Efficacy of date pits and fungi-degraded date pits as a feed ingredient for *Liza ramada* fingerlings

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ABSTRACT

This study aimed to perform a comparison between date pits (DP) and *Trichoderma. Reesei* fermented (fungi degraded) date pits (FDP) as a dietary component, to use it as a cheaper high-energy feed ingredient to increase the growth rate, and to determine the probiotic possibility of FDP supplementation for fingerlings *Liza ramada*. Diets contained different concentrations of date pit (DP), and fermented DP diets (FDP) as a replacement for dietary corn, were used to feed *Liza ramada* fingerlings (0.65 g initial weight) for 6 weeks. The specific growth rate remained unchanged at a concentration of 5% with DP, then, it was decreased by increasing the concentration of DP. A highly significant increase in growth rate was detected by increasing the concentration of FDP (p <0.001), reaching its maximum at 450 gkg^{-1}. Hemoglobin depletion occurred comparing to the control during the DP feeding, whereas, it was inclined with increasing the FDP concentrations. Serum glucose levels showed no significant change during feeding with DP but it was increased during FDP feeding. Serum triglycerides of fish fed with FDP was highly increased, that explained the increase in muscle lipid. Serum cholesterol levels were decreased in fish fed with DP, while increased with that fed FDP. It is worth mentioning that no previous studies were examined pathogen resistance of cultured *Liza ramada* associated with DP and FDP supplementation in a diet. This study enhanced the necessity to use FDP in diets to lessen the intestinal harmful bacteria (*Salmonella* spp*., Campylobacter* spp., *Shigella* spp. and *E. coli*) count, and to support fish health and increase growth rates.

INTRODUCTION

The human demand for fish consumption had increased all over the world since it became the main alternative source of the animal protein upon which one billion people

are dependent as their main protein source, and it is likely to rise further (**Assem *et al.*, 2014**).

The price of energy supplements for animal feeds has recently increased dramatically with their increasing demand and that lead up to search for cheaper high-energy feed ingredients (**Obirikorang *et al.,* 2015**). According to the principles of eco-innovation concerning a "zero waste", many wastes are now used as raw materials for many industries (**Kasapidou *et al.,* 2015**). The date palm, *Phoenix dactylifera* L., grows in many developing countries, where the supply of cereal grains for animal feeds is very limited. Egypt has been the world's largest producer of dates since 1974 (1,562,171 MT year $^{-1}$) (**FAO stat, 2018**). It represents about 20 % of the total world production (**Zaid, 2001**). Date pits are a by-product of date processing; it is known that the average weight of date pits ranges from 13% to 15% of the date weight (**Hussein *et al.,* 1998**). As a result, approximately 189,000 MT of date pits are available every year as a by-product of date processing plants in Egypt and are already used in the feeding of ruminant animals**.**

Date pits have been tested as a feed ingredient for fish feeds but fish production was not significantly affected (**Azaza *et al.*, 2009; Gaber *et al*., 2014**). The high content of indigestible carbohydrates may limit their use in the fish ration (**Rahman *et al*., 2007). Alyileili *et al.* (2020)** found that the crude fiber content of degraded date pits was found to be 20.8 %, crude fat (7.2%), protein (5.56%), total carbohydrate (87.2%), ash (2.09%). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) was found to be 74.6% and 45.7%, respectively. Monosaccharide composition of fiber revealed that the degradation with *T. reesei* significantly ($P<0.05$) enhanced the glucose and mannose content of cellulose, hemicellulose and lignin. Also pectin, total carbohydrate and mannan oligosaccharide content were increased in degraded date pits in which galactose and mannose were the major neutral sugars. And concluded that biological degradation with T. reesei significantly ($P<0.05$) improved the nutritional effect of date pits so as to be used in food industry with great improvement. **Belal (2008)** discussed the use of xylanase enzymes in feed formulations to liberate nutrients either by hydrolysis of non-degradable cellulose and hemicellulose fibers or by liberating nutrients that are blocked by these fibers. Also, **Habibi Najafi *et al.* (2016)** studied development of sourdough fermented date seed for improving the quality and shelf life of flat bread: study with univariate and multivariate analyses and results showed that fermentation with baker's yeast and/or lactic acid bacteria, improved the palatability and processability of fibers and whole meal flours and it is a potential bioprocessing technology for improving sensory aspects of bread supplemented with pulverized date seed, as a dietary fiber resource.

Because of the high cost of enzyme utilization in animal feed, a test made by **Mireles-Arriaga *et al.* (2015)** and recommended the use of the soft-rot fungus, *trichoderma. reesei*, which was able to produce the xylanase enzymes, as an economical alternative for dietary fibers degradation.*T. reesei* is an important producer of industrial enzymes, especially cellulases for conversion of cellulosic biomass (**Bischof *et al*., 2016; Paloheimo *et al.,* 2016; Schmoll *et al*., 2016**). Additionally, the fungi are approved as

save ingredients for animal (fish) feed by the U.S. Food and Drug Administration (**FDA, 1993).** It is efficient in producing large amounts of different cellulase-degrading enzymes (**Saloheimo *et al*., 2002**). Known to produce two exoglucanases (cellobiohydrolases, five endoglucanases also two β-glucosidases for cellulose degradation (**Saloheimo *et al*., 2002**). The natural substrate of this fungus found in date pits promotes induction of these enzymes. The T. reesei nucleotide sequence and annotation data have been deposited in GenBank under accession number MLIW00000000.

