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EFFECT OF DIETARY SUPPLEMENTATION OF SODIUM BUTYRATE AND / OR PROTEXIN ON THE GROWTH PERFORMANCE, SOME BLOOD PARAMETERS, AND IMMUNE RESPONSE OF *OREOCHROMIS NILOTICUS*

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SUMMARY

Two hundred *Oreochromis niloticus* fingerlings were used to explain the effects of supplementing a basal diet with sodium butyrate (SB) and / or protexin on the growth performance, some blood parameters, body composition, and immune response. *Oreochromis niloticus* fingerlings were allotted into 4 experimental groups. The control group (1) was fed the basal diet (BD), while group (2) was fed BD with SB at level 3 g/ 10 kg diet, group 3 fed BD plus protexin (probiotic) 1 g/ 10 kg diet and group 4 was fed BD with SB and protexin at 3 and 1 g/ 10 kg diet. **Results obtained showed that** the highest growth (final weight, total weight gain and SGR) of Nile tilapia were obtained with feeding diet containing SB plus probiotics followed by SB (group 2) supplemented diets ($P<0.05$) when compared with those of control group. The best FCR values observed in SB plus Protexin-supplemented diets. The results revealed that, probiotic nonsignificantly increased blood glucose and intestinal glucose absorption while sodium butyrate significantly increased blood glucose level, intestinal glucose absorption and both probiotic and SB supplementation have no any adverse effects on liver functions reflected in normal blood protein pattern and enzymatic activity. We concluded from this study that using of SB and SB plus probiotic is preferred for good performance in tilapia fish production because the beneficial effect of butyric acid on the proliferation of the intestinal epithelium.

INTRODACTION

Tilapia culture has been growing at an outstanding rate during the past two decades. As a result, the production of farmed tilapia has witness a 6-fold increase during the past 15 years, jumping from 383,654 mt in 1990 to 2,348,656 mt in 2006 (**FAO, 2008**). In addition, there has been a gradual shift in tilapia culture from traditional semi-intensive to more intensive farming systems. This has created an increasing demand for artificial feed.Tilapia, because of their enormous adaptability to a wide range of physical and environmental conditions, ability to reproduce in captivity, relative resistance to handling stress and disease-causing agents compared to other cultured finfish species, good flesh quality, feed on a low trophic level and excellent growth rate on a wide variety

of natural and artificial diets, are the most abundantly cultured species worldwide (**Welker and Lim.2011).**

Dietary sodium butyrate is a four carbon short chain fatty acid (CH3CH2CH2-COOH) and becomes sodium butyrate after receiving sodium found primarily in dairy products such as cheese and butter. It is also produced in large amounts from dietary fiber after fermentation in the large intestine, where butyric acid is generated together with other short-chain fatty acids from nondigestible carbohydrates, such as nonstarch polysaccharides, resistant starch, and miscellaneous low-digestible saccharides **(Pryde et al., 2002 and Roy et al., 2006).** Dietary sodium butyrate in absorbed in the small intestines and does not reach the large intestines and therefore there is no increase in butyrate levels found in the large intestines of sodium butyrate fed animals compared to control groups. Sodium Butyrate (SB) has many advantages for it's no pollution, no residual and distinct physiologic function. Butyrate plays an important role in homeostasis of the colonic mucosa by inducing pathways of cell maturation, including cell cycle arrest, differentiation, and apoptosis **(Heerdt et al., 1994 and 1997).**

Short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate are produced in the cecum and colon of animals via the fermentation of carbohydrates such as dietary fiber and unabsorbed starch. Numerous studies have been on effects of butyrate on colon through total parenteral nutrition, intestine perfusion and bacterial fermentation. SB can induce absorption of water and sodium and proliferation of intestinal cells (**Kripke et al., 1989; Friedel and Levine, 1992**), be used as energy resources and stimulate intestinal blood flow and the synthesis of gastrointestinal hormones (**Mineo et al., 1994**). Microencapsulated butyrate can influence the hind gastrointestinal tract (**Van Immerseel et al., 2004**). Non-protected SCFAs can have effect on the upper part of the digestive tract, but not directly further down (**Hume et al., 1993; Thompson** and **Hinton, 1997**).

