Changes in the intestinal bacterial profile and serum biochemical parameters of Nile Catfish *Clarias lazera* after feeding with probiotic.

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ABSTRACT

In this study, the experimental set-up was based on fed *Clarias lazera* commercial diet with probiotic for 45 days either 1 time/week (W_1) all over the experiment or 3 times/week for the 1st two weeks then 1 time/week till the end of the experiment (W_3). Bacteriological and biochemical examinations were done on intestinal tract and serum respectively.

Bacteriological investigation revealed the presence of *Escherichia coli* and *Klebsiella* spp. in all groups and *Pseudomonas aeruginosa* failed to be isolated from W_1 and W_3. Bacterial isolates proved to be pathogenic for mice, positive for Congo red binding activity and toxigenic in infant mice. The intestinal total aerobic bacterial count; *Enterobactereacae* count and coliform count in males and females of both W_1 and W_3 fish groups were significantly lower than the respective values in control group. Significant low values were detected in W_3 group compared to W_1 group. Significant sex differences in the three counts were detected in the three groups of fish except the *Enterobactereacae* count in W_3 group.

Concentrations of serum ammonia and urea were significantly decreased in both treated groups compared to the control group. Both serum items were significantly lower in W_3 group than that in W_1 group. Significant sex difference was recorded in serum creatinine of the three groups (females lower than males).

The activities of serum alkaline phosphatase; alanine aminotransferase and aspartate aminotransferase enzymes significantly differed according to sex with a general trend that these enzymes were significantly higher in W_3 group than that of W_1 group.

Serum total protein, albumin, globulin and A/G ratio in fish of W_1 group were more affected by probiotic administration than that in W_3 group. These serum protein items in all groups were significantly affected by sex. Serum IgM level in both sexes of W_1 group was significantly higher than that in both control and W_3 groups.

A correlation analysis was conducted to determine the strength of the association between serum biochemical parameters and viable total bacterial count; *Enterobactereacae* and coliform counts in the guts of fish. Male fish showed similarity of the correlations analysis between the three bacterial counts and the different serum biochemical parameters, while female fish detected some variations according to the type of bacterial count.

Keywords: Bacterial profile, serum biochemical, *Clarias lazera*, Probiotics

INTRODUCTION

World aquaculture has grown tremendously during the last years becoming an economically important industry (Subasinghe *et al*., 2009). Today it is the fastest growing food-producing sector in the world with the greatest potential to meet the growing demand for aquatic food (FAO, 2006). Globally, aquaculture is expanding into new directions, intensifying and diversifying (Austin *et al*., 1995). Application of probiotic for fish aquaculture has shown much potential in recent years (Irianto and Austin, 2002). Benefits across a range of fish species include improved water quality, growth performance, feed utilization, resistance to disease and immunostimulation (Burr *et al*., 2005; Abdel-Tawwab *et al*., 2008 and Kamgar *et al*., 2013). Nikoskelainen *et al*. (2003) and Panigrahi *et al*. (2004, 2005) confirmed that dietary supplementation with various indigenous and exogenous useful microorganisms "probiotics" may be an effective biotherapeutic or prophylactic means in aquaculture. In the fish farming industry, disease is a major problem with the increasing intensification and commercialization of aquaculture production (Bondad-Reantaso *et al*., 2005). The probiotics are considered as a new strategies in feeding and health management in fish aquaculture practice (Denev, 2008).

Probiotic agents exert a beneficial effect via a wide array of actions. These include: immunostimulation and increase resistance to disease (Irianto and Austin, 2002; Nikoskelainen *et al*., 2003; Panigrahi *et al*., 2004, 2005; Balcázar *et al*., 2007); improvements in nutrition and growth performance (Irianto and Austin, 2002; Abd El-Rhman *et al*., 2009). In addition, probiotics improved activity of gastro-intestinal microbiota of fish species (Burr *et al*. 2005). By the above reasons, the use of microbial probiotics in fish aquaculture is now widely accepted. The exact mode of action of the probiotic bacteria has not been fully elucidated. Very little is known about the relative importance of these mechanisms (Kesarcodi-Watson *et al*., 2008).

Commercially available probiotics not only include pure strains and defined mixture of specific strains, but also consortia of strains and undefined mixtures (Irianto and Austin, 2002; Balcázar *et al*., 2007). Probiotics that currently used in aquaculture industry include a wide range of taxa-from *Lactobacillus, Bifidobacterium, Pediococcus, Streptococcus* and *Carnobacterium* spp. to *Bacillus, Flavobacterium, Cytophaga, Pseudomonas, Alteromonas, Aeromonas, Enterococcus, Nitrosomonas, Nitrobacter, Vibrio* spp., yeast (*Saccharomyces, Debaryomyces*) and etc. (Irianto and Austin, 2002; Burr *et al*., 2005; Sahu *et al*., 2008).

The inclusion of non-pathogenic microorganisms in diet could exert beneficial physiological effects beyond the nutritional impact of food. Comprehensive research to more fully characterize the intestinal microbiota of prominent fish species, mechanisms of action of probiotics, and their effects on intestinal ecosystem, fish health and performance is warranted. Also, all probiotics must be evaluated for their safety before being used in fish nutrition. Despite its wider acceptance in aquaculture, many questions remain unanswered about its influence on fish physiology. The present work, focused on the role of certain physiological functions of Nile catfish *Clarias lazera* as influenced by a probiotic mixture (consists of strains of lactic acid bacteria and yeast cells) supplementation. Concurrent observations were also made on the changes in the composition of the gut flora before and after the probiotic feeding. Measurements of the serum chemical composition have been used as a reliable index to estimate nutritional conditions and gastrointestinal bacterial profile changes.

