

EFFECT OF GUARDIZEN-M AS A PROBIOTIC ON SOME BLOOD PARAMETERS AND HISTOLOGICAL STRUCTURE OF SOME INTERNAL ORGANS OF NILE TILAPIA *OREOCHROMIS NILOTICUS*

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ABSTRACT

This work aimed to evaluate the effect of different dietary levels of Guardizen-M as a probiotic on some blood parameters and histological structure of some internal organs of Nile tilapia. The experimental design was completely randomized; a total number of 180 Nile tilapia with average weight 68 ± 2.66 g and total length 15.41 ± 0.27 cm. were reared in 12 floating cages. The fish groups were distributed as four treatments and three replicates; a control group and three Guardizen-M treated groups (1g /kg feed, 2g /kg feed, and 3g /kg feed). All experimental groups were fed the experimental diets at a rate of 3% of the live body weight of the fish. The results of the present work revealed that no significant ($P > 0.05$) differences were recorded in blood parameters except in glucose and alkaline phosphatase. Histological studies of liver and intestine indicated that increase epithelial height of enterocytes lining the intestinal villi with increase in acidic secretion of goblet cells. The intensity of the Periodic acid–Schiff (PAS) staining increased proportionally with the increasing of the concentration of probiotics in treatment groups in comparison to control. Alkaline phosphatase activity revealed a difference among three experimental groups in comparison to control one. Results of the current study concluded that reversal effect of probiotics considering the proportion of dose. The greater area of hepatic cells increased proportionally with an increasing of the concentration of probiotics and created a greater area of nutrient storage as observed in hepatic cells.

Keywords: Probiotic, Blood Parameters, Immune response, Nile Tilapia

INTRODUCTION

It well noted that, particularly in developing countries, the shortage of human dietary protein can be provided by fish protein where protein shortage is

serious. Otherwise, this goal may be difficult due to the high cost of good quality fish meals which costs about 40-60% of total operating costs in intensive aquaculture enterprises (FAO, 1983 and Watanabe, 2002). This problem may be solved by culturing the great economic importance fish and using untraditional rations with low cost. Recently, tilapia culture is widely practiced in many tropical and subtropical regions of the world, since more than 22 tilapia species are being

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cultured worldwide, particularly the developing countries for cheap abundant and palatable protein source (Doudet, 1992 and Myers *et al.*, 1995). Moreover, tilapia is the third largest group of farmed finfish species, only after carps and salmonids (FAO, 1997).

In Egypt, the wide spread of tilapia may be due to its resistance to disease, very hard, robust fish, high tolerance to adverse environmental conditions, relatively fast growth, as well as its high potential in fish farming and their good flesh texture and taste (Head and Splane, 1979; Hulata *et al.*, 1993 and Eldar *et al.*, 1995).

Over the past decades the growth of aquaculture as an industry has accelerated; this has resulted in environmental damages and low productivity of various crops. The need for increased disease resistance, growth of aquatic organisms, and feed efficiency has brought about the use of probiotics in aquaculture practices. The first application of probiotics occurred in 1986 to test their ability to increase growth of hydrobionts (organisms that live in water). Later, probiotics were used to improve water quality and control of bacterial infections. Nowadays, there is documented evidence that probiotics can improve the digestibility of nutrients, increase tolerance to stress, and encourage reproduction. Currently, there are commercial probiotic products prepared from various bacterial species such as *Bacillus* sp., *Lactobacillus* sp., *Enterococcus* sp., *Carnobacterium* sp., and the yeast, *Saccharomyces cerevisiae* among others, and their use is regulated by careful management recommendations (Patricia Martinez Cruz *et al.*, 2012).

Some gastrointestinal bacteria have been demonstrated to enhance the growth of fish (Limsuwan and Lovel, 1981). Moreover Gatesoupe (1991) reported the benefit of using *Lactobacillus plantarum* and *Lactobacillus helveticus* in turbot, *Scophthalmus maximus* (L.), and leading to enhance growth with turbot larvae,

Scophthalmus maximus. Rengpipat *et al.* (1998) found that *Bacillus* improved the survival of larvae, increased food absorption by enhancing protease levels and gave better growth. Probiotic bacteria can be added directly to the water or administered to the larvae via live food, such as rotifers and artemia (Skjermo and Vadstein, 1999).

