0.2
10	30	3 mg/l	Basal diet	0.3

*Protexin: Commercial probiotic manufactured by International Ltd UK contain per kg: *Enterococcus faecium* (NCIMB 11181 E1708). Total Viable Count $2x10^{12}$ CFU. Ingredients: Dextrose Monohydrate. Protein 0.5%. Oil 2.0 %. Fiber 1.0 %. Ash Trace

Ammonium chloride (NH_4CL), was used as a source of ammonia by dissolving ammonium chloride powder (99.5% purity) in distilled water.

UIA-N concentrations were calculated from TAN according to pH, temperature and salinity, using the equation of Bower and Bidwell (1978): $\%NH3 = 100/ [1 + 10^{(\log Ke - pH)}]$

Where log ke as equilibrium constant, is obtained from the expression Johanson and Wedberg (1980): Log ke = (-0.467 + 0.001135S + 2877.9)/ T

where Ke is the dissociation constant, S (in gl^{-1}) the salinity and T the temperature (°K)

The concentration of the un-ionized ammonia may be obtained from the expression:

NH3 = % NH3 (NH3 + NH4+)

2. Blood collection: At the zero day,4th and 8th week of the experiment, 2ml blood samples/fish via the caudal vessels were collected from 3 fish from each group of the experiment according to (Hawak *et al.*, 1965). One ml of blood was collected with syringe containing anticoagulant (Heparin) and used for differential leucocytes count Lucky (1977) and Schalm (1986) as well as phagocytic assay (Kawahara *et al*.,1991) and the another ml of blood used for serum collection for biochemical determination (Lied *et al*., 1975). Serum total protein was determined according to Doumas *et al.* (1981). Serum albumin was determined according to Reinhold (1953). Serum globulin was determined by subtract the total serum albumin from total serum protein according to (Coles, 1974 and Khalil, 2000). Albumin/ globulin ratio was determined by division of serum albumin value on serum globulin value according to (Saffinaz, 2001) Determination of cortisol in serum according to (Gilles *et al*., 1997). Serum bactericidal activity was determined according to (Rainger and Rowley, 1993) and the results were recorded as survival index (SI) (Word Low and Unlles, 1978). Serum lysozome activity was measured with the turbidimetric method described by Engstad *et al*. (1992). The result was expressed as one unit of lysozyme activity was defined as a reduction in absorbency of 0.001/min.

3. Challenge test: At the 9th week ten fish from each group were bacteriologically tested and determined to be free from bacterial infection, were artificially infected by I/p injection with 0.2ml/fish of culture suspension of pathogenic Vibrio alginolyticus previously adjusted to 104. Specificity of death was determined by re-isolation of injected bacteria from freshly dead fish during the period of observation (one week) according to Soliman (1988).

4. Statistical analysis:
The data were statistically analyzed according to (SAS, 2000).

RESULTS

The analysis of variance indicated that although increasing ammonia concentration had a significant negative impact on the differential leucocytic count of seabass but protexin significantly improve lymphocytes and in contrary neutrophils significantly decreased in all treated groups comparing to the control but eosinophils and basophiles had no significant (Table 2) and (Fig.1). Best results were in groups fed on diet contain protexin 0.3 gm./kg feed followed by groups fed diet contain 0.2 gm./kg feed and groups fed on diet contain 0.1 gm. /kg feed respectively.

Regarding the effect of ammonia on phagocytic activity as well as phagocytic index and cortisol level of seabass (Table 3) and (Fig.2) showed, significant decrease in phagocytic activity and phagocytic index and significant increase in cortisol level of seabass by increasing ammonia concentration in comparison to the control group. Protexin improve the previous picture to the favor of nearly maintain phagocytic activity and phagocytic index and decreasing the increasing of cortisol level with increasing ammonia concentration.

Examination of serum indicated that protexin adverse the negative effects of ammonia on serum total protein, globulin and Albumin /globulin ratio in all treated groups comparing to the control specially at the 8th week. Best results showed in groups fed on diet contain protexin 0.3 gm. /kg feed (Table 4) and (Fig.3).

Concerning to the effect of protexin supplemented diet on the negative impacts of increasing ammonia concentrations on the serum bactericidal activity as well as serum lysozyme activity among different groups of seabass during experimental period revealed decrease in their activities in all protexin treated groups by increasing ammonia concentrations but their activities still higher comparing to the control. Best results were in in groups fed on diet contain protexin 0.3 gm. /kg feed (Table 5) and (Fig.4).

Mortalities of seabass challenged with Vibrio alginolyticus were significantly lower in all treated groups than the control but mortalities increased by increasing the ammonia concentrations moreover the lowest mortality percent was recorded in groups fed on diet contain 0.3 gm./kg feed of protexin followed by groups fed diet contain 0.2 gm./kg feed then groups fed on diet contain 0.1 gm./kg feed respectively (Table 6) and (Fig.5).