Zhang et al., (2011) determined the effect of dietary sodium butyrate at 0.5‰ and 1.0‰ of sodium butyrate on the growth performance and Antioxidant capacity of Anguilla rostrata. The results showed that, increase in the weight gain and reducing feed conversion ratio, both of

the 2 levels were better than the control groups. , weight gain of experimental group increased by 37% and feed conversion ratio of experimental group decreased by 26%. In feeding rate and survival rate, the three groups did not differ significantly. Compared with the control group, liver total antioxidant capacity and catalase activity of experimental group were increased by 25% and 15%, MDA was decreased by 15%, while the experimental group had little difference.

Probiotics are usually live microorganisms which when administered in adequate amounts confer a health benefits on host. Nowadays, probiotics are also becoming an integral part of the aquaculture practices to obtain high production. The common probiotics that are used for aquaculture practices include *Lactobacillus, Lactococcus,, Enterococcus, , Bacillus, Enterobacter,* and *Saccharomyces* species. The involvement of probiotics in nutrition, disease resistance and other beneficial activities in fish has proven beyond any doubt. (**Nayak, 2010**). Several studies have demonstrated that the use of probiotics improves health of larval and juvenile fish, disease resistance, growth performance and body composition, however, the mode of action in fish species may vary between farmed fish species cultured in freshwater and marine environments. The use of probiotics in feeds to improve growth of different fish species including African catfish, *Clarias gariepinu* (**Al-Dohail *et al.*, 2009**); tilapia, *O. niloticus* (**El-Haroun *et al.*, 2006**), gilthead seabream, *Sparus aurata* and Seabass, *Dicentrarchus labrax* (**Carnevali *et al.*, 2006**) has been investigated.

The effects of probiotics have been linked to modulation of gut microbiota and establishment of the beneficial microorganisms, higher specific and total digestive enzyme activities in the brush-border membrane which increases the nutrient digestibility and feed utilization (**Balcázar *et al.*, 2006; Kesarcodi- Watson *et al.*, 2008**). In addition, the production of vitamins by these gut microbiota could also increase vitamin synthesis and improve fish health (**Holzapfel *et al.*, 1998**).Growth performance and immunity of piglets were improved by dietary SB or MOS supplementation and 0.10% SB+0.10% MOS was an appropriate and feasible combination, (**You et al., 2010**).This work was conducted at the Faculty of Veterinary Medicine, Damanhour

University to study the effect of dietary supplementation of sodium butyrate and or Protexin (probiotic) on the growth performance, some blood parameters, immune response and of *Oreochromis niloticus* .

MATERIALS AND METHODS

Experimental diets

A basal diet table 1 was formulated from local ingredients to cover nutrients requirement of *Oreochromis niloticus*. It contained 24.7% crude protein, 3896.5 Kcal/ kg diet of gross energy, 8.45 % fiber as well as vitamins and minerals in the form of dry pellets.

Experimental fish

Apparently healthy *Oreochromis niloticus* (O. niloticus) with average body weight 18.85 g. Fish were brought from private farm from Kafer –Elsheikh Governorate. Fish were kept in hapas measuring (3 X 6 X 1 m).The health status was examined throughout acclimatization period.

Table 1. Ingredient composition (%) of the used basal diet in experiment

Ingredients	%
Yellow corn, ground	36
Soya bean meal (44% CP)	37
Wheat bran	13.5
Fish meal (65% CP)	7
Fish oil	2.5
Molasses	3
Mineral & Vitamin mix *	0.3
Dicalcium phosphate	0.2
Limestone, ground	0.3
Antimold	0.2

*Mineral premix contained the following minerals (kg−1 feed): CuSO4·5H2O, 0.35 g; ferric citrate, 0.2 g; ZnSO4·7H2O, 0.4 g; MnSO4·4H2O, 0.5 g; Na2SeO3, 3 mg; KI, 0.6 mg; CoCl2·6H2O, 0.7 g. Vitamin premix contained the following vitamins (kg−1 feed): vitamin A, 55,000 IU; vitamin D3, 2000 IU; vitamin E, 50 IU; menadione, 15 mg; hiamine hydrochloride, 20 mg; riboflavin, 25 mg; d-calcium pantothenate, 40 mg; pyridoxine hydrochloride, 25 mg; vitamin B12, 0.05 mg; vitamin C, 100 mg; folic acid, 10 mg; biotin, 1mg.

Table 2. Proximate chemical and calculated analysis (%) of the used basal diet in experiment.

Nutrients	Value
Chemical analysis	
Dry matter (%)	88.78
Crude protein (%)	24.7
Ether extract (%)	5.5
Crude fiber (%)	8.45
Ash (%)	9.4
Calculated analysis	
Nitrogen free extract (%)*	40.73
Gross energy(Kcal/ kg diet)**	3896.5

* Nitrogen free extract was calculated by difference

** Gross energy (GE) was estimated according **Jobiling , 1983** as 5.65, 9.45 and 4.1 kcal / g for protein, lipid and carbohydrates, respectively.