Muroga *et al.* (1987) used lactic acid bacteria (LAB) for improvements of the non-specific immune response system or the intestinal microbial flora may reduce the mortality significantly. Probiotics may provide an alternative way to reduce the use of antibiotics in aquaculture and simultaneously avoid the development of antibiotic-resistant bacteria (Salminen *et al.*, 1999).

Organs such as intestine and liver were associated with digestion, absorption of nutrients, and detoxification process in the body. Therefore, monitoring the pathological changes of these organs is considered necessary (Takashima *et al.*, 1982 and Roberts, 1989). They are the most important organs in assessing the effects of ingredients used as raw materials of animal/plant origin (Roberts, 1989). Estimation the efficiency of the digestive system and feeding habitats using histological structures is considered a good indicator of the nutritional status of fish (Hall and Bellwood, 1995, and Caballero *et al.*, 2003). Histological picture of the intestine of fish is a prominent method in assessing the effects of supplementary nutrients (Chowdhary *et al.*, 2013). Morpho-physiological condition of the liver, as well associated with feeding process (Caballero *et al.*, 2003; Ostaszewska *et al.*, 2005). It is considered as a good indicator of nutritional pathology due to its function as a detoxification of metabolizing products coming from the digestive tract. It is the key organ of metabolism, excretion, and detoxification of the blood (Surai, 2015). Furthermore, studying the intestinal changes is necessary to understand

histological picture promoted by any novel nutritional source (Gargiulo *et al.*, 1997).

MATERIALS AND METHODS

The present study was carried out at the experimental fish farm belonging to the Poultry Production Department, Faculty of Agriculture, Assiut University. This experiment was designed in order to study the effect of GUARDIZEN M on blood parameters, and histological structure of some internal organs of Nile tilapia *Oreochromis niloticus* in floating cages. The experiment started in July 2019 and lasted up to October 2019. The objective of this experiment was to assess the influence of the different inclusion levels of probiotic in the processed feed on some blood parameters, intestine and liver histomorphology of Nile tilapia, *Oreochromis niloticus*.

Management of experimental fish:

Nile tilapia *Oreochromis niloticus* with an average initial body weight of 68 ± 2.66 g and 15.41 ± 0.27 cm. total length. was used in this experiment. The fish were obtained from the experimental fish farm belonging to the Zoology Department, Faculty of Science, Alazhar University-Assiut Branch. The experimental fish were adapted for two weeks in floating cages in a water pond. At the start of the experiment, a total number of 180 healthy fish were chosen. The fish were weighed and measured for the total length, and then distributed randomly into four experimental groups (45 fish each) as follow: first group was considered as a control which fed on the basal diet, the second, third and fourth group were fed on

the basal diet with additional Guardizen-M 1g/kg, 2g/kg and 3g/kg, respectively.

Diet preparation:

The dry ingredients of the experimental diets were thoroughly grinded, mixed and made in a pelleted form by addition of tap water (20%). The paste was then extruded through a commercial mincing electric machine type Omega. The resulting spaghetti like diet (1.5 mm for small fish and 2.5 mm diameter for bigger ones) was then air dried and stored in plastic bags till used. The proximate analysis of the experimental diet is shown in (Table 1). Fish were adapted to the experimental conditions for two weeks on the diet formulated according to (Eid and El-Gamal, 1996). All the experimental groups were fed the experimental diet at a rate of 3% of the live body weight of the fish. The feed quantity was readjusted biweekly on the basis of the actual average biomass of the fish in each treatment.

GUARDIZEN M was purchased by DONG BANG CO., LTD., Korea. Which made by mixed Probiotics and Each kg contains:

1-Mixed Probiotics Concentrates 5.6 g (min. 1×10^{10} CFU) *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* *Enterococcus faecium*, *Aspergillus oryzae*, *Candida pintolopesii*

2- Lactose or Dextrose 994.4g)

Table (1): The composition of the experimental basal diet:

Ingredient	Weight (Kg)
Yellow corn grain	52
Soya bean meal	22
Fish meal (72% protein)	25
Vitamin Mix.	0.5
Mineral Mix.	0.5
Total ingredients	100
Crude protein %	30.44
Gross energy (Kcal/Kg)	4038.50

The floating cages:

The experiment was carried out in one floating cages (6mX4mX2m) which was

divided into 12 portions (2mX1mX2m) as shown in Fig1. The number of 15 fish was reared in each separate unit.



