Pathological Evaluation of Probiotic, *Bacillus Subtilis*, against Flavobacterium columnare in Tilapia Nilotica (*Oreochromis Niloticus*) Fish in Sharkia Governorate, Egypt

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Abstract: Fifteen out-of eighty-five of collected *Tilapia nilotica* fish (17.64%) showing skin lesions, were positive for *Flavobacterium columnare* with cultural, morphological and biochemical characteristics. These skin lesions were large erosions with loss of scales and red-grayish patches, particularly at the frontal head region and abdomen. All of the positive isolates (*Flavobacterium columnare*) were molecularly tested by means of PCR. With consistent with *F. columnare* standard ATCC 49512 strain, these isolates produced a 675 bp band. One hundred apparently healthy *Tilapia nilotica* fingerlings (30±5 gm) were used to evaluate the effectiveness of probiotic, *Bacillus subtilis*, in water or diet against the intramuscular challenge with *Flavobacterium columnare* infection. They were equally divided into 10 groups (10 fish for each group). Five groups were experimental control {placebo (gp 1), intramuscularly infected with 0.2 $x10^8$ *F. columnare* CFU (gp 2), received 0.1 gm/L probiotic in water (gp 3), 0.2 gm /L probiotic in fish diet (gp 4), or 1 gm/L oxytetracycline (gp 5)}; two were prophylactic experiment {received 0.1 (gp 6) or 0.2 (gp 7) gm of probiotic in water and diet, respectively 2 months before bacterial infection and continued for a week later}; and three were treated experiment {intramuscularly infected with 0.2 $x10^8$ *F. columnare* CFU and then received the probiotic in water (gp 8), diet (gp 9) or 1 gm/L oxytetracycline for a week (gp 10)}. Specimens from the skin, gills, liver, kidney and intestine were collected, fixed in 10% buffered neutral formalin solution and were routinely processed for pathological examination. Exposure of the fish to *F. columnare* infection produced focal coagulative necrosis, ulcerations besides severe hydropic and spongiosis in the epidermis, particularly at the necrotic areas of the fins and heavily infiltrated with granulocytes and few lymphocytes. The dermis was infiltrated with neutrophils and the underlying muscles were necrotic. The gills showed coagulative necrosis in the gill-filaments with neutrophils infiltration and few extravasated erythrocytes. Focal proliferation of the respiratory epithelium was noticed particularly those covering the secondary lamellae which frequently sloughed. The probiotic (prophylactic experiment) in water or diet was alleviated the lesions of the *Flavobacterium columnare* infected fish with an increase of water quality, while such changes were still similar to those described with infected fish in probiotic treated experiment. The oxytetracycline-treated group showed significant reduction of these lesions and the treated fish appeared normal. Collectively, it could be concluded that the probiotic, *B. subtilis*, in water or diet (as prophylaxis) are effective in amelioration the lesions of *F. columnare* infections that have wide spread among Egyptian freshwater fish. Oxytetracycline is the drug of choice to treat such disease and minimize the lesions of *F. columnare*.
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1. Introduction:

Columnaris disease previously referred to as myxobacterial infection and reported by Davis in 1922. It remains one of the most frequently encountered and devastating bacterial diseases of freshwater fishes. This disease is also known as saddleback disease, cotton-wool disease and fin rot. These names reflect the gross lesions of affecting fish. Columnaris disease is caused by the Gram-negative bacterium (*Flavobacterium columnare*). Such bacteria can infect fishes of any age, under a variety of water condition and during any season of the year (Griffin 1992). Acute disease is characterized by an incubation period of less than 24 hrs and the resulting mortalities are seen two to three days post exposure (Holt et al 1975).

Columnaris is a contagious disease that can be transmitted horizontally through direct contact and skin wounds as well as through orofecal route (Bullock et al 1986, Welker et al 2005 and Austin and Austin 2007). Due to the ubiquitous nature of the *F. columnare* in freshwater, an injury to the skin or gills of fish with elevation of water temperature may quickly initiate the Columnaris infection. The disease can be clinically diagnosed through its characteristic clinical picture in the affected fish. In scaly fishes, the infection initially appears as milky veiled erosions on the dorsal aspect of fish body which may progress to extensive ulcers as dull whitish or yellow necrotic areas (Sakr 1996). The infected skin loses its natural sheen and a gray, white or yellowish margin surrounds the focal lesions. The mouth and inner walls of the oral cavity may be covered with a yellowish mucoid material (Kumar et al 1986). In scaly-less fishes, the lesions start with simple ulcers which predominately end with extensive saddleback-like ulcers exposing the underlying musculatures (Morrison et al 1981). Fin and gill rot is another lesion of the progressive infection in both scaly and scaly-less fishes (Bullock et al 1986 and Latremouille 2003). The histopathology may provide useful information concerning the severity of infection. The gills showed branchial epithelial cells and goblet cells hyperplasia. These lesions rapidly progress to severe neutrophilic inflammation and gill necrosis. Erosions and ulceration of the epidermis besides necrosis and edema in the underlying muscles were recorded in the affected skin (Eissa et al 2006 and Rasha 2008).