MATERIALS AND METHODS

Fish:

Clarias lazera (average length 38.5 ± 3.4 and 35.2 ± 3.2 cm for males and females respectively) were obtained from commercial fisheries in Giza governorate. The health status was examined immediately upon arrival according to the methods described by Austin and Austin (1989). Fish were acclimatized in freshwater glass aquaria (100 X 50 X 50 cm) for 2 weeks, maintained in aerated de-chlorinated tap water and fed commercial diet (25 % protein) in a ratio of 3 % body weight. Glass aquaria were sterilized by potassium permanganate (4 mg/l) according to Post (1987).

Probiotic:

Avi-Lution® was developed by Agri-King, Inc., Fulton, Illinois, USA. This probiotic contains 21.6 % live yeast cells (*Saccharomyces cerevisiae*, $5X10^9$ CFU/g); 16.8 % *Enterococcus faecium* bacteria ($1X10^{10}$ CFU/g); 2.5 % rapidly proliferating strain of lactic-acid producing bacteria (*Bacillus subtilis*, $5X10^9$ CFU/g); 8 % non-digestible oligosaccharides [inulin (92 %), glucose + fructose + sucrose (8 %)]; 27.7 % yeast culture (corn germ meal, cane molasses and corn syrup); 22.1 % calcium carbonate as carrier and 1.3 % other ingredients [paraffin oil (1.1 %), sodium silico aluminate (0.2 %)] . The recommended Avi-Lution® supplementation rate of 0.05 % contributes about $275 X 10^6$ lactic acid bacteria/Kg of feed (Hooge, 2000). The mixture is available as a granular premix.

Experimental design:

After the acclimatization period, bloods and excreta of whole intestinal tract were collected from ten fish (initial group). The experimental set-up was based on fed fish commercial diet (25 % protein) with probiotic "1 kg/ton ration" (Agri-King, 2000) for 45 days either one time/week all over the experiment (W_1 group) or three times/week for the 1st two weeks then 1 time/week tell the end of experiment (W_3 group). Each group consists of 5 males and 5 females. The probiotic dose was dispersed into firm gelatinous capsules and pushed into the stomach of the fish. Fish of control group received the same gelatinous capsules free from probiotics.

Sampling:

Blood samples were withdrawn from caudal vein into Eppendorf tubes without anticoagulant in syringe and allowed to clot at room temperature for 2 h. The tubes were kept slop at 4 °C for overnight, then centrifuged at 3000 ×g for 10 min and the supernatant serum was collected. The serum was stored immediately at -20 °C until analyses.

After blood sampling, fish were externally washed with sterilized water and 70 % ethanol before opening the ventral surface to reduce potential contamination with skin bacteria. The whole intestinal tract of each fish was immediately removed aseptically with sterile scalpel and forceps (Soondeuk and Yeonhee, 1995 and Matyar *et al*., 2004). Each sample was put in sterile labeled plastic bags for bacteriological examination.

Bacteriological examination:

A loopful of intestinal content of each fish was streaked and cultured on blood agar, MacConkey agar, nutrient agar, Eosin methylene blue agar and pseudomonas agar base. All plates were incubated for 24 - 48 hours at 37 ºC. The developed colonies were identified morphologically and biochemically according to Carter and Cole (1990).

- **Bacterial count:**
  The intestinal content of each fish was stripped out with sterile forceps and weighed then placed in a sterile test tube having 1 % peptone water (pH 7.0) and serial dilutiton was done according to Danish Standards Association (2002).
- Aerobic plate count (ICMSF, 1982): All inoculated plates were incubated at 35ºC for 24 hours. Plates containing 30 - 300 colonies were used to calculate bacterial population results.
- *Enterobactereacae* count (ICMSF, 1978):
- Coliform count M.P.N. (ICMSF, 1978 and Peter *et al*., 1998): Tubes showing acid and gas productions were considered positive. The most probable number was estimated using table De Man.
- Detection of enteropathogenic *E. coli* (ICMSF, 1978 and Finegold and Martin, 1982):
- Some virulence factors of isolated microorganisms (*E. coli*, *Klebsiella* spp. and *Pseudomonas* spp.): including heamolytic activity (Beutin *et al*., 1989); congo red binding test (Gray *et al*., 1990); enterotoxin assay by infant mouse test according to Kirov *et al*. (1986) and Robins-Brown *et al*. (1993) as well as pathogenicity test according to Brook *et al*. (1985) and Palumbo *et al*. (1989).

Biochemical parameters:

Serum alkaline phosphatase (ALP, EC. 3.1.3.1) (Rosalki *et al*., 1993), total proteins (Burtis *et al*., 1999) and albumin (Doumas *et al*., 1971) concentrations were measured using Reactivos GPL Kits, Barcelona, España. Aspartate aminotransferase (AST, EC. 2.6.1.1) and alanine aminotransferase (ALT, EC. 2.6.1.2) (Reitman and Frankel, 1957), ammonia (Konitzer and Voigt, 1963), urea (Fawcett and Soctt, 1960) and creatinine (Bartles *et al*., 1972) concentrations were measured using Bio-Diagnostic Kits, Egypt. Serum immunoglobulin (IgM) concentrations were analyzed by radioimmunoassay (RIA) method using RIA Kits (Amersham International Ltd., Amersham, U.K.).

Statistical analysis:

In bacteriological and serum biochemical analysis, the significant differences ($P < 0.05$) between groups were determined by a one-way analysis of variance (ANOVA). Comparisons of sex were performed with *t*-tests. Correlation coefficients were used to determine the strength of the associations between serum biochemical parameters and intestinal total bacterial count. All statistics were performed using SPSS for Windows version 11.5 (SPSS Inc., Chicago, IL, USA).

RESULTS

Bacteriological findings:

Results of bacteriological investigation on Nile catfish (*Clarias lazera*) revealed the presence of *E. coli* and *Klebsiella* spp. in the four groups examined. It was clear that *Pseudomonas aeruginosa* failed to be isolated from W_1 and W_3 (Table 1). Studying virulence factors of the isolates revealed that all tested isolates (*E. coli*, *Klebsiella* spp. and *Pseudomonas* spp.) proved to be pathogenic for mice and were positive for Congo red binding activity as well as toxigenic in infant mouse by mean reading reached 0.087 for all tested strains.