Fig. 1: The floating cages in water pond in fish farming

Blood samples: At the end of the experimental period 10 fish were randomly taken from each experimental group. The blood samples from each fish of the different groups were collected by suction of the caudal peduncle. Whole blood samples were collected in small plastic vials containing heparin for determination of hemoglobin (Hb) and hematocrit (PCV %). The hemoglobin (g/dl) concentrations were determined by using commercial Kits (Spectrum Company for Biotechnology Co. Egypt) and the hematocrit (PCV %) was measured according to Stoskopf (1993).

Other blood samples were collected and transferred to centrifuge tubes and allowed to clot at room temperature. Serum was then separated by centrifugation at 3000 (rpm) for 20 minutes. The serum was stored at -20°C until analysis for total protein, albumin, alkaline 6 phosphatase, glucose. Serum total protein (g/dl), albumin (g/dl),

and glucose (mg %) were determined calorimetrically using Kits supplied by Spectrum company for Biotechnology (Egypt). Serum globulin (g/dl) levels were obtained by differences between total protein (g/dl) and albumin (g/dl). Serum alkaline phosphatase (u/l) activity was determined calorimetrically using commercial Kits purchased from Spectrum Company for Biotechnology (Egypt).

Histological Study

At the end of the experiment, three fish from each treatments and control group were taken for the microscopic structure. The middle part of anterior intestine and liver specimens about 3m³ were immediately fixed in Bouin's fluid (for 24 hours) at room temperature. One unfixed selected specimens were used for frozen sections for alkaline phosphatase staining. The fixed specimens were dehydrated in an ascending series of ethanol, cleared in

methyl benzoate and then embedded in paraplast (Sigma Aldrich). Transverse and longitudinal paraffin sections were obtained at 5 μ m thickness using a Richert Leica RM 2125 Microtome (Germany) and stained with Harris's Hematoxylin and Eosin for general histological examination (Suvarna *et al.*, 2013).

Histochemical stains: Representative sections from intestine were stained with Alcian blue (pH 2.5) demonstrates acid mucins in tissue and stains them blue. Representative section from liver were stained by PAS stain for demonstration of glycogen and mucopolysaccharide content. In addition, sections were stained with Crossomom's trichrome. The sections were examined with a light microscope. The staining protocols and used procedures were carried out following the descriptions of the histological techniques as reported by Suvarna *et al.* (2013).

Enzyme histochemistry: The histological techniques descriptions were performed according to Suvarna *et al.* (2013). Histochemical analysis of Alkaline phosphatase was performed on unfixed selected frozen sections (10 μ m) were obtained in Leica cryostat CM 1900-6-1 (Richert, Germany). Leitz Dialux 20 Microscope provided with a Canon digital camera (Canon Power shot A95) used to examine the stained sections and capture the images.

Morphometrical measurements:

Different histomorphometric parameters were performed using light microscopic images of three fish including enterocytes height is a useful histological parameter using the Fiji software (Image J) ([Http://Fiji.sc/Fiji](http://Fiji.sc/Fiji)). Using three fish from each treatment and use three different cross-sections from each fish. All the

measurements were expressed as average \pm standard deviation.

Statistical analysis:

Analysis of variance was conducted using the general linear models (GLM) procedure of SAS (SAS Institute, 1998 Licensed to UNIVERSITY OF MARYLAND USA. Proprietary Software Release 6.12 TS020). Duncan's multiple range tests was used to compare between means of the control and treated groups (Steel and Torrie, 1980). The model of analysis was as follows:

$$Y_{ij} = \mu + T_i + E_{ij}$$

μ = The overall mean.

T_i = The effect of treatment.

E_{ij} = The random error.

RESULTS

Hematological parameters:

Results in Table 2 show no significant ($P > 0.05$) impacts on all blood parameters studied except glucose and alkaline phosphatase due to dietary Guardizein-M supplementation. It is noticeable that fish fed Guardizein-M at level 3g/kg and 2g/kg had the highest values of blood glucose (98.80 and 108.05mg/dl) comparing with other treated groups and control. The lowest values of the blood glucose were found in fish fed the dietary Guardizein-M at level 1g/kg diet (65.41mg/dl). Moreover, fish fed Guardizein-M at level 1g/kg and 2g/kg had the highest values of Alkaline phosphatase (91.54 and 90.2 U/I) among the other experimental groups. The highest value of Alkaline phosphatase being 91.54 at level 1g/kg diet vs. the lowest value 28.47 was recorded in the control group.