Nowadays, we have the ability to diagnose Columnaris disease by modern molecular methods: such as polymerase chain reaction (PCR) based techniques employing species-specific primers (Wakabayashi and Riyanto 1999, Darwish et al 2004, Bader et al 2003 and Eissa et al 2010) and by DNA sequence analysis (Tiirola et al 2002, Thomas-Jinu and Goodwin 2004 and Zhang and Arias 2009).

Currently, common control measures of the diseases focused on the use of antibiotics and chemical agents. The potassium permanganate at concentration of 2 ppm for 8-10 hours (Francis-Floyd 1998) and copper sulfate 1mg/L (Thomas-Jinu and Goodwin 2004) can be treated Columnaris disease. Moreover, Columnaris disease is most effectively treated mainly with tetracycline (Hawke and Thune 1992 and Smith et al 1994). Soltani et al (1996) reported the minimal inhibitory concentration of amoxicillin (0.06µg/ml), oxytetracycline (0.06-0.12µg/ml), oxolinic acid (0.06-0.12µg/ml), norfloxacin (0.12µg/ml) and trimethoprim (>64µg/ml). However, the use of antibiotics as treatment has been problematic by acquisition of genes that result in antibiotic resistant strains of bacteria and residues of antibiotics can diffuse into the aquatic environment (Ellis 1991 and Smith et al 1994). The use of some antibiotics, disinfectants and other chemical agents to control fish diseases in pond have been prohibited because resistance can develop in environmental bacteria, negative effect on human health and restriction for export commodities (WHO 2006).

An alternative method to prevent and cure fish disease is by using probiotic. Probiotic is defined as microbial cell preparation or components of microbial cells, which have a beneficial effect on the health and well being of the host (Vivas et al 2004, Bagheri et al 2008, Zhou et al 2009 and Son et al 2009). Other benefits of probiotic in aquaculture are competitor for nutrient, source of nutrients and enzymatic contribution to digestion and improve water quality (Abraham et al 2008 and Dalmo and Bogwald 2008).

Common probiotic product used in aquaculture such as *Bacillus species* can improve water quality by reducing the number of bacteria pathogens in farm (Wang et al 2008). Furthermore, several researchers have been proved the effectiveness of probiotic in fish and shrimp to resist to pathogen (Liu et al 2010), *Aeromonas salmonicida* (Irianto Austin 2002) and enhance immunity (Randelli et al 2008 and Nayak 2010). Moriarty (1998) and Balcazar and Rojas-Luna (2007) reported that probiotic *Bacillus species* can reduce number of *Vibrio species* in penaeid cultured pond. He was observed after fed with feed containing probiotic *Bacillus subtilis* for 14 days. Significant increase in respiratory burst activity, differential leukocyte count (neutrophil and monocyte) and serum bactericidal activity are seen in comparing with the control (Aly et al 2008, Kumar et al 2008 and Marzouk et al 2008). Nayak et al (2007) reported that after Indian carp were fed with probiotic *B. subtilis* for 60 days, the total serum protein and globulin content were significantly higher in probiotic fed group than the control.

Probiotic containing in fish food can build up the beneficial bacterial flora in skin and intestine while they grow competitively over certain bacteria (Ziaei-Nejad et al 2006 and Abd El-Rhman et al 2009). *Flavobacterium columnare* grows by stacking adhesion layer on fish skin and the consequence of colonization caused skin damage lesion and thus, including fish mortality. However, manipulation of the composition of the bacterial community on fish skin may be used in prevention to Columnaris disease. This studyencourages work on more ecological and natural method to prevent *F. columnare* infection in Nile tilapia by using commercial probiotic product *B. subtilis*.

The objective of this work was to survey the prevalence of natural infection with *F. columnare* and to evaluate the effectiveness of commercial probiotic product, *Bacillus subtilis* as competitor in prevention against experimental infection on *Tilapia nilotica*, employing the pathology besides the PCR for confirmative diagnosis in naturally infected fish.

2. Materials and methods

Fish:

A total of 85 tilapia species, showing skin-lesions (erosions, ulceration and fin or tail rot), were collected from Abbassa Fish Farm in Sharkia governorate. The clinical signs were recorded and fish were transferred alive in plastic bags to the laboratory and subjected to full microbiological and Real time PCR-isolation of *Flavobacterium columnaris*.

Bacteriological Isolation and Identification:

Sterile bacteriological swabs from skin lesions were cultured into tryptic soy broth (TSB) and onto Hsu-Shotts agar (Bullock et al 1986) or Cytophaga agar plates (Farmer 2004). The inoculated broth and agar plates were inoculated at 25ºc for 48-72 hours. Bacterial isolates were presumptively identified using broth cultural characteristics and conventional biochemical tests recommended by Griffin (1992).