Table 1: Bacterial isolates from different *Clarias lazera* groups

Bacterial isolates	After acclimatization	After experiment		
		Control	W₁	W₃
E. coli	+	+	+	+
Klebsiella spp.	+	+	+	+
Pseudomonas spp	+	+	-	-

Table (2) demonstrated a significant sex difference ($P < 0.05$) for the mean intestinal total aerobic bacterial count after the acclimatization group ($314.00 \pm 6.0 \times 10^4$ for males and $4.40 \pm 0.07 \times 10^4$ for females).

Table 2: Intestinal total bacterial count (T.B.C.) and some biochemical serum parameters of *Clarias lazera* after 2 weeks acclimatization period.

Parameters	Male		Female	
	Mean	± S.E.	Mean	± S.E.
T.B.C. ($\times 10^4$)	314.00	6.00	4.40	0.07*
Ammonia (µg/dl)	258.90	7.34	253.40	35.31
Urea (mg/dl)	52.93	3.58	47.61	1.46
Creatinine (mg/dl)	0.55	0.03	0.41	0.03*
ALP (U/L)	57.34	9.41	34.03	2.14*
ALT (U/ml)	11.50	0.67	7.88	0.50*
AST (U/ml)	55.75	1.01	82.53	3.09*
Total Protein (g/dl)	4.25	0.09	5.02	0.37
Albumin (g/dl)	1.67	0.08	1.88	0.08
Globulin (g/dl)	2.58	0.12	3.14	0.30
A/G Ratio	0.66	0.06	0.62	0.04
IgM (IU/ml)	11.76	1.14	4.29	0.53*

Significant values between the two sexes are represented by *: $P < 0.05$.
n = 5/sex

The results of intestinal total aerobic bacterial count; *Enterobactereacae* count and coliform count of the three groups of *Clarias lazera* after 45 days as experimental period were illustrated in Table (3). The three counts in males and females of both W₁ and W₃ fish groups were significantly lower than the respective values in control group. Also significant differences were detected between both groups with lower values in W₃ group than W₁ group. Significant sex differences in the three counts were detected in the three groups of fish except the *Enterobactereacae* count in W₃ group.

Table 3: Intestinal total aerobic bacterial count (T.B.C.); *Enterobactereacae* count and coliform count of *Clarias lazera* fed on probiotic either 1 time/week for 45 days (W₁) or 3 times/week for 14 days then 1 time/week for the rest of 45 days (W₃).

Parameter	Sex	Control		W₁		W₃	
		Mean	± S.E.	Mean	± S.E.	Mean	± S.E.
Intestinal T.B.C. ($\times 10^4$)	Male	2502.00 A	1.30	185.20 aB	0.37	9.45 ab	0.11
	Female	234.40 A	9.87*	27.44 aB	2.76*	2.11 ab	0.01*
Enterobactereacae count ($\times 10^4$)	Male	35.40 A	0.11	2.96 aB	0.01	0.15 ab	0.01
	Female	13.95 A	13.01*	0.91 B	0.08*	0.30 ab	0.25
coliform count ($\times 10^4$)	Male	1.97 A	0.01	1.08 aB	0.01	0.05 ab	0.005
	Female	0.90 A	0.63*	0.33 B	0.03*	0.02 ab	0.001*

Significant values between the two sexes are represented by *: $P < 0.05$.
Small letters (a & b) represent significant values to capital letters (A & B) respectively by LSD using ANOVA test at $p < 0.05$.
n = 5/sex/group

Biochemical findings:

Table (2) shows the values of serum biochemical parameters of *Clarias lazera* after 2 weeks acclimatization period. Significant sex differences were detected in which serum creatinine; alkaline phosphatase (ALP); alanine aminotransferase (ALT) and immunoglobulin (IgM) contents were higher in males than females, while serum aspartate aminotransferase (AST) activity was higher in female fish than males.

Tables (4 – 6) illustrate the results of the control, W_1 (fish fed on probiotic 1 time/ week for 45 days) and W_3 (fish fed on probiotic 3 times/ week for 14 days then 1 time/ week for the rest of the experiment). Concentrations of serum ammonia and urea in both sexes were significantly decreased in both treated groups compared to the control group (Table 4). Serum ammonia values in both sexes of W_3 group were significantly lower than that in W_1 group. The significant low value of serum urea between W_3 and W_1 groups was detected only in male fish. For male fish, serum creatinine content was significantly higher in W_1 group and lower in W_3 group comparing to control and it was significantly lower in W_3 group compared to W_1 group (Table 3). Significant sex differences were recorded in serum urea of the control group (female higher than male) and creatinine of the three groups (females lower than males).

Table 4: Serum ammonia, urea and creatinine levels of *Clarias lazera* fed on probiotic either 1 time/week for 45 days (W_1) or 3 times/week for 14 days then 1 time/week for the rest of 45 days (W_3).

Parameters	Sex	Control		W_1		W_3	
		Mean	± S.E.	Mean	± S.E.	Mean	± S.E.
Ammonia (µg/dl)	Male	615.60 A	4.59	320.90 aB	4.60	179.10 ab	13.88
	Female	584.40 A	26.11	313.20 aB	7.01	159.20 ab	8.48
Urea (mg/dl)	Male	38.12 A	0.48	9.42 aB	0.04	7.01 ab	0.58
	Female	54.45 A	2.89[*]	8.51 a	0.48	7.73 a	0.24
Creatinine (mg/dl)	Male	0.76 A	0.01	1.01 aB	0.05	0.71 ab	0.01
	Female	0.64	0.02[*]	0.65	0.07[*]	0.61	0.03[*]

Significant values between the two sexes are represented by [*]: P < 0.05.
Small letters (a & b) represent significant values to capital letters (A & B) respectively by LSD using ANOVA test at p < 0.05.
n = 5/sex/group

Table (5) reveals that serum ALP enzyme activities were significantly lower in male fish and higher in female fish of groups W_1 and W_3 compared to control group. ALP activity in males of W_3 group was significantly higher than that of W_1 group. ALP activities in female fish were significantly lower and higher than males of control and W_1 groups respectively. Serum ALT activities in male fish were significantly lower and higher in W_1 and W_3 groups respectively compared to control group. Serum ALT activities in both sexes of W_3 group were significantly higher than that of W_1 group. Female fish of W_3 group showed significant lower value than that of males (table 5). AST enzyme activities were significantly lower in both sexes of both treated groups compared to control group. AST value in male fish of W_3 group was significantly higher than that in W_1 group. Sex difference in AST activity was detected only in W_1 group where its value in female fish was significantly higher than that in male fish (Table 5).