Table 2: Effects of different treatments on differential leucocytic counts of sea bass during experimental period:

Groups		N	Lymphocytes	Monocytes	Basophils	Eosinophil	Neutrophils	Thrombocytes
Zero day	G1	3	62.33±1.2 a,c	1±0 b,c	4.67±0 b	8.33±0.33 a	12.33±0.33 a	2.33±0.33
	G2	3	62.67±1.33 a	1±0 b,c	4±0 b	7.33±0.33 b,c	13±0 a	2.67±0.33
	G3	3	61.67±0.88 a,b	1.33±0.33 a,c	4±0 b	7±0 b,c	14.33±0.33 a	2.67±0.33
	G4	3	61.33±0.33 a,b	1.33±0.33 a,c	6±0.58 a	7.67±0.33 a,c,d	24±0.58 b	2.67±0.33
	G5	3	60.33±0.33 b,c	1±0 b,c	4±0 b	7.33±0.33 b,c,d,e	23.33±0.33 b,c	2.67±0.33
	G6	3	61±0.58 a,b	1.67±0.33 a,c	4.33±0 b	7.67±0.33 a,c	21.67±1.67 c,d	2.33±0.33
	G7	3	62±0 a,b	1.33±0.33 a,c	5.67±0.33 a	7.67±0.33 a,c	21.67±0.88 c,e	2.33±0.33
	G8	3	61±0.58 a,b	1±0 b,c	4±0 b	6.67±0.33 b,e	23±0.58 b,d,e	2.67±0.33
	G9	3	60±0 b	1.67±0.33 a,c	4±0 b	6.67±0.33 b,e	24±0.58 b	2.67±0.33
	G10	3	61.33±0.88 a,b	2±0 a	5.67±0.33 a	6.67±0.33 b,e	23±0 b,d,e	2.67±0.33
4th week	G1	3	62±0.58 e	1±0 b	5.33±0.33 a,b	7±0.58 a,b	12.67±0.33 c	2±0 b
	G2	3	63.67±0.88 c,d	1±0 b	6±0 a	6.33±0.33 b,c	15.33±0.33 b	3±0 a
	G3	3	64.67±0.33 c	1±0 b	5.67±0.33 a,b	7.33±0.33 a	15±0.58 b	3±0 a
	G4	3	66.33±0.33 b	1.67±0.33 a,b	5±0 b	7.33±0.33 a	21±0.58 a	3±0 a
	G5	3	62.67±0.33 d,e	1.33±0.33 a,b	5.67±0.33 a,b	5.67±0.33 c	15.67±0.67 b	3±0 a
	G6	3	64.67±0.33 c	1.33±0.33 a,b	5±0 b	7±0 a,b	12.33±0.33 c	3±0 a
	G7	3	64.67±0.88 c	1.67±0.33 a,b	5±0 b	7±0 a,b	20.67±0.67 a	3±0 a
	G8	3	66.33±0.33 b	2±0.58 a	6±0 a	6.67±0.33 a,b	15±0.58 b	2.33±0.33 b
	G9	3	67.0.33 a,b	1±0 b	6±0.58 a	7±0 a,b	12.67±0.33 c	2.33±0.33 b
	G10	3	68.67±0.33 a	1.67±0.33 a,b	5.33±0.33 a,b	7.33±0.33 a	21±1 a	2.33±0.33 b
8th week	G1	3	61±1 g	1.33±0.33 b	6.33±0.33 a,b	7±0 b,c,d,e	13.33±0 c	3±0 c
	G2	3	66.67±0.33 f	1.33±0.33 b	6.67±0.33 a,b	7.33±0.33 a,c	13.67±0.88 c	3±0 c
	G3	3	68.67±0.33 d,e	1.67±0.67 b	6±0 b,d	7.67±0.33 a,b,d	16.67±0.88 b	3.33±0.33 b,c
	G4	3	70±0.58 c,d	1±0 b	6±0.58 b,c	7±0 b,c,f	21±0.58 a	3.67±0.33 a,b
	G5	3	68.33±0.33 e	1.67±0.33 b	7±0 a	7±0 b,c,g	11±0 d	3±0 c
	G6	3	71.33±0.33 b,c	1.67±0.67 b	6±0 b,e	8±0 a	7±0 e	4±0 a
	G7	3	73.67±0.33 a	1.33±0.33 b	6.33±0 a,b	6.67±0.33 c,h	15.67±1.2 b	4±0 a
	G8	3	68±0 e,f	2±0 a,b	6.33±0.33 a,b	6.33±0.33 e,f,g,h,i	10.33±0.58 d	3±0 c
	G9	3	70.33±0.33 c	3±0 a	5.33±0 c,d,e	7.33±0.33 a,d,f,g,i	6.67±0.33 e	3.33±0.33 b,c
	G10	3	72.33±0.33 a,b	1±0 b	6.33±0.33 a,b	6.67±0.33 c,i	16.67±0.88 b	3.33±0.33 b,c

Means in the same column with the different small litter at the same week are significantly different at (P≥0.05)