In aquaculture, infectious diseases are the major cause of economic losses. Modulate the immune defenses and nutritional performance (**Ramos *et al*., 2013).** Probiotic supplementation may change the microbiota of the digestive tract and accordingly the goal of present study is to evaluate the effect of fungi degraded date pits as a feed ingredient and its role in decreasing the bacterial infection, via estimation of the intestinal bacterial count of the fish, that leading to health benefits to *L. ramada*.

MATERIALS AND METHODS

Preparation of the fish for experiments
The grey mullet, *L. ramada*, fingerlings ranging in length from 6.0 - 8.0 cm used in all experiments were obtained from local hatcheries, and maintained in 20 L fiberglass tank under a 12 L: 12 D photoperiod. The tanks were provided with a continuous flow of aerated dechlorinated tap water at $25 \pm 1^{\circ}$C. The fish was fed daily with fish diet (35% protein) at a rate of 3% body weight, feeding was interrupted 24hr before the start of experiments and throughout their duration. During the experiments, fishes were transferred to 12 L glass aquaria, its water was changed every 24hr by siphon technique to minimize disturbance.

Preparation of fungal culture and process of degradation
Fungal culture
Four ampoules of dried (vacuum) culture of *T. reesei*DSM 678 were purchased from DSMZ (Braunschweig, Germany). As described by the company (DSMZ) manual, ampoules are opened, and re-hydration of dried cultures occurred. Transferee of a subsample from the re-hydrated fungi culture applied to potatoinfusion media.

Fermentation of date pits by Trichoderma reesei
Five kilograms of freshly separated date pits of *P. dactylifera* were finely grounded using a feed grinder, sieved to pass through 0.6 mm diameter, and stored in labelled containers until used. Half of the grounded date pits were exposed to a degradation process using *T. reesei* fungus by feed batch fermentation process as described by **Belal (2008).**

Experimental diets
Date Pit diets (DP) and Fermented Date Pit diets (FDP)
The five isocaloric-isonitrogenous diets (300 g kg^{-1} crude protein, and 107 kJ gross energy / 100 g) contained 0, 5, 10, 15 and 20 g kg^{-1} Date pit (DP).

The four isocaloric-isonitrogenous fermented diets (300 g kg^{-1} crude protein, and 107 kJ gross energy /100g) contained 0, 150, 300 and 450 g kg^{-1} FDP. All diets were prepared as described by **Belal (2008)** as follows: All feed ingredients were grounded and mixed in a commercial mixer (Spar mixer, 3 HP, Taiwan) for 20 min. Vitamin and mineral mixes were gradually added with continuous mixing. Distilled water (60 $^{\circ}$C) was slowly added while mixing to achieve a consistency suitable for pellet production. The wet mix was then passed through a kitchen meat grinder and dried for 24 h at 60 $^{\circ}$C in a forced air drying oven. The dried diet was chopped into pellets in a blender and then passed through laboratory test sieves (mesh 2.00 and 0.88 mm) to ensure homogeneity of particle size. All feeds were stored at - 20 $^{\circ}$C until used.

Table 1. Composition and proximate analyses of the tested diets (g kg^{-1} dry matter)

Feed ingredient	Degraded date pits (g kg^{-1})			
	0	150	300	450
Casein	236.7	224.5	214.9	207.7
Gelatin	106	106	106	106
Yellow corn	340	238	137	35
Date pits	0	150	300	450
α-cellulose	207.3	184.5	158.1	121.3
Soybean oil	30	17	4	0
Fish oil	20	20	20	20
Vitamins premix[1]	20	20	20	20
Minerals premix[2]	40	40	40	40
Crude fat	51	48	51.3	50.5
Crude protein	308	305	308	303
Crude fiber	228.6	208.2	167.6	135.1
Total ash	128.1	128.8	131.2	135.5
NEF[3]	284.3	306. 7	345.2	375.9
Energy[4] (kcal g^{-1})	34	34.8	36.2	37.4

[1]Vitamins include: Thiamine 2.5 g kg^{-1}, riboflavin 1 g kg^{-1}, pyridoxine 2 gkg^{-1}, pantothenic acid 5 g kg^{-1}, inositol 100 g kg^{-1}, biotin 0.32.5 g kg^{-1}, folic acid 0.75gkg^{-1}, para aminobenzoic acid 2.5 g kg^{-1}, choline 200 g kg^{-1}, niacin 10 g kg^{-1}, cyanocibalmin 0.005 g kg^{-1}, atocopherol acetate 20.1 g kg^{-1}, ascorbic acid, retinol palmitate   100 000 IU, acid 50 g kg^{-1}, menadione 2 g kg^{-1}, cholecalciferol 500 000 IU. Similar to that in **Jauncey (1998).**