Molecular Identification:

Chromosomal DNA was extracted from 100 µl of bacterial suspension (a single colony of each of the isolated bacteria suspended in 100 µl of sterile saline) using DNeasy tissue extraction kit (QIAGEN, Valencia CA) according to the manufacture's instructions. The extracted DNA was amplified using specific primer set specifically for *F. columnare* (Darwish et al 2004). Two specific primers were efficiently used {Forward (598-CAGTGGTGAAATCTGGT-614) and Reverse (1260-GCTCCTACTTGCGTAGT-1276)}. The negative controls consisted of a PCR mixture with molecular grade water (negative QC control) and another with *Pseudomonas florescence* DNA template (negative QA control). Positive control (FC+) consisted of a PCR mixture with DNA extracted from known *F. columnare* (ATCC 49512). Thermal cycling and amplification procedures were done according to the method described by Darwish et al (2004).

Experimental Design:

One hundred apparently healthy *Tilapia nilotica* fingerlings (30±5 gm) were equally divided into 10 groups (10 fish for each group). Five groups were experimental control {placebo (gp 1), intramuscularly infected with 0.2 x10^8 *F. columnare* CFU (gp 2), received 0.1 gm/L probiotic in water (gp 3), 0.2 gm /L probiotic in fish diet (gp 4), or 1 gm/L oxytetracycline (gp 5)}; two were prophylactic experiment {received 0.1 (gp 6) or 0.2 (gp 7) gm probiotic in water and diet, respectively 2 months before bacterial infection and continued for a week later}; and three were treated experiment {the fish firstly infected with 0.2 x10^8 *F. columnare* CFU and then received the probiotic in water (gp 8), diet (gp 9) or 1 gm/L oxytetracycline for a week (gp 10)} as shown in table (1). All fish were daily observed for the clinical signs and mortalities and then they were sacrificed at the end of each experiment.

Probiotic and its Safety:

The probiotic, *Bacillus subtilis* (ATCC 6633), was obtained as lyophilized cells from Sigma. This probiotic was prepared according to previous studies (Liu et al 2009 and Tseng et al 2009). The fish were daily immersed in the probiotic at 3 constant times (bath exposure) or thoroughly mixed with the diet (*ad libitum*). The safety of such probiotic was evaluated by absence of the clinical sign, lesions and mortalities (Aly et al 2008).

Water quality assessment:

Water temperature and pH were measured using a waterproof digital combo pH meter and thermometer {HI98127 (pHep 4)-Hanna instruments Inc., RI, USA}. Dissolved oxygen (DO) concentration were measured using a digital dissolved oxygen meter (HI 9142-Hanna instruments Inc., RI, USA). Total ammonia nitrogen (TAN mg/L) was determined following the method described by Chattopadhyay (1998).

Histopathological Examination:

Specimens from the examined organs (skin, gills, liver, kidney and intestine) were collected after necropsy and fixed in 10% buffered neutral formalin solution. Five-micron thick paraffin sections were prepared, stained by hematoxylin and eosin HE and then examined microscopically for histopathology (Bancroft and Stevens 1996).

3. Results

Prevalence and Microbiological Findings:

Fifteen out-of eighty-five of collected *Tilapia nilotica* fish (17.64%) showing skin lesions, were positive for *Flavobacterium columnare* with cultural, morphological and biochemical characteristics (table, 2). All of the positive isolates (*Flavobacterium columnare*) were molecularly tested by means of PCR. Consistent with *F. columnare* standard ATCC 49512 strain, these isolates produced a 675 bp band (Fig 1).

Water Quality Parameters:

Water quality parameters of the affected aquaria including pH, temperature, DO and TAN, were determined in different groups and compared to the control one (table, 3).

Table (1): Groups and treatments of experimental study.

Gps	Classifications	Treatments				
		Flexibacter Infection 0.2 x 10^8 CFU	Prob. in water 0.1 gm/ L	Prob. in ration 0.2 gm/ Kg	Oxytetracycline 1 gm/ 10 L	Withdrawal 2 weeks later
1	Experimental control	-	-	-	-	-
2		+	-	-	-	-
3		-	+	-	-	-
4		-	-	+	-	-
5		-	-	-	+	-
6	Prophylactic	+	+ *	-	-	+
7		+	-	+ *	-	+
8	Treatment	+	Simultaneously	-	-	-
9		+	-	Simultaneously	-	-
10		+	-	-	Simultaneously	-

2 months before infection and a week after (simultaneously) then 2 weeks withdrawal.

Pathological Findings:

Groups (1, 3 and 4): control or received probiotic, *Bacillus subtilis*, in the water (0.1gm/L) and diet (0.1gm/L).

Neither gross nor microscopic abnormalities were seen in the skin, gills, liver and kidney.

Groups (2, 8 and 9): *Tilapia nilotica* infected with 0.2 x 10^8 CFU of *Flavobacterium columnare* or they simultaneously received the probiotic in the water and ration as treatment.