Table 5: Serum enzyme activities of *Clarias lazera* fed on probiotic either 1 time/week for 45 days (W$_1$) or 3 times/week for 14 days then 1 time/week for the rest of 45 days (W$_3$).

Parameters	Sex	Control		W$_1$		W$_3$	
		Mean	± S.E.	Mean	± S.E.	Mean	± S.E.
ALP (U/L)	Male	40.72 A	0.48	24.22 aB	0.45	29.33 ab	1.60
	Female	22.37 A	3.67[*]	43.83 a	2.46[*]	40.98 a	6.65
ALT (U/ml)	Male	9.02 A	0.47	7.16 aB	0.04	11.90 <u>ab</u>	0.82
	Female	7.84 B	0.48	6.05 B	1.08	8.84 b	0.91[*]
AST (U/ml)	Male	44.86 A	0.46	22.06 aB	0.46	37.68 ab	2.79
	Female	43.51 A	0.62	36.55 a	0.38[*]	36.80 a	1.83

Significant values between the two sexes are represented by *: P < 0.05.
Small letters (a & b) represent significant values to capital letters (A & B) respectively by LSD using ANOVA test at p < 0.05.
n = 5/sex/group.

Table (6) demonstrated that serum protein levels in fish of W$_1$ group were more affected by probiotic administration than that in W$_3$ group. Serum total protein, albumin, globulin and A/G ratio in males of W$_1$ group were significantly lower than that in control group, whereas it were significantly higher in females except A/G ratio. In W$_3$ group, the significant lower values were observed only in albumin and A/G ratio of males compared to both control and W$_1$ groups.

Table 6: Serum proteins levels of *Clarias lazera* fed on probiotic either 1 time/week for 45 days (W$_1$) or 3 times/week for 14 days then 1 time/week for the rest of 45 days (W$_3$).

Parameters	Sex	Control		W$_1$		W$_3$	
		Mean	± S.E.	Mean	± S.E.	Mean	± S.E.
Total Protein (g/dl)	Male	3.89 A	0.05	3.41 a	0.05	3.32	0.27
	Female	3.45 A	0.37	4.67 a	0.16[*]	3.96	0.23
Albumin (g/dl)	Male	1.32 A	0.04	0.98 aB	0.01	0.79 <u>ab</u>	0.09
	Female	1.14 A	0.11	1.57 A	0.06[*]	1.22	0.04[*]
Globulin (g/dl)	Male	2.57 A	0.02	2.43 A	0.04	2.53	0.21
	Female	2.31 A	0.27	3.10 A	0.16[*]	2.74	0.21
A/G Ratio	Male	0.51 A	0.01	0.40 aB	0.01	0.31 <u>ab</u>	0.03
	Female	0.50	0.03	0.51	0.04[*]	0.45	0.03[*]
IgM (IU/ml)	Male	7.00 A	0.19	13.94 aB	0.45	6.93 <u>b</u>	0.89
	Female	4.37 A	0.21[*]	14.00 a	2.56	11.72 a	1.80[*]

Significant values between the two sexes are represented by *: P < 0.05.
Small letters (a & b) represent significant values to capital letters (A & B) respectively by LSD using ANOVA test at p < 0.05.
n = 5/sex/group

In female fish, serum IgM levels were significantly higher in W_1 and W_3 groups than control. Female fish had significantly lower and higher IgM values than male fish in control and W_3 groups respectively.

Female fish in the W_1 group had significantly higher values of the four parameters than males, while albumin and A/G ratio were significantly higher in females of W_3 group than males. Serum IgM level in males of W_1 group was significantly higher than that in both control and W_3 groups.

A correlation analysis was conducted to determine the strength of the association between serum biochemical parameters and viable total bacterial count; *Enterobactereacae* count and coliform count in the guts of fish (Table 7). The correlations between serum ammonia, urea and AST and TBC in the guts of both W_1 and W_3 fish-supplemented with probiotic revealed significant (P < 0.01) positive correlations in both sexes. Significant positive and negative correlations were recorded between serum ALP (P < 0.01) and total protein (P < 0.05) in one side and the TBC in the intestine – on the other side – of both male and female fish respectively.

Table 7: Correlations between intestinal total bacterial count; *Enterobactereacae* count and coliform count and different serum biochemical items of *Clarias lazera*.