[2] Minerals: $CaHPO_4.2H_2O$ 727.775 g kg^{-1}, $MgSO_4.7H_2O$ 127.5 g kg^{-1}, NaCl 60 g kg^{-1}, KCl 50 g kg^{-1}, $FeSO_4.7H_2O$ 2gkg $^{-1}$, $ZnSO_4.4H_2O$ 5.5 g kg^{-1}, $MnSO_4.4H_2O$ 2.5375 gkg^{-1}, $CuSO_4.2H_2O$ 0.7850 g kg^{-1}, $CoSO_4.6H_2O$ 0.4775 g kg^{-1}, $CaIO_3.6H_2O$ 0.295 g kg^{-1}, $CrCl_3.6H_2O$ 0.127 g kg^{-1}. Similar to that in **Jauncey (1998).**

[3] Nitrogen-free extract, determined by difference.

[4]Gross energy, calculated based on protein (5.64 kcal g^{-1}), carbohydrate (4.11 kcal g^{-1}) and lipids (9.44 kcal g^{-1} (**Belal, 2008).**

Growth experiment

Experimental diets were fed to triplicate groups of fish (1.88 g), to satiation, twice a day, for 6 weeks. At the end of the experiment, all fish from each tank were separately weighed, killed, grounded in a commercial blender and stored at -20 $^\circ$C for subsequent body composition analysis. Feed efficiency performance including fish weight gain percentage (WG), feed conversion ratio (FCR), protein deposition value (PDV), calculated energy deposition value (EDV), protein efficiency ratio (PER), and specific growth rate (SGR) were calculated with the following equations:

$WG = (W_f - W_i)/W_i \times 100$.

Where W_f is the mean final weight (g) per fish, and W_i is the mean initial weight (g) per fish.

$SGR = (LN\ W_2 - LN\ W_1) / (T_2 - T_1)$

Where W_2 is weight (g) at time T_2

W_1 is weight (g) at time T_1

FCR = feed (dry) intake (g)/wet weight gain (g).

PDV= final body protein (g) per fish - initial body protein (g) per fish

PER=live weight gain (g) / protein intake (g)

EDV= final body energy (kJ) per fish-initial body energy (kJ) per fish

Experimental design and Culture system

Groups of ten fish were randomly stocked into each aquarium, with four replicates per each treatment. To avoid the influence of any systematic stress factors, fish groups were randomly redistributed halfway through the experiment. In each trial, one aquarium was allocated to fish fed on the diet without DP or FDP to serve as the control. Each aquarium was considered as an experimental unit. Fish were fed ad-lib at 09.00, 12.00, and 15.00 h. Approximately 15 to 20 min after all feeding activity had subsided, the uneaten feed was removed and weighed to determine the amount of eaten feed. Fecal wastes were siphoned from each aquarium every day. Group weight measurements were done at weekly intervals. Experimental periods lasted for 6 weeks for all the tested diets.

Blood and tissue samples collection:

Three groups of 8 fish were killed at the end of the experiment (6 weeks), for each of the experimental diets. Handled controls were subjected to the same amount of disturbance as the experimental fish but fed FDP free diet. Fish were caught by hand net quickly to minimize the disturbance. Then they were placed upside down and the blood was obtained by incision directly into the heart using heparinized glass pipette, then the blood taken was divided for blood hemoglobin and serum analysis. Serum was separated directly by centrifugation to avoid hemolysis and stored at -20 $^\circ$C till analysis was done. After blood sampling, the fish were decapitated and after removal of the skin, a piece of white epaxial muscle was taken from a definite area bellow the dorsal fin then stored

frozen as the serum at -20°C. The intestine was dissected and prepared for bacterial analysis. In addition, the muscle was dissected and frozen for further analysis of the body composition.

Analytical techniques

Hemoglobin content in blood was determined by using the commercial kit Diamond diagnostic haemoglobin kit. Serum glucose concentration was determined in using glucose-liquizyme GOD-PAP kit. The determination of cholesterol in serum is by CHOD/POD method kit. The determination of triglyceride in serum is by GPO/PAP method kit. Muscle protein concentration was measured by the method of biuret (**Gornall *et al.,* 1949**). Muscle lipid content was determined with an adaptation of the sulphophosphoanilin method described by **Knight *et al.* (1972).** Muscle water content was analyzed by taking a piece of a specific weight of white epaxial muscle and drying it to a constant weight at 100°C for 24h. It was then reweighed after drying and then the water content was measured as a percentage to the muscle weight.