Table 2: The average blood Parameters \pm S.E of Nile Tilapia *Oreochromis niloticus* fed dietary Guardizein-M for 12 weeks.

Parameters	Control		Guardizein-M		Pro
	C	T1 (1g/kg)	T2 (2g/kg)	T3 (3g/kg)	
Blood components					
a) Hematology					
1) Hemoglobin (g/dl)	7.83 \pm 0.57	6.96 \pm 0.33	7.54 \pm 0.35	7.33 \pm 0.34	0.5351
2) Hematocrit %	27.5 \pm 1.35	29.38 \pm 1.5	27.35 \pm 1.11	26.91 \pm 0.73	0.6901
b) Serum constituents					
1- Total protein (g/dl)	5.91 \pm 0.35	5.05 \pm 0.10	5.59 \pm 0.38	5.15 \pm 0.34	0.1819
2- Albumin (g/dl)	4.34 \pm 0.27	3.71 \pm 0.08	4.15 \pm 0.38	4.13 \pm 0.23	0.4177
3- Globulin (g/dl)	1.57 \pm 0.11	1.35 \pm 0.17	1.45 \pm 0.07	1.37 \pm 0.8	0.2510
4- Glucose (mg %)	65.41 b \pm 1.16	56.2 b \pm 0.8	98.80 a \pm 3.26	108.05 a \pm 7.92	<0.0001
5- Alkaline phosphatase (U/l)	28.47 c \pm 1.89	91.54 a \pm 2.35	90.2 a \pm 7.57	41.94 b \pm 2.66	<0.0001

a, b, c Means within rows differ (P<0.05) when superscripts differ

Histological parameters:

Intestinal histology: The intestinal villi showed an increase in epithelial height lining the intestinal villi in treatments groups when compared to the control one (Table 3 and figure 2). The morphology of

the middle intestine of treatment groups when compared to control group show increase in acidic mucous secretion of goblet cells when stained by Alcian blue pH2.5 (fig. 3).

Table 3: The epithelial height lining the intestinal villi in treatments groups (Mean \pm SD)

	Control	T1(1g/kg)	T2(2g/kg)	T3(3g/kg)
Mean \pm SD	27.89 \pm 6.49	37.92 \pm 5.26	46.54 \pm 5.84	50.64 \pm 4.83

Liver histology: Histological analysis of the liver showed that the intensity of the PAS staining increased proportionally with an increasing of the concentration of probiotics (fig. 4) and alkaline phosphatase

reaction (figure 5) in treated groups in comparison with control. In figure 4, the greater area of hepatic cells increased proportionally with the increasing of the concentration of probiotics.

LEGENDS

Figure 2: Photomicrographs of the paraffin section of anterior intestine of *Oreochromis niloticus* at the middle showing the different in epithelial height between groups. A: control group, B: group1: C: group 2, D: group 3. A and B: Hematoxylin and eosin stain. B and D: Crossmon's trichrome

Figure 3: Photomicrographs of the paraffin section of anterior intestine of *Oreochromis niloticus* at the middle fed different levels of probiotics showing increase in mucus production of goblet cells A and B: control group, C and D: group 1, E and F: group2, G and H: group 3. Arrowheads indicates kocation of goblet cells.

Figure 4: Photomicrographs of the paraffin section of Liver from different groups revealed a difference among three types of treatment in PAS reaction. A and B: control group, C and D: group 1, E and F: group2 .G and H: group 3. The intensity of the PAS staining increased proportionally with the increasing of the concentration of probiotics in treated groups in comparison to control group. Note: Greater area in hepatic cells increased proportionally with the increasing of the concentration of probiotics (B, D, F, and H).

Figure 5: Photomicrographs of the frozen section revealed a difference among three groups of treatment in alkaline phosphatase activity in comparison to control group. The intensity increase in groups 1 and 2 in comparison to control and decrease in group 3.