Clinical Signs and Postmortem Examination:

The infected fish showed loss of appetite and respiratory distress. They swam near the water surface, gasping and engulfing the atmospheric air. The fish showed rapid movement of the opercula with nervous manifestation and 70% mortalities. The postmortem lesions were similar to natural infected fish. The skin lesions were presented as large erosions with loss of scales and red-grayish patches (plaques), particularly at the frontal head region and abdomen. Some frontal ulcers were deeply penetrated the skull to form hole-in-the head-like lesion (head cysts). Hemorrhagic small ulcers surrounded by red eroded zone were visualized on the pelvic and anal areas. Sometimes, the ulcers appeared yellow or orange in color. Hemorrhage was noticed at the base of the pectoral fins besides fragmented or frayed caudal fin with edematous, grayish discolored margin. Necrosis of the membranous portion of the caudal fin (fin-rot) was also detected. The gill filaments were congested, swollen and covered with profuse mucus. The visceral organs (liver, spleen and kidney) were congested. Sometimes, corneal opacity was recorded (Fig 2).

Histopathological Findings:

The significant lesions were restricted to the skin and gills besides septicemic lesions in the other organs. The skin showed focal coagulative necrosis and severe spongiosis in the epidermis, particularly at the necrotic areas of the fins and heavily infiltrated with granulocytes, EGC and few lymphocytes (Fig 3). Sometimes, the epidermal necrosis and pustules (Fig 4) was serious in the affected areas, sloughing off to induce erosions or ulcerations (Figs 5 and 6). These ulcers showed masses of basophilic bacterial colonies. Spongiosis and hydropic degeneration were observed with mucous cell proliferation and mucous cysts formation, which showed eosinophilic mucus (Fig 7).The ulcerations spread by radial expansion and may penetrate into deeper tissues, producing necrotic dermatitis, myositis and perimyosititis (Fig 8). The affected dermis was infiltrated with neutrophils and the underlying muscles were necrotic (Fig 9). Excessive edema and hemorrhage were seen among the necrotic skeletal muscle fibers (Fig 10). The latter revealed central myolysis and gradually disappeared and replaced by cavitations that empty or filled with fine eosinophilic granular material. The edematous fluid was usually entrapped leukocytes. The dermal capillaries were congested, especially in the areas of ulcerations. Some ulcers were undergoing healing by granulation tissue formation which was infiltrated by leukocytes and a great number of melanin-carrying cells and extended to the underlying necrotic muscles. Inflammatory cell aggregations together with numerous EGC were noticed, particularly near the bony elements. The gill infections are less common but more serious. Columnaris begins at the tips of the lamellae and causes a progressive necrosis that extends to the base of the gill arch. The affected gills showed coagulative necrosis in the gill-filaments with neutrophils infiltration and few extravasated erythrocytes (Fig 11). Focal proliferation of the respiratory epithelium was noticed particularly those covering the secondary lamellae which frequently sloughed (Fig 12). Telangiectasia of the branchial blood capillaries and hemorrhages besides congestion of the lamellar blood capillaries and focal aneurysms were detected (Fig 13). The interlamellar spaces were obliterated by epithelial and mucous cells hyperplasia as well as neutrophils infiltrations (Fig 14). Large basophilic bacterial clumps were tightly adhered to the primary lamellae, within the mucous cells and on the necrotic areas. Sometimes, lamellar epithelial degeneration, exfoliation, fusion and clubbing were mildly visualized besides few inflammatory cells infiltrations that leading to lamellar thickening. The most severe lesions were seen in the gill arches. These lesions were represented by multifocal ballooning degeneration, spongiosis, and hydropic degenerations and necrosis (Fig 15). Hypertrophy and hyperplasia of the mucous cells were noted. Edema, congestion, hemorrhages and leukocytic infiltrations mainly with neutrophils and lymphocytes were detected. These cells were extended to the submucosa and lamina propria. The basement membrane of the superficial epithelium of the gill-arch was hyalinized. Sometimes, the gill rakers showed necrosis and complete sloughing of the lining epithelia. The liver revealed coagulative necrosis and neutrophils infiltrations (Fig 16). Focal vacuolations and hydropic degeneration were seen besides congestion of the hepatic blood vessels. The pancreatic acini were atrophied and showed focal coagulative necrosis (Fig 17). The blood sinusoids and the central veins were highly congested. Sometimes the epithelial lining of the bile ducts showed vacuolar degeneration and others revealed hyperplasia in the lining epithelia. Focal replacements of the hepatic parenchyma with fibrous connective tissue infiltrated with numerous macrophages, lymphocytes and polymorphnuclear cells were detected. The kidney revealed multifocal areas of coagulative necrosis and hemorrhages (Fig 18). Moderate hydropic degenerations in the lining epithelium of the renal tubules and shrunken glomeruli were seen. Sometimes, the glomeruli were contracted with dilated Bowman's capsule with eosinophilic material. Focal congestion, hemorrhages and depletion of the hemopoietic elements were uncommon besides activation of the melanomacrophages centers. Focal area of fibrosis and mononuclears were seen replacing the renal tissue. Some renal tubules showed hyaline droplet degeneration and few interstitial leukocytes infiltrations.

Group (5): received 1 gm / 10 L of oxytetracycline.

Clinical Signs and Postmortem Examination:

Loss of balance, loss of appetite and sluggish movement were the most common clinical signs. No mortalities were recorded.