Intestinal bacterial count

The bacterial count of the intestine samples of fish fed with concentrations 5 and 10 gkg^1 of dietary DP and 300 and 450g kg^{-1} of FDP were estimated using the dilution plate method (**Johnson and Curl, 1972**). Intestines were immersed in 100 ml of distilled water and minced using a sterile mortar and pestle under aseptic conditions, and then shaken for 20 min on a shaker at 250 rpm at 25 °C. Aliquots (0.2 ml) were spread with a sterile glass rod over the surface of different general purposes and selective agar media in sterile plastic petri dishes (90 mm diameter). Plates were dried in a laminar flow cabinet for 20 min before incubation at 37 °C in the dark for 4 days and colony counts were carried out from day 2 onwards. Five plates per dilution were made for each sample. Population densities were expressed as $\log_{10}$ colony-forming units (CFU)/g dry intestine weight (**Hallmann *et al.,* 1997**). Organisms selected for enumeration and the media used were as follows; (i) total aerobic bacteria on 1/5 M32 medium (**Sivasithamparam *et al.,* 1979**), (ii) *Escherichia coli* on Eosin methylene blue (EMB) (M317) (HiMedia Laboratories Limited, Mumbai, India) (iii) *Salmonella* spp. on (Bismuth sulphite agar (modified Wilson and Blair medium, CM0201) (Oxoid Limited, Basingstoke, Hampshire, UK), and (iv) *Shigella* spp. on xylose lysine deoxycholate agar (XLD agar M031) (HiMedia Laboratories Limited, Mumbai, India) all are incubated for 2–4 days. Also controls with and without antibiotic were done.

Statistical analysis

Data were analyzed using SPSS (SPSS 20.0 for Windows, SPSS Inc., USA). Normal distributions were checked by the Shapiro–Wilk test. A one-way analysis of variance (ANOVA) was carried out. Statistical significance was judged on an overlap of 95% confidence intervals ($p<0.05$). Multiple Comparisons were made by Turkey test. Handled controls were subjected to the same amount of disturbance as the experimental fish,

including transfer to another aquarium but remained in their original medium. Values in the tables were expressed as mean ± standard error of the mean (SEM).

RESULTS

1. Growth Performance and Feed Utilization

The specific growth rate of fish during feeding with dietary (DP) remained unchanged compared to the control at the 5 g kg^{-1} diet concentration and decreased significantly at all the other concentrations. Conversely, a highly significant increase in the growth rate compared to the controls, was recorded with increasing the concentration of dietary FDP at all concentrations, with the lowest recorded increase at the concentration 150 (Table 2 and Table 3).

Table **2**. Biochemical parameters and Growth Performance of *Liza ramada* fingerling fed with Date pits.

| Parameters | Control | Date pits | | | | *P*-value between groups |
		5%	10%	15%	20%	
Glucose (mg/dl)	46.4±1.22	44.6±1.17	47.3±1.17	47±1.28	50±1.54	0.070
Total cholesterol(mg/dl)	275±11.49	190±12.25 [a]	183.3±13.06 [a]	240±12.71[c]	237±15.15[c]	<0.001
Triglycerides (mg/dl)	123.1±11.65	111.5±7.07	128.2±7.07	114.7±8.91	112.8±5.09	0.552
Hemoglobin(g/dl)	1.8±0.02	1.5±0.02[a]	1.5±0.04[a]	1.51±0.05[a]	1.7±0.06[b,c,d]	<0.001
Weight gain(g)	1.96±0. 12	1.13±0. 11[a,c]	0.79±0. 20[a,b]	0.97±0. 42 [a]	0.7±.092[a,b,d]	<0.001
SGR(g/fish)	0.012±0.0012	0.012±0.0013	0.008±0.0012	0.008±0.0008	0.006±0.0015[a,b]	0.003
FI(g/fish)	0.87±0.012	0.53±0.011 [a]	0.58±0.020 [a]	0.62±0.042 [a]	0.61±0.003 [a]	<0.001
FCR(g/fish)	0.45±0.005	0.47±0.004[c,d]	0.64±0.087[a,b]	0.64±0.004[a,b]	0.61±0.003 [a]	<0.001
PER	7.49±0.013	7.05±0.004[a,c,d]	4.54±0.020[a,b,d]	5.22±0.042[a,b,c]	3.71±0.093[a,b,c,d]	<0.001
EDV	3.25±0.012	3.6±0.043	3.12±0.445	3.43±0.042	3.59±0.092	0.388
PDV	0.37±0.005	0.22±0.004[a,c,d]	0.16±0.006[a,b,d]	0.20±0.004[a,b,c]	0.14±0.003[a,b,c,d]	<0.001
Protein(g/100g tissue)	20.31±1.16	22.33±1.56	20.69±1.56	20.35±2.04	17.1±1.36	0.231
Lipid(g/100g tissue)	5.66±0.012	5.37±0.57	5.37±0.038	4.63±0.56	4.38±0.01	0.133
Water(g/100g tissue)	74.0±0.96	75.3±1.87	75.4±2.04	75.2±1.71	75.1±2.19	<0.001
Ash(g/100g tissue)	1.43±0.012	1.48±0.011	1.52±0.042	1.52±0.042	1.58±0.092	0.417

Data are expressed as mean ± SEM. n = 8 for each experimental group
[a] p < 0.05 *vs*. control group
[b] p < 0.05 *vs*. 5% date pits treated group
[c] p < 0.05 *vs*. 10% date pits treated group
[d] p < 0.05 *vs*. 15% date pits treated group