Parameters	Intestinal total bacterial count (T.B.C.)				*Enterobactereacae* count				coliform count			
	Male		Female		Male		Female		Male		Female	
	r =	P <	r =	P <	r =	P <	r =	P <	r =	P <	r =	P <
Ammonia	0.961	0.01	0.936	0.01	0.964	0.01	0.572	0.05	0.966	0.01	0.673	0.01
Urea	0.998	0.01	0.965	0.01	0.998	0.01	0.574	0.05	0.878	0.01	0.603	0.05
Creatinine	- 0.265	N.S.	0.103	N.S.	- 0.257	N.S.	0.229	N.S.	0.180	N.S.	0.234	N.S.
ALP	0.895	0.01	- 0.704	0.01	0.892	0.01	- 0.492	N.S.	0.616	0.05	- 0.481	N.S.
ALT	- 0.333	N.S.	- 0.004	N.S.	- 0.328	N.S.	- 0.145	N.S.	0.025	N.S.	- 0.257	N.S.
AST	0.660	0.01	0.788	0.01	0.654	0.01	0.455	N.S.	0.252	N.S.	0.470	N.S.
Total Protein	0.618	0.05	- 0.556	0.05	0.618	0.05	- 0.572	0.05	0.566	0.05	- 0.472	N.S.
Albumin	0.849	0.01	- 0.479	N.S.	0.851	0.01	- 0.578	0.05	0.872	0.01	- 0.422	N.S.
Globulin	0.155	N.S.	- 0.545	0.05	0.153	N.S.	- 0.524	0.05	0.049	N.S.	- 0.456	N.S.
A/G Ratio	0.823	0.01	0.160	N.S.	0.826	0.01	- 0.069	N.S.	0.893	0.01	0.038	N.S.
IgM	- 0.412	N.S.	- 0.711	0.01	- 0.405	N.S.	- 0.465	N.S.	0.045	N.S.	- 0.446	N.S.

N.S.: Not significant

Serum albumin and A/G ratio showed significant (P < 0.01) positive correlations with TBC in the intestine of males, while serum globulin (P < 0.05) and IgM (P < 0.01) showed significant negative correlations in females. The correlation analysis between *Enterobactereacae* count and different serum biochemical parameters in male fish detected the same correlations recorded with TBC, while female fish detected significant (P < 0.05) positive correlations with serum ammonia and urea and negative correlation with serum albumin (table 7). Contrary to TBC, serum ALP, AST and IgM showed insignificant correlations with *Enterobactereacae* count. Also, the correlation analysis between *Coliform* count and different serum biochemical parameters in male fish detected the same correlations recorded with TBC except that serum ALP and AST showed significant (P < 0.05) positive and insignificant correlations respectively (table 7). In female fish, significant positive correlations were recorded with serum ammonia (P < 0.01) and urea (P < 0.05) whiles the rest of serum parameters did not show any significant correlations.

DISCUSSION

The alimentary tract of fish represents an interface between the external environment and the body. It's a complex ecosystem, containing large number of microorganisms and has an important influence on health and disease. The intestine is a complex multifunctional organ. In addition to digesting and absorbing feedstuff, it is critical for water and electrolyte balance, endocrine regulation of digestion, metabolism and immunity (Denev *et al.*, 2009). Microbial populations in the intestinal contents are much higher than those in the surrounding water; this indicates that the intestine provides favorable ecological niches for microorganisms. In addition, some normal bacterial microflora in water such as *Pseudomonas* spp. can be found in the intestinal tract of fish under normal conditions but may be under environmental stress produce disease (Al-Harbi and Uddin, 2003).

It is revealed isolation of *E. coli* and *Klebsiella* spp. from all studied fish groups. However, *Pseudomonas* spp. was isolated only from acclimatization and control groups (table 1). Examination of acclimatization group showed $314.00 \pm 6 \times 10^4$ in male and $4.40 \pm 0.07 \times 10^4$ in female (Table 2). The total aerobic count, *Enterobacteriacaea* count and coliform count of control group were: $2502 \pm 1.3 \times 10^4$ (♂) and $234.4 \pm 9.87 \times 10^4$ (♀); $35.40 \pm 0.05 \times 10^4$ (♂) and $13.95 \pm 5.82 \times 10^4$ (♀) and $1.97 \pm 0.005 \times 10^4$ (♂) and $0.90 \pm 0.28 \times 10^4$ (♀) respectively (table 3). Several studies have demonstrated many bacteria spp. encountered in intestine of different fish. It is generally accepted that the gastrointestinal tract of fish is an important route of infection (Birkbeck and Ringø, 2005). The present results are agreement with Cahill (1990) who isolated *E. coli*, *Klebsiella* spp. and *Pseudomonas* spp. from intestinal tract of fish. Also Soondeuk and Yeonhee (1995) proved that gram negative bacteria constituted 85 % of total isolates among these is *Pseudomonas* spp. Emikpe *et al.* (2011) recorded heavy contaminated *Clarias gariepinus* with high incidences of total aerobic count and *Enterobacteriacaea* count isolated from their stomach. In addition, Sanaa and Yagoub (2009) isolated *Enterrobacteriaecae* from intestinal content of fish and verified that the most dominant isolates were *E. coli*, *Klebsiella* spp. and *Pseudomonas* spp. Isolation of *Enterobacteriacaea* indicated public health hazards and concerns particularly isolation of some highly pathogenic agents as *E. coli* which are not usually associated with normal intestinal flora of fish and potential pathogenic organisms as *Klebsiella* spp. Moreover, isolation of *Pseudomonas* spp. is of highly importance because this bacterium plays a considerable role as potential pathogenic bacteria for human and as indicator of food quality as spoilage organism.

Bacteria attached to epithelial surfaces have been demonstrated in the gut of a variety of fresh- and saltwater fish species, and it has been suggested that this attachment is an important factor in determining whether a particular organisms colonizes in the intestinal tract (Ringø *et al.*, 2003).

The effect of probiotic was studied on W_1 and W_3 groups, in addition control group was used to clarify the effect of probiotic after and before fed on it (table 3). Examination of group W_1 revealed that total aerobic count, *Enterobacteriacaea* count and coliform count were: $185.2 \pm 0.37 \times 10^4$ (♂) and $27.44 \pm 2.76 \times 10^4$ (♀), $2.96 \pm 0.005 \times 10^4$ (♂) and $0.91 \pm 0.4 \times 10^4$ (♀) and $1.08 \pm 0.005 \times 10^4$ (♂) and $0.33 \pm 0.02 \times 10^4$ (♀) respectively. It is clear that fish fed on probiotic one time a week for 45 days showed lower bacterial count than those of control group. So probiotic affect positively the bacterial count either total aerobic count or *Enterobacteraecaea* count or even coliform count. Regarding to the second group (W_3), the total aerobic count, *Enterobacteriacaea* count and coliform count were $9.45 \pm 0.11 \times 10^4$ (♂) and $2.11 \pm$

0.01 X 10^4 ($\female$), 0.15 ± 0.006 X 10^4 ($\male$) and 0.30 ± 0.11 X 10^4 ($\female$) and 0.05 ± 0.002 X 10^4 ($\male$) and 0.02 ± 0.003 X 10^4 ($\female$) respectively. This group showed the least bacterial count as they fed on probiotic 3 times per a week for 14 days and then one time per a week for the other 45 days.