Histopathological Findings:

The liver of treated fishes showed diffuse vacuolations (Fig 19) and hydropic degeneration besides necrotic changes in the pancreatic acini. Multiple areas of interstitial aggregations of mononuclear cells were observed throughout the hepatic parenchyma (Fig 20). The hepatic blood vessels and sinusoids were congested. The kidney revealed focal hydropic degeneration and individual-cell necrosis in the affected renal tubular epithelium (Fig 21). The hemopoietic cells were focally necrotic with activation of the melanomacrophage centers. The gills showed severe congestion of lamellar capillaries (Fig 22). The skin was normal.

Groups (6 and 7): received the probiotic in the water and diet then infected with 0.2×10^8 CFU of *Flavobacterium columnare* (prophylactic experiment).

Clinical Signs and Postmortem Examination:

The affected fish were mostly normal. Sometimes, they showed dullness and darker-colored skin with slight frayed tail (Fig 23). Focal petechial hemorrhages were rarely encountered besides easily detached scales. The liver and kidneys were slight congested with congested gills. No mortalities were recorded.

Histopathological Findings:

The probiotic in water or diet was alleviated the lesions of the challenged *Flavobacterium columnare*, where it activated the skin immune system. The latter was represented by thickening of the epidermis with acanthosis and mild spongiosis with no evidence of ulcerations (Fig 24). Sometimes, the apical layers of the epidermis were sloughed and numerous melanomacrophages were detected at the junction with the underlying dermis (Fig 25). Proliferation of mucus secreting cells was focally noticed in the epidermis of some cases. The dermis revealed few lymphocytes and polymorphnuclear cells infiltrations among normal or mild degenerated muscle fibers (Fig 26). The gills showed mild proliferative changes in the respiratory epithelium with focal fusion of the secondary lamellae (Fig 27). Congestion, telangiectasis and hemorrhage were also frequent in gill filaments, rakers and arches. The liver showed moderate vacuolations of the hepatocytes and focal necrosis in the pancreatic acini (Fig 28) in some cases. Severe congestion of some hepatic blood vessels and hemorrhage were also visualized. The liver of most fishes was nearly normal. The kidney was normal except for hydropic degeneration in some renal tubular epithelia (Fig 29).

Group (10): infected with *F. columnare* and treated with oxytetracycline.

Clinical Signs and Postmortem Examination:

The experimental fish were emaciated and showed no other clinical signs. The mortality rate was 10% (1 out-of 10 fish).

Histopathological Findings:

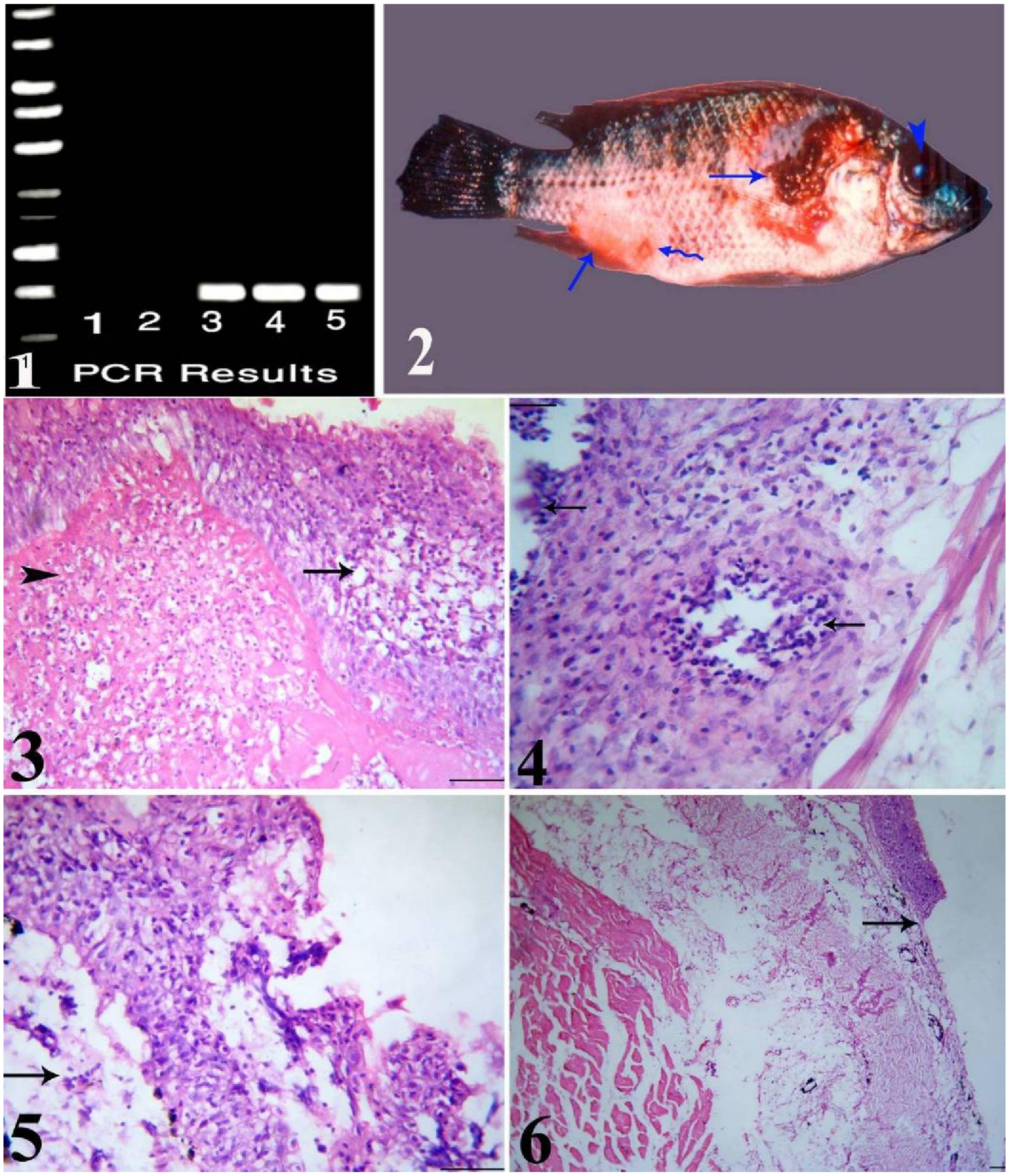
Almost all columnaris-lesions were reduced to become normal. The skin showed mild Zenker's degeneration and edema in the dermal skeletal muscles. Few round cells aggregations were also noticed. The gills were severely congested or hemorrhagic with hyperplasia in the lining epithelium and lymphocytes infiltrations (Fig 30). The liver showed mild hydropic degeneration and glycogen depletion. Mild congestion of the hepatic blood vessels was noticed. Few round cells infiltrations were detected in the portal areas and interstitial tissue. The pancreatic acini were normal. The kidney revealed few polymorphnuclear cells and lymphocytes among the renal tubules. The latter showed mild hydropic degeneration. The hemopoietic elements were normal besides activation of the melanomacrophage centers particularly in the anterior kidney.