Table 3. Biochemical parameters and growth performance of *Liza ramada* fingerling fed with fermented date pits

| Parameters | Fermented Date pits (g kg^{-1}) | | | | *P*-value |
	Control	150	300	450	between groups
Glucose (mg/dl)	46.4±1.22	66.70±1.41[a,c]	51.80±1.41[a,b]	61.60±1.06[a,b,c]	<0.001
Total cholesterol (mg/dl)	275±11.49	358±13.48[a,c]	408±12.25[a,b]	263±13.19[b,c]	<0.001
Triglycerides (mg/dl)	123.1±11.65	162±12.40[a]	134±10.31	167±14.65[a]	0.044
Hemoglobin (g/dl)	1.8±0.02	1.9±0.021	2.0±0.042[a]	2.31±0.014[a,b,c]	<0.001
Weight gain (g)	1.96±0.12	1.35±0.011[a,c]	1.62±0.020[a,b]	2.1±0.042[a,b,c]	<0.001
SGR (g/fish)	0.012±0.0012	0.014±0.0013	0.018±0.0012[a]	0.027±0.0008[a,b,c]	<0.001
FI (g/fish)	0.87±0.012	0.49±0.011[a]	0.49±0.020[a]	0.38±0.042[a,b,c]	<0.001
FCR (g/fish)	0.45±0.005	0.36±0.0062[a,c]	0.30±0.0058[a,b]	0.180.0043[a,b,c]	<0.001
PER	7.49±0.013	9.11±0.044[a,c]	10.94±0.038[a,b]	18.77±0.042[a,b]	<0.001
PEV	3.25±0.012	3.69±0.043[a]	3.79±0.02[a]	4.26±0.042[a,b,c]	<0.001
PDV	0.37±0.005	0.31±0.004[a,c]	0.36±0.006[b]	0.47±0.004[a,b,c]	<0.001
Protein (g/100g tissue)	20.31±1.16	21.14±1.15	21.45±1.16	21.08±1.38	0.923
Lipid (g/100g tissue)	5.66 ±0.012	5.77±0.012[a,c]	5.52±0.038[a,b]	6.31±0.012[a,b,c]	<0.001
Water (g/100g tissue)	74.0±0.96	72.36±1.15	74.96±1.11	74.99±1.16	0.308
Ash (g/100g tissue)	1.43±0.012	1.4±0.020	1.53±0.042	1.54±0.098	0.196

Data are expressed as mean ± SEM. n = 8 for each experimental group

[a] p < 0.05 *vs*. control group

[b] p < 0.05 *vs*. 150 g kg^{-1} date pits treated group

[c] p < 0.05 *vs*. 300 g kg^{-1} date pits treated group

2. *Hematological and Biochemical Parameters*

Serum glucose levels showed no significant change compared to the control during feeding with the dietary DP.

On the contrary, it increased significantly (p<0.01) at the fish fed with dietary FDP compared to the control and was at its highest level at the concentration 150 g kg^{-1} (concentration 150 g kg^{-1} significant with control and concentration 300 gkg^{-1}; p<0.001).

Hemoglobin content during feeding with DP showed a significant depletion compared to the control at all concentrations $p < 0.001$ except at the concentration of 20 g kg^{-1} diet where there was no significant change compared to control but significant decrease with concentrations 5%, 10% and 15% (p-value 0.015, 0.015, and 0.021 respectively). But at feeding with FDP a direct correlation occurred, by increasing the concentration of dietary FDP the hemoglobin content increased in blood of fish ($p<0.001$).

Cholesterol levels were significantly decreased in serum during feeding with dietary unfermented date pit at concentrations 5 and 10 g/dl compared to the control group ($p < 0.001$, and $p<0.03$ respectively), while showed less depletion at concentrations 15 and 20 g/dl compared to concentration 10% treated group (p-value 0.03, and 0.042 respectively). Using FDP, cholesterol levels in serum increased significantly compared to controls ($p< 0.01$) at concentrations 150 and 300 gkg^{-1}, and concentration 300 gkg^{-1} treated group was significantly with increased compared to concentration 150 treated group (p-value 0.04) concentration Whereas, concentration 450 treated group was significantly decreased when compared to concentration 150 and 300 gkg^{-1} treated groups ($p<0.001$).

Triglycerides showed no significant change in serum during feeding with dietary DP at all concentrations. Its level was increased significantly during feeding with dietary FDP at concentrations 150 and 450 g kg^{-1} (p-value 0.04) and showed no significant change at the other concentrations. No significant change of muscle lipid content occurred at all dietary fermented date pit contents comparative to control.

Muscle lipid in fish fed FDP was significantly increased at concentration 150 and 450 gkg^{-1} when compared to control (p-value 0.024, <0.001 respectively) and a depletion occurred at concentration 300 gkg^{-1} compared to control (p-value 0.002).

Muscle protein content of fish fed with DP showed no significant change at all concentrations and only a significant decrease occurred at dietary of 20% diet. No significant change in muscle protein content occurred at all concentrations of FDP compared to control. There was no change in muscle water content during feeding with both DP and FDP (Table 2).