Many studies confirmed our results as probiotics currently used as feed additives of fish (Irianto and Austin, 2002 and Sahu *et al.*, 2008). Using *Bacillus subtilis* as food additives stimulate the gut immune system enhance immune and health status (Mesalhy *et al*, 2008). In addition adding *Enterococcus faecium* in food's fish increase growth performance and improve immune response (Wang *et al.*, 2008), also they enhance the digestive enzymes activities in the gut. Moreover, using *Saccharomyces cerevisiae* in fish food inhibit potential harmful bacteria, stimulate beneficial bacteria and enhance the non specific immunity (He *et al.*, 2009). Tovar *et al.* (2004) found that live yeast cell have a better growth and feed efficiency. In general, the uses of those potential probiotic stimulate the growth of preferred microorganisms that out compete potentially harmful bacteria and reinforce the organism's natural defense mechanisms. It also increases the digestive enzyme activities and enhances the growth of fish (Shunpei *et al.*, 2009).

In this study all isolates (*E. coli*, *Klebsiella* and *Pseudomonas*) were tested for virulence (the ability to produce haemolysis, to bind with simple dyes and production of enterotoxin in suckling mouse and pathogenicity to mice). The Congo red is a simple dye that can be incorporated into agar media and uptake of dye has been proved to be a virulence marker for several pathogenic bacteria (Statner and George, 1987). All isolates throughout this study gave Congo red positive with different degree in red colour. The detection of enterotoxins in isolates was considered as an indicator of enteropathogenicity. Experimental infection of the mice lead to death of infected mice and the death occurred within 1 - 4 days with mean reading reached 0.087 for all tested strains.

Probiotic agents exert a beneficial effect via a wide array of actions. These include competition for adhesion sites and resistance to colonization; competition for essential nutrients; production of antagonistic compounds against pathogens; enhancement of the immune response and diseases resistance. In addition, probiotics improving enzyme activity for feed digestibility and utilization, fish health and performance (Verschuere *et al.*, 2000 and Wang *et al.*, 2008). Adhesion to the intestinal mucosa has been suggested to enhance the ability to stimulate the immune system (Nikoskelainen *et al.*, 2003). Adhesion of some pathogens to intestinal mucus of fish was significantly reduced by probiotics.

Ammonia is a major nitrogenous metabolic waste produced continuously in the animals. It becomes highly toxic if allowed to accumulate *in vivo* even to a very low concentration. It is either excreted out or converted to urea via ornithine-urea (o-u) cycle present in their liver (Cohen, 1976). Most freshwater teleosts lacked a complete and functional o-u cycle and were incapable of effective ureogenesis from ammonia (Wilson, 1973). Two siluroid species of freshwater air-breathing teleosts, *Hetropneustes fossilis* and *Clarias batrachus,* which are primarily ammoniotelic in their aquatic habitat (Saha *et al.,* 1988), have a complete o-u cycle in their liver and kidney (Saha and Ratha, 1989). When both sexes of *C. lazera* in the present work were fed either W_1 or W_3 by the probiotic, both serum ammonia and urea were significantly decreased compared to the control (Table 4) and significant positive correlations were existed between the concentrations of both parameters and the intestinal total aerobic bacterial count; *Enterobactereacae* count and coliform count (table 7). These results may indicate the decrease of protein catabolism in fish and no

stress indices due to the action of the probiotic feeding. Working on the water for the same fish species, Abonorag *et al*. (2011) added probiotic consists of mixture of two strains of lactic acid bacteria "*Pediococcus acidilactis*, *P. pentosaceus*" and *Bacillus amylolique faciens* in addition to two strains of yeast cells "*Pichia farinose* and *Dekera anomala*". Comparison of the present results (Table 4) with Abonorag *et al*. (2011) work showed that the present results of serum ammonia and urea are significantly decreased by probiotic feeding, while probiotic addition in fish water significantly increased both serum parameters. No sex differences were observed in both ammonia and urea values in probiotic fed groups, while Abonorag *et al*. (2011) detected significant sex difference in serum ammonia of fish exposed to probiotic in water.

Enzymatic activity is mainly dependent on the transport mechanism in the liver, kidney and intestinal mucosa (Vasudevan and Sreekumari, 1998). The assessment of multiple enzymes is considered a valuable tool for indications of tissue damage, which can complement microbiological and histopathological techniques (Racicot *et al*., 1975). Very little information is available regarding serum enzymes in fish fed probiotics. According to the sex of fish, serum alkaline phosphatase (ALP) activity changed differently in the present work after feeding probiotic. In both fed-groups significant lower and higher values were recorded in the male and female fishes respectively than its respective control groups (table 5) and the significant difference of ALP values between W_1 and W_3 fed-groups was observed in male fish only. On the same fish species, Abonorag *et al*. (2011) observed significant higher values of serum ALP were observed in both sexes of fish exposed to probiotic in water and significant sex difference was agreement with the present work. References on different fish species demonstrated a variety of changes in serum ALP due to probiotic feeding. Hoseinifar *et al*. (2011) found that serum ALP levels were not affected by dietary prebiotic oligofructose. Also, Kumar *et al*. (2006) reported no significant change in serum ALP activity during feeding Indian major carp (*Labeo rohita*) with *Bacillus subtilis* as probiotic and the decreased ALP activity in the *B. subtilis* fed groups attributed to the effect of *B. subtilis* on the immune system, which conferred sufficient protection to the vital organs of fish. Khattab *et al*. (2004) showed significant decrease in plasma ALP of Nile tilapia (*Oreochromis niloticus*) with increasing probiotic levels in the diet. On the other hand, Panigrahi *et al*. (2010) observed that the viable freeze-dried probiotic *Lactobacillus rhamnosus* significantly elevated plasma ALP activity in rainbow trout after feeding for 20 and 30 days. They attributed this elevation to the up-regulation of this enzyme resulting from the increased availability of certain minerals or their retention. Highly significant ($P < 0.01$) positive and negative correlations between the intestinal total aerobic bacterial count and serum concentration of ALP were demonstrated in both male and female fishes respectively (table 7). The significant positive correlations were also detected with both *Enterobactereacae* count and coliform count in male fish only.