Fig. 1: Differential leucocytic counts of seabass during experimental period

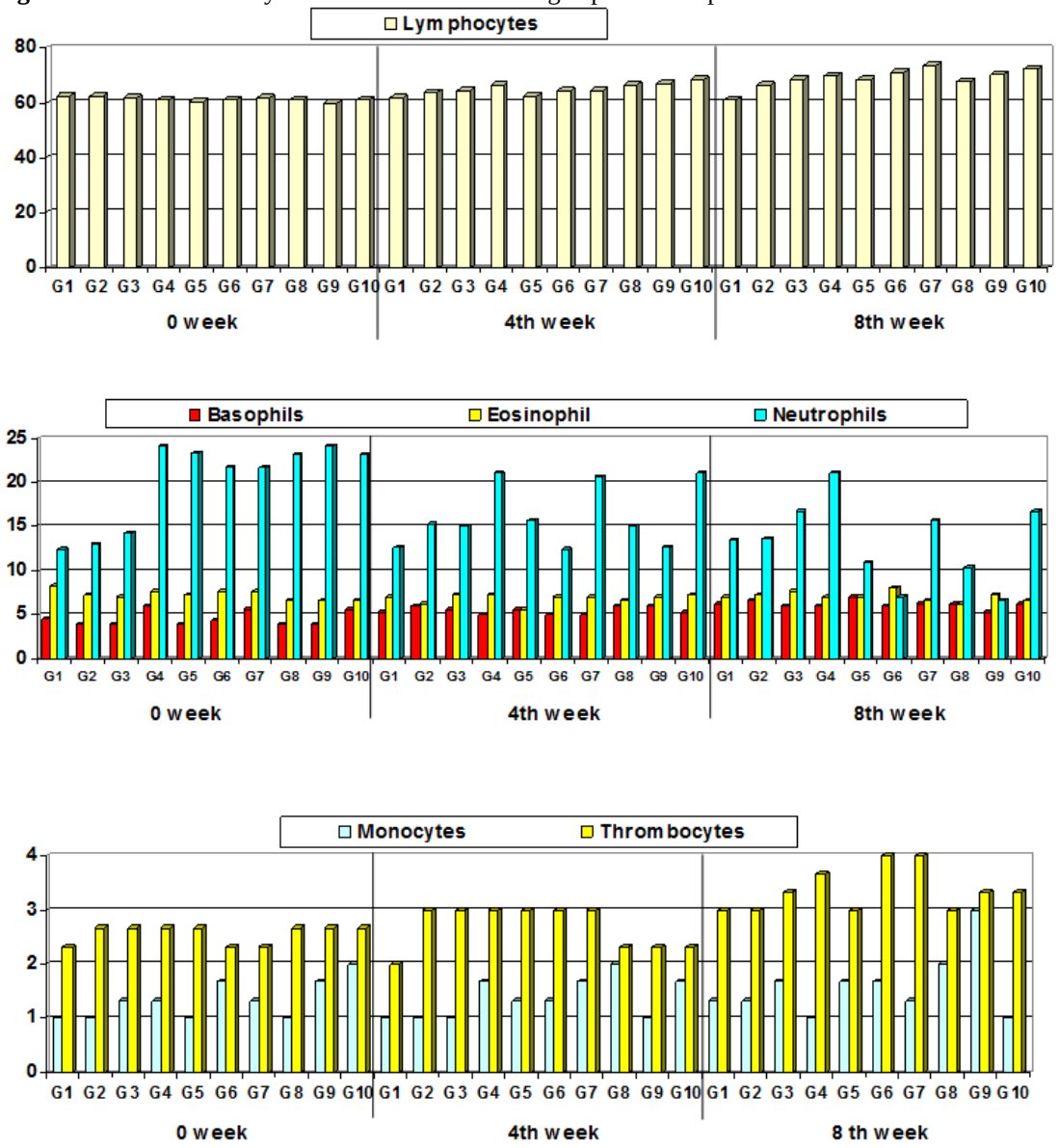


Fig. 2: Cotrisol level, phagocytic activity and phagocytic index of seabass during experimental period.

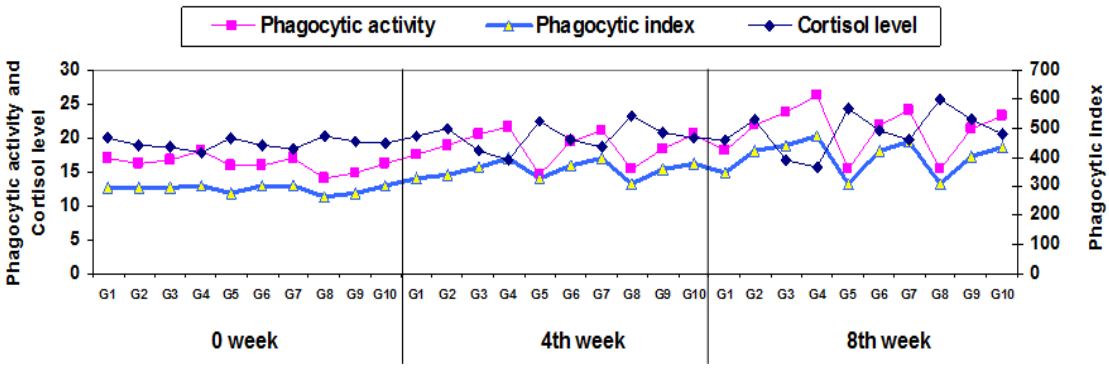


Table 3: Effects of different treatments on cortisol level, phagocytic activity and phagocytic index of seabass during experimental period:

	Groups	N	Cortisol level	Phagocytic activity	Phagocytic index
Zero day	G1	3	468.52±0.56 b	17±0 d	12.67±0.33 a
	G2	3	443.2±1.15 e	16.33±0.33 e,f	12.67±0.33 a
	G3	3	434.05±1.39 f	16.67±0.33 d,f	12.67±0.33 a
	G4	3	418.82±0.52	18±0 a	13±0 a
	G5	3	468.48±1.32 b	16±0 e	12±0 c
	G6	3	440.93±1 e	16±0 e	13±0 a
	G7	3	431±0.68 g	17±0 d	13±0 a
	G8	3	475.33±1.59 a	14±0 b	11.33±0.33 b
	G9	3	455.59±1.12 c	15±0 c	12±0 c
	G10	3	447.34±0.81 d	16.33±0.33 e,f	13±0 a
4th week	G1	3	470±3.08 e	17.67±0.33 e	14±0 f,g
	G2	3	497.98±0.83 c	19±0 c,d	14.67±0.33 e,f
	G3	3	420.59±0.89 h	20.67±0.33 b	15.67±0.33 b,d
	G4	3	394.01±1.17 i	21.67±0.33 a	17±0 a
	G5	3	526.14±2.95 b	14.67±0.33 f	14±0 f,g
	G6	3	457.85±0.79 f	19.33±0.33 c	16±0 b,c
	G7	3	434.03±1.06 g	21±0 a,b	17±0 a
	G8	3	542.64±1.33 a	15.33±0.33 f	13.33±0.33 g
	G9	3	485.89±2.35 d	18.33±0.33 d,e	15.33±0.33 c,d,e,f
	G10	3	466.66±2.69 e	20.67±0.33 b	16.33±0.33 a,b
8th week	G1	3	459.23±1.05 g	18±0 e	15±0 e
	G2	3	532.41±1.89 c	22±0 c,d	18±0 c,d
	G3	3	392.78±1.28 h	23.67±0.33 b	19±0 b
	G4	3	364.53±1.42 i	26.33±0.33 a	20.33±0.33 a
	G5	3	570.04±0.58 b	15.33±0.33 f	13.33±0.33 f
	G6	3	494.14±0.52 e	22±0 c,d	18±0 c
	G7	3	457.73±1.7 g	24±0 b	19.33±0.33 b
	G8	3	597.22±1.76 a	15.33±0.33 f	13.33±0.33 f
	G9	3	526.63±2.8 d	21.33±0.33 d	17.33±0.33 d
	G10	3	480.08±0.25 f	23.33±0.33 b,c	18.67±0.33 b,c

Means in the same column with the different small litter at the same week are significantly different at ($P \geq 0.05$)

Table 4: Effects of different treatments on total serum protein, albumin, globulin and A/G ratio of seabass during experimental period.

	Groups	N	Total protein	Albumin	Globulin	A/G ratio
Zero day	G1	3	4.12±0.02 h	2.14±0.01 d,e,f	1.98±0.01 e	1.08±0 a
	G2	3	4.55±0.01 c	2.13±0 e,g	2.42±0.01 b	0.88±0.01 d
	G3	3	4.59±0.01 b	2.17±0 c,e	2.43±0.01 b	0.89±0.01 d
	G4	3	4.89±0.01 a	2.2±0.01 c	2.69±0.01 a	0.82±0 e
	G5	3	4.35±0.01 f	2.05±0.01 i	2.3±0.02 c	0.89±0.01 d
	G6	3	4.47±0 d	2.12±0.01 f,g,h	2.35±0.01 c	0.9±0.01 d
	G7	3	4.56±0.01 b,c	2.23±0 b	2.34±0.01 c	0.95±0 c
	G8	3	4.29±0.01 g	2.1±0.01 h	2.19±0.01 d	0.96±0 c
	G9	3	4.4±0.02 e	2.17±0.03 c,d	2.23±0.05 d	0.97±0.04 c
	G10	3	4.53±0.01 c	2.3±0.01 a	2.23±0.01 d	1.03±0 b
4th week	G1	3	4.2±0.01 f	2.41±0.01 g	1.79±0.02 h	1.34±0.02 a
	G2	3	4.39±0.01 e	2.25±0.01 i	2.14±0.02 d,e	1.05±0.01 g
	G3	3	4.76±0.03 c	2.5±0.01 f	2.25±0.04 b,c	1.11±0.02 e,f
	G4	3	5.07±0.02 a	2.74±0 a	2.34±0.02 a	1.17±0.01 d
	G5	3	4.39±0.01 e	2.35±0.01 h	2.04±0.01 g	1.15±0.01 d,f
	G6	3	4.69±0.01 c,d	2.54±0.01 e	2.15±0.01 e,f	1.18±0.01 c,d
	G7	3	4.92±0.02 b	2.64±0.03 c	2.28±0.04 a,b	1.16±0.03 d,e
	G8	3	4.41±0.04 e	2.4±0.01 g	2.01±0.04 g	1.19±0.03 b,d
	G9	3	4.67±0.02 d	2.59±0.01 d	2.09±0.01 f,g	1.24±0.01 b
	G10	3	4.88±0.02 b	2.69±0.01 b	2.19±0.01 c,d,f	1.23±0 b,c
8th week	G1	3	4.22±0.01 d	2.2±0.01 e,f	2.02±0.01 d,e	1.09±0.01 b
	G2	3	4.17±0.04 d	2.14±0.02 f,g	2.02±0.04 d,e	1.06±0.02 b,d
	G3	3	5.19±0.01 b	1.79±0.06 h	3.4±0.07 a	0.53±0.03 g
	G4	3	5.43±0.03 a	2.81±0.02 a	2.62±0.04 b,c	1.07±0.02 b,c
	G5	3	4.24±0.08 d	2.12±0.09 g	2.12±0.09 d	1±0.04 d,e
	G6	3	4.91±0.02 c	2.32±0.02 c,d	2.59±0.01 b,c	0.9±0.01 f
	G7	3	5.18±0.01 b	2.51±0.01 b	2.67±0.01 b	0.94±0.01 f
	G8	3	4.18±0.01 d	2.26±0.02 d,e	1.92±0.01 e	1.18±0.02 a
	G9	3	4.9±0.02 c	2.38±0.02 c	2.51±0.01 c	0.95±0.01 e,f
	G10	3	5.09±0.01 b	2.57±0.02 b	2.52±0.03 c	1.02±0.02 c,d

Means in the same column with the different small litter at the same week are significantly different at (P>0.05)

Fig. 3: Total serum protein, albumin, globulin, and A/G ratio of sea bass during experimental period.