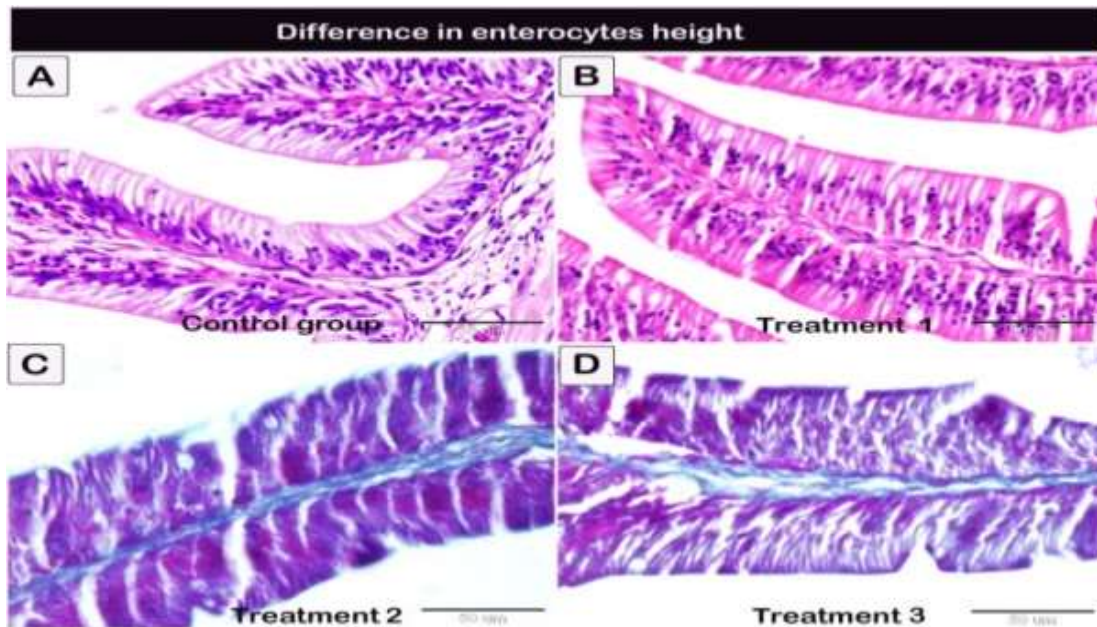


Fig. (1)

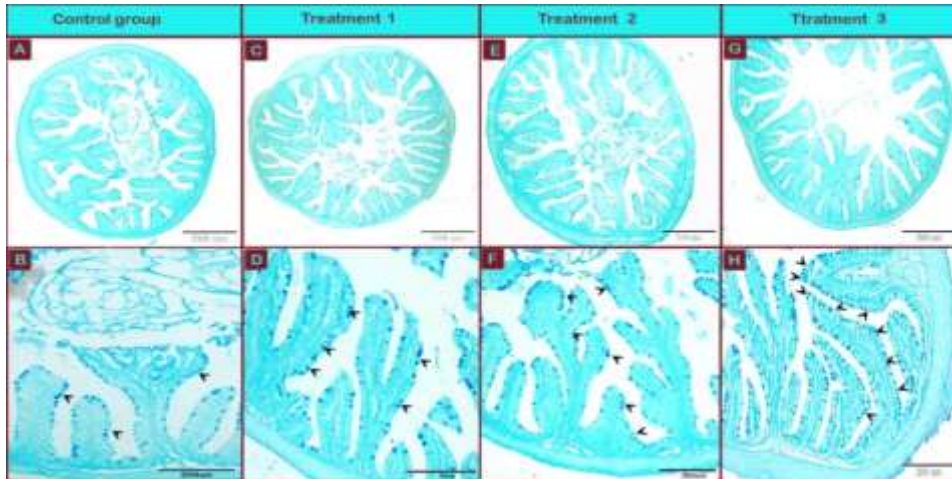


Fig. (2)