Values of serum alanine aminotransferase (ALT) were significantly lower and higher in male *C. lazera* fed W1 and W3 respectively compared to control, while in female fish both treatments did not indicate significant values (table 5). On the other hand, serum aspartate aminotransferase (AST) in both sexes showed significant lower values than controls in the two feeding groups. Abonorag *et al*. (2011) mentioned that no serious changes occurred in the serum ALT and AST after exposing *C. lazera* to the probiotic in water. Khattab *et al*. (2007) recorded significant decrease in plasma AST and ALT in *Oreochromis niloticus* fed with diet containing mixture of *Micrococcus luteus* and *Pseudomonas* species as probiotic. Also, Palikova *et al*.

(2004) and Abd El-Rhman *et al.* (2009) reported the same observation in *Cyprinus carpio* and *O. niloticus* fed diet containing extract of *Cyanobacteria* and *Pseudomonas* species respectively as probiotics. Mahmoud (2012) mentioned that the highest values of plasma AST and ALT were observed in *O. niloticus* fed on a diet supplemented with yeast (*Saccharomyces cerevisiae*), while the lowest value of AST was recorded in fish group fed on a diet supplemented with *Bifidobacterium bifiduim*. The increase in the activities of serum AST and ALT during feeding Indian major carp (*Labeo rohita*) with *Bacillus subtilis* as probiotic was reported by Kumar *et al.* (2006). In the present work, serum AST in both sexes were obviously ($P < 0.01$, + ve) affected by the intestinal total aerobic bacterial count and by the *Enterobactereacae* count in male fish only (table 7).

Sex of fish plays an important role in the changes of serum total protein, albumin, globulin and albumin:globulin ratio of *C. lazera* after feeding with the present probiotic. Males of W_1 feeding group showed significant lower values of these serum items than the control ones, contrary to that females showed significant higher values (table 6). Serum total protein level is measurable humoral component of the non-specific defense mechanism, and the elevated levels of the total serum protein and globulin indicates that fish are immunologically strong (Nayak *et al.*, 2004). Very low levels of serum total protein have significance in relation to infectious disease, kidney damage and nutritional imbalance in fish (Wedemeyer and McLeay 1981). Wang *et al.* (2008) indicated no remarkable difference in the total serum protein, albumin content, globulin concentration and A/G ratio between the *Enterococcus faecium* fed tilapia and the control fish. Kumar *et al.* (2006) described an increase in the serum protein and globulin levels in the *Bacillus subtilis* feeding major carp and thought to be associated with a stronger innate response in fish and the decrease in the A/G ratio is indicative of better immunity of the animal. Khattab *et al.* (2007) mentioned that the plasma total protein was significantly decreased in *Oreochromis niloticus* fed with diet containing mixture of *Micrococcus luteus* and *Pseudomonas* species as probiotic. Mahmoud (2012) mentioned that the highest value of plasma albumin was recorded in fish fed on a diet supplemented with *Lactobacillus acidophilus*, while the highest value of globulin was achieved with fish group fed on a diet supplemented with bacterial mixture (*Lactobacillus acidophilus*, *Streptococcus thermophilus* and *Bifidobacterium bifiduim*) and yeast (*Saccharomyces cerevisiae*). On the other hand, the lowest values of total protein and albumin were recorded in fish group fed on a diet supplemented with *Bifidobacterium bifiduim*. The results of Bandyopadhyay and Das Mohapatra (2009) supported the use of probiotic (*Bacillus circulans*) to be an important immunostimulant in *Catla catla*. They concluded that the lowest significant A/G ratio observed in sera of fish fed *B. circulans* might be due to increased lymphocyte proliferation and subsequent immunoglobulin production.

IgM is an important immunoglobulin class. It is the first immunoglobulin to appear in evolution and commonly the only immunoglobulin class described in fish (Magnadóttir, 1998). The concentration of IgM in serum from several fish species has been surveyed (Israelsson *et al.*, 1991) and the results obtained by Magnadóttir (1998) fall within the range reported ($0.7–17$ mg ml^{-1}). Several studies have also demonstrated considerable individual variations in serum IgM levels amongst fish. This may be related to size/age (Klesius, 1990; Magnadóttir *et al.*, 1999$_b$), the environmental conditions (Olesen and Vestergard-Jørgensen, 1986; Klesius, 1990; Magnadóttir *et al.*, 1999$_a$) or the disease status (Magnadóttir *et al.*, 1995). Our study indicated that probiotic feeding significantly raised serum IgM in both sexes of *C. lazera* fed either W_1 or W_3 (table 6). The probiotic effect on serum IgM was more