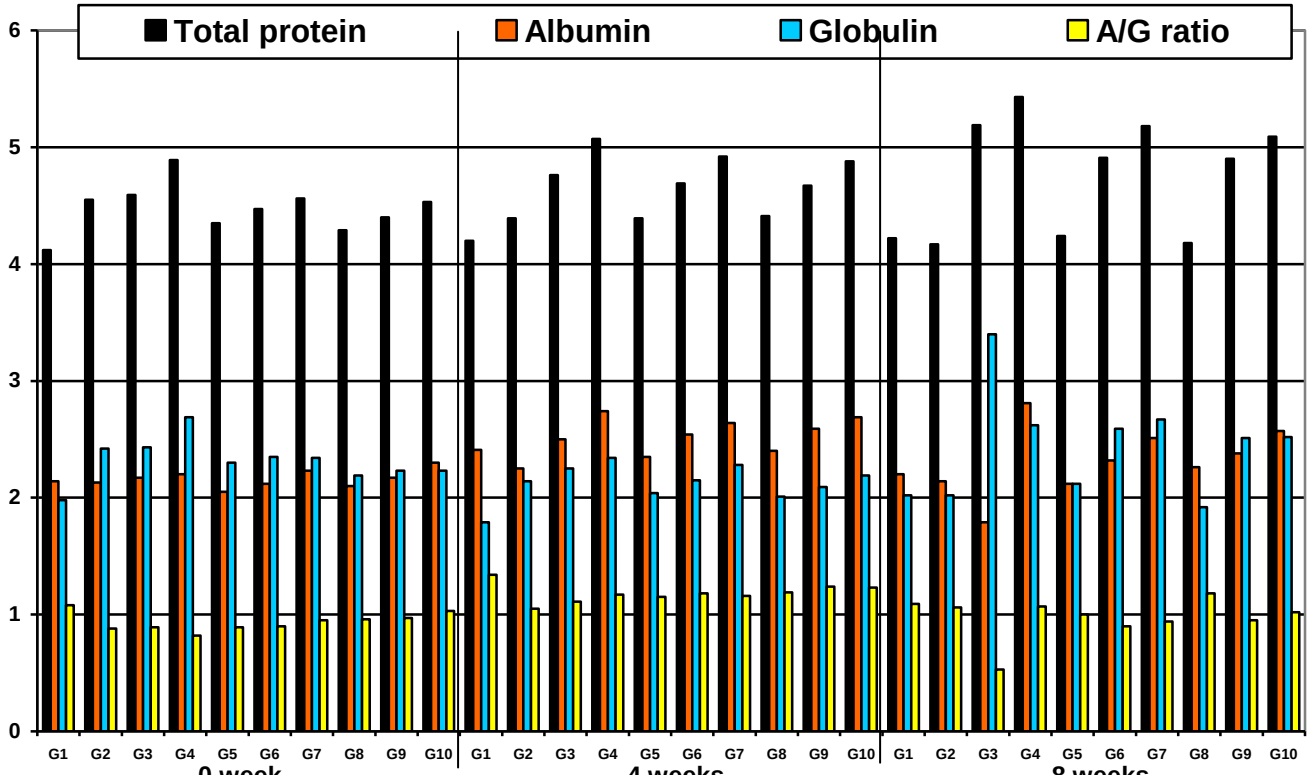


Fig. 4: Serum lysozyme and bactericidal activity of seabass during experimental period

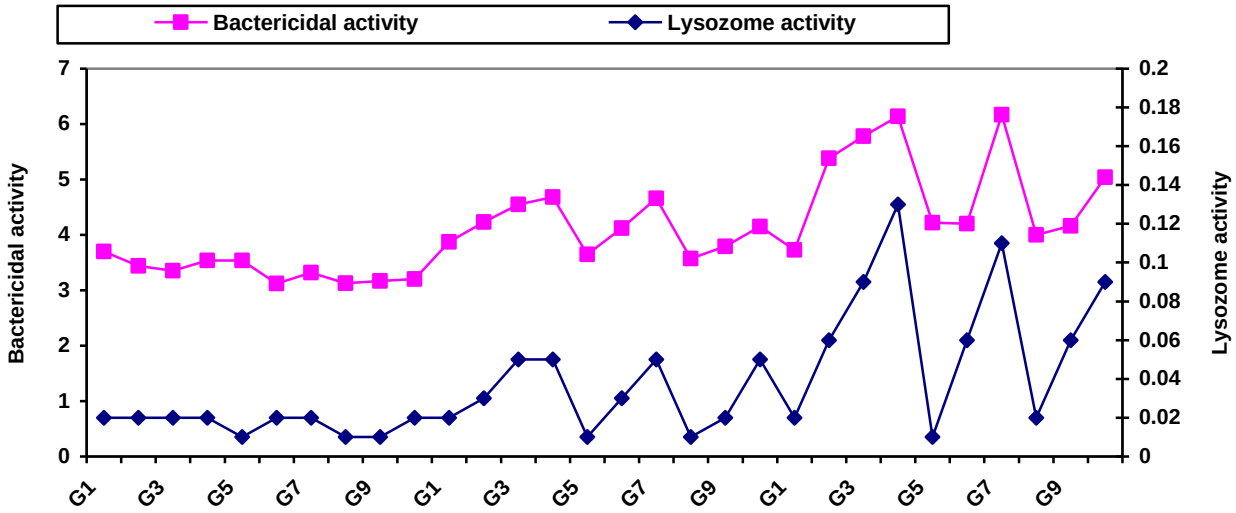


Table 5: Effects of different treatments on serum lysozyme and bactericidal activity of seabass during experimental period:

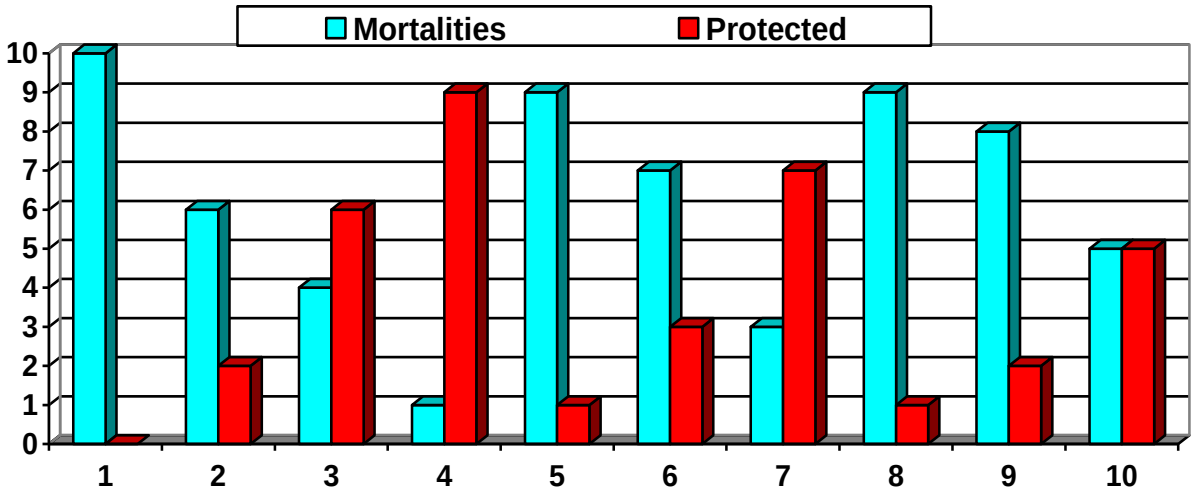
	Groups	N	Lysozome activity	Bactericidal activity
Zero day	G1	3	0.02±0 a	3.7±0.04 a
	G2	3	0.02±0 a	3.44±0 b
	G3	3	0.02±0 a	3.35±0.01 c
	G4	3	0.02±0 a	3.54±0.05 d
	G5	3	0.01±0 b	3.54±0 d
	G6	3	0.02±0 a	3.12±0 e
	G7	3	0.02±0 a	3.32±0 c
	G8	3	0.01±0 b	3.13±0.01 e
	G9	3	0.01±0 b	3.17±0.01 e,f
	G10	3	0.02±0 a	3.2±0.01 f
4th week	G1	3	0.02±0 c	3.87±0 e
	G2	3	0.03±0 b	4.23±0.01 c
	G3	3	0.05±0 a	4.55±0.01 b
	G4	3	0.05±0 a	4.68±0.01 a
	G5	3	0.01±0 d	3.65±0.01 g
	G6	3	0.03±0 b	4.12±0.01 d
	G7	3	0.05±0 a	4.66±0.01 a
	G8	3	0.01±0 d	3.57±0.01 h
	G9	3	0.02±0 c	3.79±0.01 f
	G10	3	0.05±0 a	4.15±0.01 d
8th week	G1	3	0.02±0 e	3.73±0.03 h
	G2	3	0.06±0 d	5.38±0.03 d
	G3	3	0.09±0 c	5.78±0.03 c
	G4	3	0.13±0 a	6.14±0.01 b
	G5	3	0.01±0 f	4.22±0.01 f
	G6	3	0.06±0 d	4.2±0.01 f
	G7	3	0.11±0 b	6.17±0.03 a
	G8	3	0.02±0 e	4±0.02 g
	G9	3	0.06±0 d	4.16±0.01 f
	G10	3	0.09±0 c	5.04±0.04 e

Means in the same column with the different small litter at the same week are significantly different at (P≥0.05).

Table 6: Effects of different treatments on mortality percent of seabass after challenge with *Vibrio alginolyticus*

Group	N	Mortalities		Protected	
		Number of fish	%	Number of fish	%
1	10	10	100	0	0
2	10	6	60	2	20
3	10	4	40	6	60
4	10	1	10	9	90
5	10	9	90	1	10
6	10	7	70	3	30
7	10	3	30	7	70
8	10	9	90	1	10
9	10	8	80	2	20
10	10	5	50	5	50

Fig. 5: Mortality percent of seabass after challenge with *Vibrio alginolyticus*



DISCUSSION

Ammonia exposure may act as a stressor and induce some adaptive endocrine responses and complex metabolic adaptations (Mommsen *et al*., 1999). Fish experiencing stress show a number of physiological changes that are expressed through a number of particular indicators.