Table (2): Morphological and biochemical characteristics of isolated *F. columnare*.

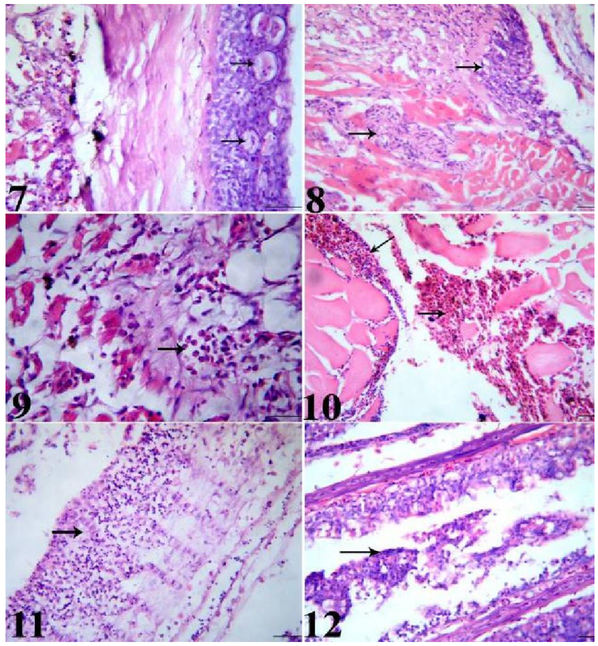
No	Morphological and biochemical characteristics	Results
1	Gram stain	Gram –ve bacilli (rod-like)
2	Gliding Motility	+ ve
3	Colony on Hsu-Shotts agar	Round, yellow and strongly adherent to the agar.
4	Chromo-shift (adding 3% KOH)	The yellow pigmented colonies change to pink
5	Glucose fermented	+ ve
6	Raffinose	- ve
7	Citric utilization	+ ve
8	Flexirrubin-type pigment	+ ve
9	Congo Red test	+ ve
10	Catalase test	+ ve
11	Gelatin hydrolysis	+ ve
12	Urea hydrolysis	+ ve
13	Indole production	- ve
14	Casein hydrolysis	+ ve
15	Starch hydrolysis	- ve
16	Nitrate reduction	+ ve
17	H2S production	+ ve
18	Growth on Trypticase Soy broth	- ve
19	Growth with 0.5% NaCl	+ ve
20	Growth with 1.0 % NaCl	- ve
21	Growth in the presence of Neomycin sulfate	+ ve

Table (3): Water quality parameters in different groups. (probiotic in water or diet was similar).