obviously in W_1 group. In fish little has been done in terms of studying, how the immune system is affected upon probiotic stimulation. Working on rainbow trout (*Oncorhynchus mykiss)*, both Panigrahi *et al*. (2004) and Kamgar *et al*. (2013) found that IgM rate was increased in the *Lactobacillus rhamnosus*- and *Bacillus subtilis*-fed fish respectively. Panigrahi *et al*. (2005) reported that the plasma total Ig level of all the probiotic fed rainbow trout groups irrespective of the form of lactic acid bacteria (LAB) showed an increasing trend at 20 days. Thereafter, the level of total immunoglobulin decreased in all LAB fed groups reaching levels similar to that in control group of fish at 30 days suggesting that the stimulation of the immune system was a short-term phenomenon attributable to the probiotic. Upon withdrawal of the probiotic diets, the LAB disappeared from the intestine and the elevated immune parameters returned to the prefed level. It has been demonstrated that viable LAB are more efficient at enhancing certain aspects of immune function than the non-viable heat-killed form. The heightened immune response observed by Panigrahi *et al*. (2004; 2005) can be concluded that the probiotic *L. rhamnosus* elicits the fundamental non specific immune parameters like phagocytosis, lysozyme and serum alternate complement activities in rainbow trout. Besides, the gut colonization is influenced by the level and duration of feeding. The results of Nikoskelainen *et al*. (2003) and Tukmechi *et al*. (2007) showed that the total plasma immunoglobulin level was increased significantly in rainbow trout (*O. mykiss*) that supplemented the *Lactobacillus rhamnosus* in the diet. The ability of *Lactococcus lactis*, *Lactobacillus sakei* and *Leuconostoc mesenteroides* to modify the intestinal microbiota and stimulate the plasma total immunoglobulin level in brown trout (*Salmo trutta*) was elucidated by Balcázar *et al*. (2007). Also, Wang *et al*. (2008) showed the bacterial strain *Enteroccus faecium* treated as probiotic succeeded in improving certain immunological parameters of tilapia. In the present work, no significant correlations were detected between serum IgM and any types of bacterial counts (table 7) except that female fish showed significant negative correlation between serum IgM and intestinal total bacterial count. The last decade have been demonstrated that many probiotic agents stimulate certain cellular and humoral functions of the immune system and have a positive effect on fish health (Panigrahi *et al*., 2004, 2005; Balcázar *et al*., 2007).

CONCLUSION

Avi-Lution probiotic has good effect on the catfish *Clarias lazera* not only by reducing the counts of *E. coli, Klebsiella spp.* and *Pseudomonas* spp. but also by enhancing the physiological functions and the immunity of the fish.

ACKNOWLEDGEMENT

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ARABIC SUMMARY

تغيرات المحتوى البكتيرى للأمعاء والمؤشرات البيوكيميائية لمصل أسماك قرموط النيل المغذاة بالبروبيوتيك.

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تم تصميم التجربة على أساس تغذية أسماك قرموط النيل بعلف تجارى مضاف له البروبيوتك لمدة ٤٥ يوم إما مرة واحدة كل أسبوع طوال فترة التجربة (W_1) أو ثلاث مرات كل أسبوع لمدة أسبوعين ثم مرة واحدة كل أسبوع لباقى فترة التجربة (W_3). تم أجراء الأختبارات البكتريولوجية والبيوكيميائية على القناة الهضمية ومصل الدم على التوالى.

أوضحت الأختبارات البكتريولوجية وجود ميكروبى *E. coli* و *Klebsiella* spp. فى كل مجموعات الأسماك المفحوصة ولم يتم عزل ميكروب *Pseudomonas aeruginosa* فى المجموعتى W_1 و W_3 وكانت كل السلالات المعزولة مرضية للجرزان وإيجابية للأتحاد بصبغة الكونجو الأحمر بالأضافة للتأثير السمى على الجــرزان الرضــيعة. وكــان العــد الأجمــالى للبكتيريــا الهوائيــة بالقنــاة الهضــمية والعــد لميكروبــات *Enterobactereacae* والعد لميكروبات coliform فى كل من ذكور وأناث الأسماك لكلتا المجموعتين (W_1 و W_3) أقل معنوياً من مثيلتها فى المجموعة الضابطة. وكان هناك أيضا أختلافاً نوعيا بين كل من المجموعتين مع قيم أقل فى المجموعة W_3 عنها فى المجموعة W_1. وتم تباين أختلافاً معنوياً فى قيم الثلاث أعداد من البكتريا بين جنسى السمكة فى كل المجموعات المدروسة ماعدا عد ميكروب *Enterobactereacae* فى مجموعة W_3.

ووجد أن تركيز كلاً من الأمونيا واليوريا فى المصل أقل معنوياً فى المجموعتين المغذاة بالبروبيوتك مقارنة بالمجموعة الضابطة وكانت المجموعة W_3 أقل معنوياً عن المجموعة W_1. كما تم تسجيل أختلافاً معنوياً فى تركيز الكرياتينين بالمصل بين الجنسين فى المجموعات الثلاثة بحيث كان التركيز فى الأناث أقل من الذكور.

وقد أختلفت أنشطة أنزيمات الفوسفاتيز القلوى والألنين أمينوترانسفيريز والأسبارتات أمينوترانسفيريز فى المصل معنوياً تبعاً لجنس السمكة. وكان الأتجاه العام لنشاط هذه الأنزيمات عالياً فى المجموعة W_3 عنها فى المجموعة W_1.

كــان تــأثير أضــافة البروبيوتيـك علـى البروتين الكلـى والألبيومين والجلوبيولين ونسبة الألبيـومين للجلوبيولين فى مصل أسماك المجموعة W_1 أكثر من المجموعة W_3. وتأثرت هذه المكونـات البروتينيـة فى كل المجموعات معنوياً بجنس السمكة. وكان الأميونوجلوبيولين م "IgM" فى مصل كلا الجنسين للمجموعة W_1 أعلى معنوياً عنها فى كل من المجموعتين الضابطة و W_3.

وتـم أجراء تحليـل أرتبـاط لبيـان قوة الأرتبـاط بيـن المؤشـرات البيوكيميائيـة فـى المصـل والعـد الكلـى للميكروبات الحية والعد لميكروبات *Enterobactereacae* والعد لميكروبات coliform فى أمعاء الأسماك. فكان هناك تشابه فى تحاليل الأرتباط بين الثلاث أعداد للبكتيريا والمؤشرات البيوكيميائية المختلفة فى ذكور الأسماك بينما تم أستبيان بعض الأختلافات فى الأناث تبعا لنوع العد البكتيرى.