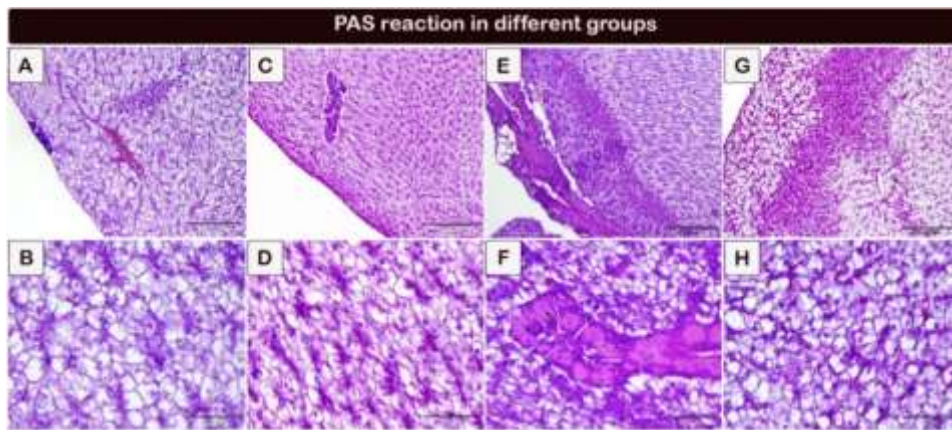


Fig. (3)

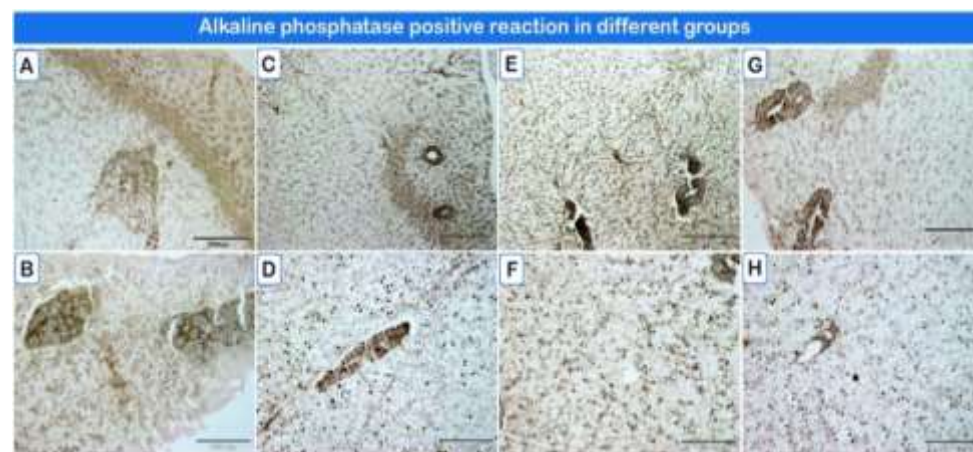


Fig. (4)

DISCUSSION

The obtained results of the experiment exhibit that blood glucose and alkaline phosphatase were increased by Guardizein-M than that control group. These results were agree with those obtained by Al-Dohail *et al.* (2009) who found that the probiotic group had higher nutrient absorption rates and improved enzyme activates than the control group. Recently, Yang *et al.* (2019) reported that serum glucose was increase due to dietary bacillus supplementation. They found that dietary bacillus supplementation could elevated the activity of amylase in intestine together with an increase in serum glucose of red sea bream (Zaineldin *et al.*, 2018). Also Yang (2019) indicated that *b. cereus* supplementation had positive impact on the carbohydrate metabolism and promote the use of carbohydrate.

In the same trend the utilization of various strategies in the assessment of the impacts of feed on intestinal and liver histology is essential. Strategies for decision in numerous investigations are semi-quantitative techniques, since they are not tedious, and are simple for assessment of tests, yet they are not objective as others. Morphometric techniques are more pertinent than semi-quantitative (Rašković *et al.*, 2011).

The outcomes show the probiotic increment increase in the height of enterocytes or epithelial height of lamina epithelials of musousa of middle intestine in treatments group in comparison to control group. The obtained data agreed with findings of Medri *et al.* (1999) and Sliva *et al.* (2005). According to Hisano *et al.* (2006) who explained the rise in epithelial height promoting a rise in area for absorption within the mucosa of intestine of Nile Tilapias fed diet supplemented with yeast.