Water quality parameters	Control	Infected	Probiotic	Prob + Inf	Inf + Prob	Inf + Oxyt
Water pH	6.6	8.7	6.5	6.6	8.0	7.2
Water temperature	24º C	30º C	26º C	25º C	28º C	27º C
Water dissolved oxygen / ppm	6.5	3.1	6.6	6.0	4.5	5.0
Total ammonia nitrogen / ppm	0.4	3.2	0.65	0.9	2.1	1.6

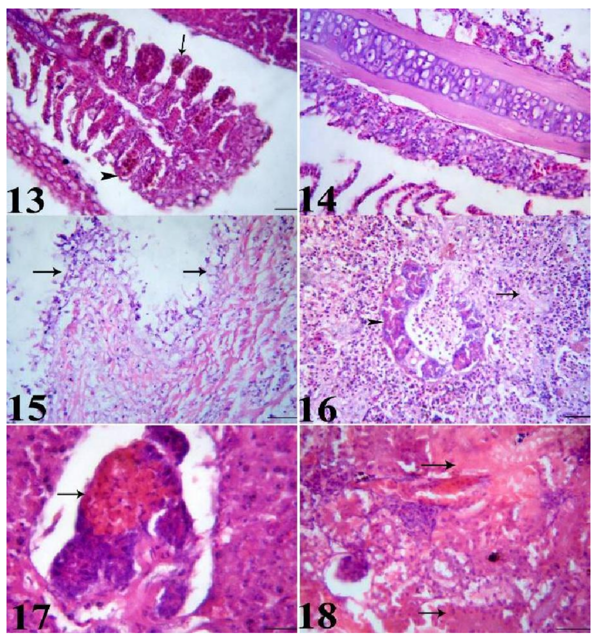


Figs (1-6): Groups (2, 8 and 9) are similar.
1-PCR-results. 2-Tilapia showing skin erosions, ulcers and grayish hemorrhagic patches. 3-Skin showing focal coagulative necrosis in the epidermis. 4-Skin showing pustule. 5-Skin showing hydropic degeneration and spongiosis with dermal edema. 6-Skin showing ulceration of the epidermis. (HE: Bar = 100 µm).



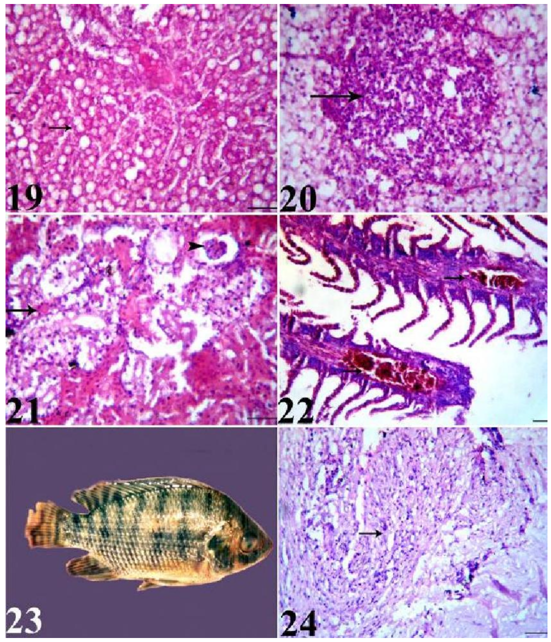
Figs (7-12): Groups (2, 8 and 9) are similar.

7-Skin showing proliferation of mucus cells with cyst formation. 8-Skin showing deep ulcer with necrotic dermatitis. 9-Skin showing neutrophils infiltrations among necrotic muscles. 10-Skin showing excessive edema and hemorrhage among necrotic muscles. 11-Gill showing coagulative necrosis in the gill-filaments with neutrophils infiltration and few extravasated erythrocytes. 12-Gill showing sloughing of the epithelial lining of the secondary lamellae. (HE: Bar = 100 µm).

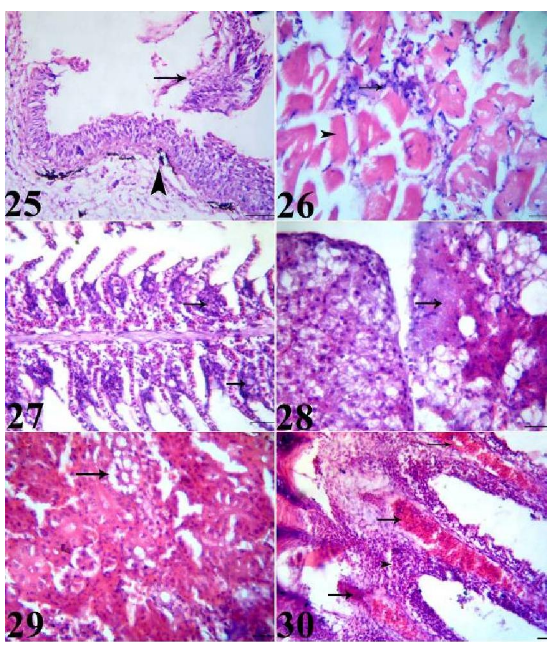


Figs (13-18): Groups (2, 8 and 9) are similar.

13-Gill showing congestion of the lamellar blood capillaries and focal aneurysms. 14-Gill showing interlamellar spaces obliterated by epithelial and mucous cells hyperplasia and neutrophils infiltrations. 15-Gill-arch showing multifocal ballooning degeneration, spongiosis, and hydropic degenerations and necrosis. 16-Liver showing coagulative necrosis and neutrophils infiltrations. 17-pancreatic acini showing atrophy and focal coagulative necrosis. 18-Kidney showing coagulative necrosis in tubular epithelia. (HE: Bar = 100 µm).