In general stress affects aquatics through weakening immune system (Mahshid and Leila, 2013).

A lot of bacterial cultures containing nitrifying bacteria to control the ammonia level in culture water are available commercially. Nitrifies are responsible for the oxidation of ammonia to nitrite and subsequently to nitrate (Asaad *et al*., 2013).

Some Lactic acid bacteria (LAB) have proven to be very useful probiotics in different animals including fish / shellfish. The promising probiotic strains of LAB include the members of genera *Lactobacillus, Lactococcus* and *Enterococcus* from the representative species *Enterococcus faecium (E.faecium)*. Similarly LAB can improve haematological indices such as hemoglobin concentration, haematocrit percentage, total erythrocytes counts, total leucocytes counts, total serum protein, globulin, glucose, and cholesterol level in fish. Several immunological studies have confirmed the ability of some LAB to stimulate fish immune systems and have been found to elevate several immunological parameters like phagocytic activity, lysozyme level, peroxidase/anti-peroxidase level, complement activity and respiratory burst activity in teleosts (Sukanta, 2013).

Haematological and biochemical parameters have been acknowledged as valuable tools for monitoring fish health.
The results indicated that although increasing ammonia concentration had a significant negative impact on the differential leucocytic count but protexin significantly improve lymphocytes and incontrary neutrophils significantly decreased in all treated groups comparing to the control. These findings were nearly obtained by Manal A. A. Essa *et al*. (2012) after supplementation of *E.faecium* in the diet of *Oreochromis niloticus* but without using ammonia as stress.

The present study showed, significant decrease in phagocytic activity and phagocytic index; similar results were obained by Winton *et al*. (2004) where ammonia caused a depression in immune Parameters as phagocytic activity of *H. diversicolor supertexta* but Protexin improve the previous picture to the favor of nearly maintain phagocytic activity and phagocytic index as the control group these results nearly supported by (Sukanta, 2013).

It is well known that cortisol plays an adaptive function against stressors. However, chronically elevated cortisol levels may become damaging for several physiological functions e.g. suppression of the immune system (Person *et al*., 2003). In the present study, serum cortisol was measured as an indicator of primary stress response. The analysis of serum cortisol levels carried out in this study gave the evidence that the groups fed with protexin showed a better tolerance to ammonia as stress rearing conditions. This was evidenced by the lower cortisol levels detected in the treated experimental groups with comparing to control nearly these results were supported by (Carnevali and Sulpizio 2006) where cortisol levels obtained in the probiotic treated European seabass juveniles (*Dicentrarchus labrax*,L.), fishes were significantly lower than those in the control that untreated with probiotic.

With increasing concentration of Total Ammonia Nitrogen (TAN), haemoglobin and serum protein content were reduced in mrigal (*Cirrhinus mrigala*) (Hamilton) (Das *et al*., 2004) similar results were observed in the present study but with increasing protexin concentration in seabass feed the serum total

proteins as well as total globulin levels were significantly improved in all treated groups than the control. Similar results were also obtained after *E.faecium* supplementation in the diet of *O.niloticus* by Manal A. A. Essa *et al*. (2012) but without using ammonia as stress.

Lysozyme, being an enzyme with antibacterial activity, can split peptidoglycan in bacterial cell walls especially of the gram positive species and can cause lysis of the cells. The Lysozyme increased with activation of immune system.

The present study revealed decreasing lysozyme activity as well as Bactericidal activity during the experimental period in all treated groups by increasing ammonia concentrations but using Enterococcus faecium relatively improve this picture to be better than the control, These results supported by Kim *et al*. (2012) in investigation of the effect of a probiotic, *Enterococcus faecium*, on olive flounder (*Paralichthys olivaceus*) moreover also (Taoka *et al*., 2006) showed that viable probiotics administered to Japanese flounder (*Paralichthys olivaceus*), increased nonspecific immune response, determined by parameters such as lysozyme activity, neutrophil migration and bactericidal activity, which improved the resistance of fish to infection. On contrary, dietary supplementation of probiotics like *Enterococcus faecium* in *O.niloticus* failed to elevate lysozyme level (Wang *et al*., 2008) and (Zhou *et al*., 2009). This is may be due to different fish species and water.

Concerning mortalities of ammonia stressed seabass challenged with *Vibrio alginolyticus* were significantly lower in all treated groups than the control but mortalities increased by increasing the ammonia concentrations moreover the lowest mortality percent was recorded in groups fed on diet contain 0.3 gm./kg feed of protexin. These results supported by Liu and Chen (2004) as well as Cheng *et al*. (2004) and (Winton *et al*., 2004) where ammonia in water caused a depression in the immune response and an increase in mortality of *Litopenaeus vannamei* from the *V. alginolyticus* infection and mortality of *H. diversicolor supertexta* from *V. parahaemolyticus* respectively. The obtained results in the present study may be attributed to that ammonia caused suppression of the cellular or the non-specific defense mechanisms or to the effect of ammonia on other physiological systems (vascular, respiratory, etc.) Hurvitz *et al*. (1997) where studying the effect of sublethal concentrations of ammonia on rainbow trout (*Oncorhynchus mykiss* Walbaum), vaccinated against and challenged with *Streptococcus iniae* survival and the antibody response referred decreasing of protection against *S. iniae* in ammonia-exposed trout. Inclusion of protexin in seabass feeding have positively impacted the resistance of fish to *Vibrio alginolyticus* infection as was indicated by significantly lower mortality rates of the ammonia treated fish challenged by *Vibrio alginolyticus* in comparison to the control. These results were supported by Krummenauer *et al.* (2009) after dietary application of *E.faecium* to shrimp challenged by *Vibrio parahaemolyticus* as well as Gopalakannan and Arul (2011) after challenging *E.faecium* MC13 supplemented *Cyprinus carpio* by *Aeromonas hydrophila.*