3. *Total and intestinal bacterial count:*
Depletion of total bacterial count occurred at DP fish compared to control. Total intestinal bacterial counts of fish treated with FDP decreased compared to both control and unfermented date pit. Mainly the bacterial count decreased by increasing the dietary date pit or dietary fermented date pit concentration. At FDP concentration 300 gkg^{-1}; bacterial counts decreased significantly showing a similar response to that showed with antibiotic- treated control Table (4).

Table 4. Microbial population densities in $\log_{10}$ colony-forming units (CFU) per g dry fish intestine tissue for total bacterial counts (TBC).

Treatment	TBC	*Salmonella*	*Campylobacter*	*Shigella*	*E. coli*
Control without antibiotic	8.53 ± 0.88	2.03 ± 0.13	2.74 ± 0.09	2.12 ± 0.07	7.19 ± 0.14
Control with antibiotic	4.21 ± 0.14	0.03 ± 0.03	0.08 ± 0.05	0.53 ± 0.06	2.06 ± 0.18
DP (5 %)	7.07 ± 0.91^{abdef}	1.41 ± 0.08^{abef}	2.01 ± 0.10^{abdef}	1.90 ± 0.07^{bef}	5.53 ± 0.14^{abdef}
DP (10 %)	6.02 ± 0.12^{abcef}	1.48 ± 0.11^{abef}	0.95 ± 0.07^{abcef}	1.67 ± 0.09^{abef}	4.61 ± 0.16^{abcef}
FDP(150gkg^{-1})	4.99 ± 0.11^{abcdf}	0.61 ± 0.10^{abcdf}	0.10 ± 0.06^{acdf}	0.78 ± 0.11^{abcdf}	3.06 ± 0.17^{abcdf}
FDP (300gkg^{-1})	3.95 ± 0.12^{acde}	0.05 ± 0.05^{acde}	0.06 ± 0.04^{acde}	0.25 ± 0.09^{abcde}	2.21 ± 0.13^{acde}

Microbial population densities in $\log_{10}$ colony-forming units (CFU) per g dry fish intestine tissue for total bacterial counts, Salmonella spp., Shigella spp. and Escherichia coli. The values in Table 3 are means of 6 replicates for each treatment and the values in parentheses are the standard error of the mean.

[a] $p < 0.05$ *vs*. control *without antibiotic* group

[b] $p < 0.05$ *vs Control with antibiotic* group

[c] $p < 0.05$ *vs.Date pit (5 %)* group

[d] $p < 0.05$ *vs. Date pit (10 %)* group

[e] $p < 0.05$ *vs. Fermented Date pit (150g kg^{-1})* group

[f] $p < 0.05$ *vs. Fermented Date pit (300g kg^{-1})* group

DISCUSSION

Dietary carbohydrate sources (maize, sorghum, wheat, rice, soybeans, date and barley) are important sources of dietary energy for herbivorous fishes such as carp, *Liza ramada*, and *tilapia* as they can utilize high levels of digested carbohydrates (**krogdahl *et al*., 2005**; **Enes *et al.,* 2011**). In this case, aquaculture industries are with high input cost as feeding diets cost, low income and environmental issues (**Deb *et al., 2017***). The replacement of cereal in a diet with a cheap bi-product such as date pit and fermented date pit is an excellent outcome purpose (**Obirikorang *et al.,* 2015**).

In this study, specific growth rate of *L. ramada* remained unchanged at the 5% concentration DP diet and then decreased significantly by increasing the concentration of dietary date pit agreeing with **Belal and Al-Owaifer (2005)** showed a growth reduction in *O. niloticus* fed with different levels of date pits (150, 300 and 450 gkg^{-1}) compared with the control group. Also, **El-Sayed *et al*. (2006)** found that date pits based diets replaced up to 75% wheat bran resulted in reduced growth rates and feed utilization

efficiency of Nile tilapia. They reported that, fungi degraded pits (FDP) could replace 300 gkg[-1] dietary corn. This result revealed that, although the high reduction in the performance of Nile tilapia feeding date pits, the reduction in the cost of DP diets may justify the use of this bi-product in feeding Nile tilapia. However, they recommended for a further work to improve the quality of DP for tilapia, using suitable processing treatments. On the other hand, **Ali *et al*. (1999)** indicated that feeding male rats with date pits for 28 days, at levels of 7% and 14%, have increased the final body weight significantly**. Aldhaheri *et al*. (2004)** reported that adding 12.5% of date pits in the dietary treatment had no significant effect on total body weight gain of Wistar rats compared to the control diet. **Al-Asgah (1988)** found that the growth of carp *Cyprinus carpio* fed different dietary levels of date pits (up to 283 g kg^{-1}) was similar to that of the control group. That was probably due to differences in digestion between species. On the other hand, **Ahmed *et al. (*2017)** found that the date palm seeds powder have a positive effect on growthperformance of common carp.