Figs (19-24): Groups (5, 6 and 7).
19-Liver (5) showing severe vacuolations in the hepatocytes. 20-Liver (5) showing interstitial aggregation of mononuclears. 21-Kidney (5) showing focal hydropic degeneration and individual-cell necrosis in the affected renal tubular epithelium. 22-Gill (5) showing severe congestion of lamellar capillaries. 23-Tilapia (6) was darker in color with slight frayed tail. 24-Skin (6) showing thickening of the epidermis by acanthosis and mild spongiosis with no evidence of ulcerations. (HE: Bar = 100 µm).



Figs (25-30): Groups (6, 7 and 10).
25-Skin (6) showing sloughed apical layers of the epidermis and numerous melanomacrophages at the junction with the underlying dermis. 26-Skin (7) showing few lymphocytes and polymorphnuclear cells among mild degenerated muscle fibers. 27-Gill (7) showing mild proliferative changes in the respiratory epithelium. 28-Liver (6) showing moderate vacuolations and focal necrosis in the pancreatic acini. 29-Kidney (7) showing hydropic degeneration in some renal tubular epithelia. 30-Gill (10) showing severe congestion and hemorrhage. (HE: Bar = 100 µm)

4. Discussion:

It is evident that the columnaris is primarily an epithelial disease of freshwater fish. It causes erosive / necrotic skin and gill lesions that may become systemic. It often presents as large erosions with loss of scales and red-grayish patches that may have a red margin on the head, back (saddleback lesion), and / or fins (fin rot) especially the caudal fin. Fragments of the fin rays may remain after the epithelium has sloughed, leaving a ragged appearance. Ulcerations with yellow or orange color were deeply seen in underlying tissue due to masses of pigmented bacteria growth (Sakr 1996 and Latremouille 2003). The gill infections are less common but more serious. Columnaris begins at the tips of the lamellae and causes a progressive necrosis that extends to the base of the gill arch (Bullock et al 1986). The gill filaments were congested, swollen and covered by profuse mucus. Such lesions were the main cause of large mortalities among infected fish. The previous findings could be attributed to the adherence and irritation of the bacteria with gill structure (Eissa et al 2010).

When the probiotic, *Bacillus subtilis,* included in the diet and in water of aquaria for 2 months before the challenge with *Flavobacterium columnare*, the fish showed no clinical signs with rare focal petechial hemorrhage and easily detached scales. No mortalities were recorded. The addition of probiotic to the water or diet of fish could improve the water quality (Eissa et al 2010) and the immune system (Randelli et al 2008 and Nayak 2010). Balcazar et al (2006) reported that the use of probiotics, which control pathogens through a variety of mechanisms, is increasingly viewed as an alternative to antibiotic treatment. Nevertheless, some possible benefits linked to the administering of probiotics have already been suggested as: competitive exclusion of pathogenic bacteria (Balcazar 2003 and Balcazar et al 2004), source of nutrients and enzymatic contribution to digestion (Sakata 1990 and Prieur et al 1990) direct uptake of dissolved organic material mediated by the bacteria (Moriarty 1997) enhancement of the immune response against pathogenic microorganisms (Irianto and Austin 2002) and antiviral effects (Girones et al 1989 and Direkbusarakom et al 1998).

The histopathological findings observed with the *F. columnare* were similar to those described by Roberts (1989) and Sakr (1996). The skin and gills, primary target organs, showed necrosis with intense neutrophilic response. Erosions and ulcerations were seen as sloughing the necrotic tissue. Such reaction was deeply penetrated the underlying tissue as dermis and muscle (myositis and perimyositis) in skin and gill-arch. The dermal lesions besides those of the epidermis are probably the reasons for easily detached scales. The aforementioned lesions were milder or completely absent with probiotic treated fish with numerous melanomacrophages infiltrations in these organs. The melanin pigment inside these cells is supposed to share in the defense mechanism as it is a response to probiotics. Melanin pigments are also found in the melanomacrophages within lysosomes; such pigments are considered to have a bactericidal effect through the production of free radicals (Ellis 1981). The treated groups with simultaneously received the probiotic during infection were similar to those the control (gp 2), where the probiotic has no time to enhance the immune system or improve the water quality (Eissa et al 2010). Meanwhile the use of oxytetracycline antibiotic was ameliorated the lesions of *F. columnare*. The previous findings could indicate that the oxytetracycline is sensitive and a drug of choice in treatment of columnaris disease in fishes (Hawke and Thune 1992 and Smith et al 1994).

Collectively, it could be concluded that the probiotic, ***B. subtilis***, in water or diet (as prophylaxis) are effective in amelioration the lesions of ***F. columnare*** infections that have wide spread among Egyptian freshwater fish. Oxytetracycline is the drug of choice to treat such disease and minimize the lesions of *F. columnare*.

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