CONCLUSION

It could be concluded that supplementation of protexin (*Enterococcus faecium*) as probiotic in seabass feeding have positively impacted the resistance of fish to stress (sub-lethal concentrations of ammonia) as was indicated by significant increasing of lymphocytes, total protein, globulin, phagocytic activity, index of phagocytes, lysozyme activity and bactericidal activity which enhanced the resistance of challenged fish to *Vibrio alginolyticus* as was indicated by significant decrease in mortalities rates in protexin treated groups than the control. The highest dietary level 0.3 gm. /kg feed of protexin showed best results than 0.2 gm./kg feed then 0.1 gm./kg feed. For seabass ammonia stress tolerance *Enterococcus faecium* supplement should be recommended.

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دراسات مناعية علي التحكم في الآمونيا في أسماك القاروص المستزرعة

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استهدف البحث دراسة التأثيرات المناعية للتركيزات المختلفة تحت المميتة للآمونيا ومحاولة التحكم في تلك التأثيرات المناعية بإستخدام تركيزات مختلفة مضافة في علف اصبعيات أسماك القاروص لمركب البروتكسين كبروبيوتك (إنتيروكوكس فيكم) من خلال دراسة كل من :العد النوعي لكرات الدم البيضاء، النشاط البلعمي ، مؤشر البلعمة، مستوي الكورتيزول، البروتين الكلي، الألبيومين، الجلوبيولين، نشاط البكتيريسيدال، نشاط الليسوزوم وكذلك مدي مقاومة الأسماك لميكروب الفيبريو ألجينوليتكس بعد عمل اختبار تحدي بهذا الميكروب للأسماك الخاضعة للبحث. تم تقسيم عدد ٣٠٠ سمكة من أسماك القاروص الي عشر مجموعات بحثية متساوية العدد وهي: المجموعة الضابطة، المجموعة الثانية والثالثة والرابعة وتخضع هذه المجموعات الي ضغط باستخدام نسبة ٠.٠١مجم/لتر من الآمونيا الغير متأينة بواسطة كلوريد الأمونيوم بتركيز ١مجم /لتر وتتغذي علي علف يحتوي علي ٠.١ جرام/كجم ، ٠.٢ جرام/كجم و ٠.٣ جرام /كجم علف علي التوالي من مركب البروتكسين. أما المجموعة الخامسة والسادسة والسابعة فتخضع كل من هذه المجموعات الي ضغط باستخدام نسبة ٠.٠٢مجم/لتر من الآمونيا الغير متأينة بواسطة كلوريد الأمونيوم بتركيز ٢مجم /لتر وتتغذي علي علف يحتوي علي ٠.١ جرام/كجم ، ٠.٢ جرام/كجم و ٠.٣ جرام /كجم علف علي التوالي من مركب البروتكسين. بينما كل من المجموعات الثامنة والتاسعة والعاشرة فتخضع كل من هذه المجموعات الي ضغط باستخدام نسبة ٠.٠٣مجم/لتر من الآمونيا الغير متأينة بواسطة كلوريد الأمونيوم بتركيز ٣مجم /لتر وتتغذي علي علف يحتوي علي ٠.١ جرام/كجم ، ٠.٢ جرام/كجم و ٠.٣ جرام /كجم علف من مركب البروتكسين علي التوالي. تم تقديم العلف للأسماك يوميا بنسبة ٣% من وزن الأسماك يوميا لمدة عشرة أسابيع. أظهرت النتائج أن إضافة مركب البروتكسين لعلف الأسماك أدي إلي مقاومة التأثيرات السلبية لزيادة تركيزات الآمونيا مقارنة بالمجموعة الضابطة، علي كل من: خلايا الليمفوسيت، النشاط البلعمي ومؤشر البلعمة، البروتين الكلي، الجلوبيولين، نشاط البكتيريسيدال ونشاط الليسوزوم في كل المجموعات الخاضعة للبحث مقارنة بالمجموعة الضابطة علي العكس كان هناك نقص في كل من خلايا النيتروفيل و نسبة كل من الألبيومين والجلوبيولين في كل المجموعات الخاضعة للبحث مقارنة بالمجموعة الضابطة. كذلك النتائج بينت أن نسبة النفوق في الأسماك في كل المجموعات كانت أقل من المجوعة الضابطة وذلك بعد إجراء اختبار التحدي بإستخدام ميكروب الفيبريو ألجينوليتكس الذي خضعت له كل المجموعات البحثية. مما سبق نجد أن هذه الدراسة توضح أن كلما زادت نسبة إضافة مركب البروتكسين (إنتيروكوكس فيكم) كلما كانت النتائج أفضل في كل الموشرات المدروسة في التحكم في الآمونيا وكانت إضافة مركب البروتكسين (إنتيروكوكس فيكم) بنسبة ٠.٣ جرام /كجم علف في أعلاف أسماك االقاروص يحسن من كفاءة الأداء المناعي لهذه الأسماك مما يزيد مقاومتها لزيادة الضغط الواقع عليها بزيادة نسبة الأمونيا.