During the feeding with fermented date pits, the specific growth rate of thin lip mullet fish increased directly with a high significance by increasing the concentration of dietary FDP. We suggest that the high levels of growth rates are due to many related factors that occurred due to the fermentation and degradation of date pit cellulose by fungi. This process resulted in releasing digestible nutrients and carbohydrates, and to a high level of the free sugar mannose, which worked as a growth promoter. Since date pits contain natural substrates for *T. reesei* such as lignocellulose and galactomannan fibers (**Lopes *et al*., 2017**) that promote good cellular growth and induction of both xylanases and cellulose-degrading enzymes as reported by **Dienes *et al*. (2006).**

Kamali-Sanzighi *et al.*(2018) showed improvement of fish growth and feed utilization by replacing 10% date waste meal, while higher substitution levels could not provide further enhancement. **Belal (2008)** was the first to introduce FDP into the tilapia diet, aimed to improve the fish growth rate. During his experiment, fish weight increased than controls when fed diets with 150 and 300 gkg[1] FDP but when the FDP was increased in tilapia diets above 300 g kg-1, the growth performance was negatively affected. **Assem *et al*. (2014)** also recorded a highly significant growth increase only in fish fed with the lowest FDP concentration, and the fish growth remained unchanged when FDP percentage was increased in the diet. **Hosseinifar *et al*. (2015, 2017)** reported that feeding carp fry by date palm fruit were significantly improved growth performance, immune efficacy and increase the antioxidant activity ($P<0.05$) in early stages of common carp culture. Also, **Azaza *et al.* (2009)** noted that, no feed-related mortality was observed during the entire experimental period. Final body weight and specific growth rate in the different treatments were statistically not significantly different ($P>0.05$). While, waste date meal could be substitute with soybean meal up to 300 g/kg[-1] without compromising growth of Nile tilapia. Also, feed intake (FI), food conversion ratio (FCR), protein

deposition value (PDV), protein efficiency ratio (PER), and protein energy value (PEV) confirm the results of the growth rate.

For more understanding of this situation, blood parameters were measured. Fish peripherical blood analysis is used as a diagnosis to assess its physiological state, effect of hazardous substances, external stress and to establish the quality of food **(Kamali-Sanzighi** *et al*.**, 2018).**

The hemoglobin (Hb) content in the blood has a very important role and acts as a transportation element of oxygen to body tissues **(Vahedi** *et al*.**, 2017)**. The hemoglobin content of fish during feeding with dietary DP showed a significant depletion compared to the control at all concentrations, this anemic condition may be due to the deficiency in the dietary content of unfermented date pit.

This is in line with **Zheng** *et al*. **(2012)**, who reported significant decrease in hemoglobin level in grass carp fed with a high cottonseed meal (CM) diet (48.94%) were significantly lower than those of the fish fed with low CM diets. In channel catfish, the dietary free gossypol (FG) of 900 mg/kg decreased the hemoglobin content (**Yildirim** *et al*.**, 2003**). A decreased Hb resulting from the high level of dietary CM or FG were also reported in rainbow trout (**Blom** *et al.,* **2001; Rinchard** *et al*.**, 2003**) and tilapia (**Yue and Zhou, 2008**).

The hemoglobin content of fish during feeding with dietary FDP increased by increasing the concentration of dietary FDP. **Kamali-Sanzighi** *et al*. **(2018)** found that the Hb content was significantly higher in 5% DWM treated groups than the control. The increment in blood hemoglobin indicates the better oxygen supply and good health of fish which was described by **Vahedi** *et al*. **(2017**), and this resulted in high growth rates.

In the present study, serum glucose levels of fish during feeding with dietary DP showed no significant change compared to the control treatment. It may be due to the production of glucose by the liver to compensate the decrease in the dietary supplement of sugar in DP. The increment in glucose production in fish through gluconeogenesis and glycogenolysis pathways**)** to cope with the energy demand due to the poor dietary sugar supply (**Iwama** *et al*.**, 1999)**. This glucose production is mostly mediated by the action of cortisol which stimulates liver gluconeogenesis and also halts peripheral sugar uptake (**Wedemeyer** *et al*.**, 1990**). Glucose is then released (from liver and muscle) toward blood circulation and enters into cells through insulin action to compensate the need for glucose (**Nelson and Cox, 2005**). Agreeing with **Kamali-Sanzighi** *et al*. **(2018)** who found that the addition of DWM to treatment diets resulted in a linear insignificant increase (P>0.05) of blood glucose concentration. The fibrous content in diet helps to increase in gluconeogenesis activity (**Masoudi** *et al*. **2011**). Since date pit (DP) has a high fibrous content which supports low glucose than corn, so date pit affect on blood parameters so as to increase lipid metabolism for supporting the energy requirements of common carp fish (**Masoudi** *et al*.**, 2011**).

Serum glucose levels of fish during feeding with dietary FDP increased significantly compared to the control treatment by increasing the dietary FDP. This is explained through the release of free sugar from the date pit resulted from the fermentation process by the fungus. In line with our results, **Zhou *et al*. (2016)** found that serum glucose in green tea waste (GTW) fish group were significantly higher than control group and that GTW supplementation has positive impact on health of fish without affecting the growth of fish. Also**, Assem *et al*. (2014)** reported an increment of serum glucose in tilapia fed FDP, which explains the increment of growth.