Salinas *et al.* (2008), Ringo *et al.* (2010) and Merrifield *et al.* (2010) demonstrated enhancements in the morphology of the intestinal microvilli of the salmonides when added probiotics to the fish feed. Sweetman *et al.* (2008) reported that the interactions between the intestinal microflora, morphology of the intestine, immune system and absorption of nutrients may influence the health and performance of the fishes. Jesus *et al.* (2017) emphasized that the probiotic did not induce tissue damage and/or intestinal inflammation, demonstrating the power to enhance and maintain healthy intestinal structure. Mucosal surfaces of the fish gut considered the crucial first line of defense against the threats present in the immediate environment (Lazado and Caipang, 2014).

Previous studies demonstrated the role of mucosa to the overall health and welfare of fish and at securing optimum conditions in controlled aquaculture environments (Beck and Beatmon 2015; Lazado *et al.*, 2015). This mucosal surface secreted by mucous and goblet cells contains inhibitory activity against pathogens, with potent molecules present in the matrix including mucins, lysozymes, complement proteins, lectins, antimicrobial peptides, and immunoglobulin, among many others (Nigam *et al.*, 2012; Ellis, 2001). The mechanism of the mucosal surfaces involves the concerted action of physical barriers such as mucus, scales, and epithelium, which orchestrate the first line of defense by trapping and by direct elimination of pathogens (Cabillon and Lazado, 2019).

Liver of fish fed on probiotic supplemented diets showed the PAS staining increased proportionally with the increasing of the concentration of probiotics in treatment groups in comparison to control group. The intensity of alkaline phosphatase (AP) activity increase in-group 1 and 2 in comparison to control and decrease in-

group 3 indicate reversal effect of probiotics considering with proportion of dose and created a greater area of nutrient storage as observed in hepatic cells. It could be concluded that the best level of Guardizein-M as growth promoter in Nile Tilapia diet was 1g/kg feed.

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تأثير الجوارديزين-م كمعزز حيوي على بعض صفات الدم والتركيب الهستولوجي لبعض الأحشاء الداخلية في البلطي النيلي

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استهدفت الدراسة تقييم أثر إضافة الجوارديزين-م كمعزز حيوي الى علائق اسماك البلطي النيلي على بعض صفات الدم والتركيب الهستولوجي لبعض الأحشاء الداخلية . اجريت هذه الدراسة في الوحدة السمكية بمزرعة كلية الزراعة جامعة اسيوط خلال الفترة من يوليو الى اكتوبر ٢٠١٩. تم اختيار ١٨٠ سمكة من البلطي النيلي بوزن $2,66 \pm 68$ جم وطول $15,41 \pm 0,27$ سم حيث تم توزيعها على ١٢ قفص عائم وتم اجراء اربع معاملات ثلاث معاملات (١ جم/كجم علف ٢, ٣ جم/كجم علف) والآخرى كنترول . تم تغذية اسماك التجربة على اساس ٣% من الوزن الحي.

أوضحت النتائج المتحصل عليها انه لا توجد فروق معنوية بين المعاملات بالنسبة لصفات الدم ما عدا جلوكوز الدم والفسفات القاعدي . أما الدراسة الهستولوجية أظهرت ان هناك تغيرات واضحة في الكبد والامعاء ترجع لتأثير استخدام الجوارديزين-م كمعزز حيوي ومنتشط للنمو . حيث أشارت الدراسات النسيجية للكبد والامعاء إلى زيادة الارتفاع الظاهري للخلايا المعوية المبطننة للزغابات المعوية مع زيادة الإفراز الحمضي للخلايا الكأسية وكذلك زادت شدة كثافة PAS بالتناسب مع زيادة تركيز البروبيوتيك في المعاملات المضاف لها الجوارديزين-م مقارنة بمجموعة الكونترول. كذلك كشف نشاط الفوسفاتيز القاعدي عن اختلاف بين ثلاث المجموعات المعاملة بالجوارديزين مقارنة بمجموعة الكونترول. لكن مع زيادة الجرعة المقررة ونظرا إلى التأثير العكسي للبروبيوتيك (معاملة ٣ جم/كجم علف) حدث التأثير العكسي. وكذلك زادت المساحة المسطحة في خلايا الكبد بشكل متناسب مع زيادة تركيز البروبيوتيك ووجود مساحة أكبر لتخزين المغذيات كما لوحظ ذلك في خلايا الكبد ولذلك يوصى باستخدام الجوارديزين-م كمنتشط للنمو عند مستوى ١ جم/كجم علف.