   The lipid metabolism of the liver includes the secretion, transport, and uptake of lipid. A balance between the secretion and uptake of lipids occurred under normal physiological conditions. **Karavia *et al*. (2013)** used rats as an experimental animal and attributed the hepatic lipid accumulation to the blocking of lipid secretion.

Muscle lipid content during feeding with dietary DP showed no significant change relative to control. In addition, TG showed no significant change in serum of fish. This may be due to poor lipid uptake by the gut in fish fed dietary unfermented date pit. Agreeing with **Kamali-Sanzighi *et al*. (2018)** replacement of plant sources by DWM led to negligible effect on the glucose, triglycerides, cholesterol of plasma of the treatment group (P>0.05), But disagreeing with this study where Serum cholesterol was decreased during feeding with dietary unfermented date pit. This may be due to the depletion in lipid uptake by the gut. In line with our results, the inclusion

of black cumin seeds also decreased cholesterol concentrations in broiler, human, rat, rabbit and mouse cells (**Bamosa *et al*., 2002; Ibraheim, 2002; Zaoui *et al.,* 2002; Le *et al*., 2004; Morikawa *et al*., 2004; Miraghaee *et al*., 2011**).

Serum triglycerides of fish during feeding with dietary FDP increased with high significance at concentrations 150 and 450 g kg^{-1} and showed no significant change at other concentrations, which may explain the increase in muscle lipid at those concentrations. Thus, it may be involved in the increment of growth rates. The increment of TG may be due to increasing the absorption of lipids by gut at FDP diets (**Assem *et al*., 2014**). Besides, the serum TG concentrations may indicate a more active endogenous lipid transport (**Du *et al*., 2005; Gatesoupe *et al*., 2014**). These results might indicate that the ability for transporting lipids out of the liver increased after the intake of a high FDP diet.

In his investigation on tilapia**, Belal (2008)** explained the negative effects of FDP in diet higher than 300 g kg^{-1} as a result of significant reduction of feed intake as indicated by an increase of body water content and the reduction of body fat. Contrarily to our results, the body water content remained unchanged while serum triglycerides increased with increasing FDP in diet.

In line with our results, **Assem *et al*. (2014)** indicated that lower serum cholesterol concentrations in fish by increased percentage of FDP in diets, in odd with our results where Serum cholesterol of fish during feeding with dietary FDP increased with high

significance at concentrations 150 and 300 g kg^{-1} but showed no significant change at the other concentration. This is because of the increase in dietary uptake of lipids by the gut. Research is now being directed towards the vast unexplored source of plant-based antimicrobials and immune-stimulants for disease management, many of which are without the negative side effects associated with synthetic chemotherapy (**Hutson *et al*., 2012; Militz *et al*., 2013**). **Asheg *et al*. (2014)** studied the effect of *Arbutus pavarii*, *Salvia officinalis* and *Zizyphus Vulgaris* plants on the growth performance and the intestinal bacterial count of broiler chickens, and emphasized the potential biotic role of such plants together with the immune-modulating effects on treated birds. The general concept that the use of probiotics in aquaculture may produce various beneficial effects has been proven in many studies (**Gaggı̀a *et al*., 2010; Oliva-Teles, 2012**). The scientific application of this concept to fish health and disease, although still at the beginning, has already produced some positive results (**Ramos *et al*., 2013; Ingerslev *et al*., 2014; Huu *et al*., 2016; Jahanian *et al*., 2016; Augustine and Joseph 2018**).

It must be mentioned that there were no previous studies were examining the immunoregulatory response or pathogen resistance of cultured *L. ramada* associated with DP and FDP supplementation in the diets.

In the current study, the intestinal total bacterial counts, *Salmonella* spp., *Campylobacter* spp., *Shigella* spp. and *E. coli* of fish treated with FDP were decreased compared to the control and DP. At the concentration of 300g kg^{-1} FDP; bacterial counts decreased significantly showing a similar response to that shown with the antibiotic-treated control mainly the magnitudes of reductions were FDP or DP concentrations dependent on the diet. These data supported the suggestion that the FDP may have a probiotic action and significantly affects the intestinal microbiota in *L. ramada*, and the response may be FDP concentration-dependent. In contrary to our results, **Assem (2014)** indicated that the changes in the intestinal bacterial count were not translated into increased weight gain and reflected no improvements in fish performance, but our study showed an increase in growth rates and the improvement in fish performance could be related to the decrease of the intestinal bacterial count. Additionally, we detected a small depletion of bacterial count that occurred at fish fed with the DP compared to the control, and these results indicated that fermented date pit diets helped in the resistance against bacteria in *L. ramada* intestine.

The results of this study showed the possibility of using FDP in diets to lower the intestinal harmful bacteria, which could support the fish health and increase growth rates.

CONCLUSION

FDP and DP are often accustomed to replace the cereal grains for fish (animal) feed. They are less expensive high-energy feed ingredient for fish. DP is often used as a replacement of cereal within the fish diet at limited concentration. But the FDP showed

far better results when used as a replacement to cereals in fish diets. FDP is often used as supplementation to enhance the fish resistance to intestinal harmful bacteria, and then improve the animal health and growth rates. So as improve fish aquaculture to extend fish production in Egypt.

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