Experimental design

Two hundred O. niloticus were distributed into 4 haba and acclimatized for the experimental conditions for 2 weeks prior to the start. During that period fish were adapted on feeding of control diet (without any additives). The experimental design is to be seen in table (3).

Fish group	treatment	Dose (g/10 kg diet)
Group 1 (control)	Basal diet	0
Group 2	Sodium butyrate*	3
Group 3	Protexin**	1
Group 4	Sodium butyrate+ Protexin	3+1

*Sodium butyrate $C_4H_7O_2Na$, molecular weight 110.09. It was used as a feed additive at a rate 300 g/ton feed. Manufactured by Singao Co., LTD, china

**Protexin® (protexin probiotics, *enteroccus faecum* manufactured in the UK by probiotics international LTd. It was used as a feed additive at a rate 100 g/ton feed.

Feeding rate

During the experimental period, a fixed feeding rate of 3% of the fish wet weight per day (dry feed/whole fish) was supplied. The quantity of feed related to fish weight was adjusted through biweekly fish weighing at early morning before feeding. The diets were offered at two equal meals per day.

A-Evaluation of growth performance

1- Growth weight: estimated biweekly throughout the experimental period

2- Body weight gain: Final fish weight (g) - Initial fish weight (g) (**Annet , 1985**).

3- Specific Growth Rate %: It was calculated as the percentage increase in weight per fish per day as suggested by ***Jauncy and Ross (1982)***, using the following equation:

$$SGR = \frac{Log_e W_2 - Log_e W_1}{T_2 - T_1} X\ 100$$

Where:

SGR%=Percentage increase in body weight per fish per day.

Ln WT=natural log of weight at time T.

Ln Wt= natural log of initial weight.

T=time T, t=initial time

4- Feed Conversion Ratio (FCR)

$$FCR = \frac{\text{Total feed consumption (g)}}{\text{Total weight gain (g)}}$$

As reported by **De Silvia and Anderson (1995)**.

5- Protein Efficiency Ratio (PER)

$$FER = \frac{\text{Weight gain (g)}}{\text{Protein intake (g)}}$$

As reported by **De Silvia and Anderson (1995)**

6. Body length: The whole body length (cm) of each fish was measured from the anterior part of fish to the end of its tail. The body length increment *LI* (cm) was estimated according to the following equation:

$$LI = L1 - L0$$

Where:

LI = Length increment (cm) *L1* = Average final length (cm)

L0 = Average initial length (cm)

7. Condition factor (K)

The condition factor, which relates body length of the fish to the body weigh; was computed for fishes according to **Higgs et al. (1982)** as follows:

$$CF = \frac{W}{L^3}$$

Where:

W= Body weight in grams. L = Body length in cm.

8. **Survival rate was** calculated as the following equation:

$$SR = \frac{\text{End number of live fish}}{\text{The beginning number of fish}} \times 100$$

B. Blood analysis parameters

At the end of each experiment about 1.5 ml blood samples were collected from different groups via the caudal vessel from 3 fishes using disposable tuberculin syringe. Blood samples were taken without anti-coagulant and used for serum separation by centrifugation of blood at 3000 rpm for 15 minutes. The clear serum was transferred carefully to clean and dry vials and kept in deep freezer until analysis for determination of serum total protein, albumin, globulin, alkaline phosphatase, glucose, GPT and GOT

1. Determination of serum total protein:

Serum total protein was determined according to ***Doumas et al. (1981)*** using commercial kits produced by ELI TECH Company

2. Determination of serum albumin:

Serum albumin was determined according to **Reinhold (1953)** using commercially available kits produced by ELI TECH Company.

3. Calculation of serum globulin content:

Serum globulin was determined by subtracting the albumin value from the total protein value of the same sample according to **Coles (1974)**.

4-Determination of serum alkaline phosphatase:

Serum alkaline phosphatase was estimated according to modified method of ***Kind and King (1954)*** using commercial kits produced by Pasteur Lab.

5- Determination of serum Glucose: Glucose present in the sample is determined according to **Kaplan, (1984).**

6- Determination of serum GPT: Serum GPT activity was determined photometrically according to the method described by **Reitman and Frankel, (1957).**

7- Determination of serum GOT: Serum GOT activity was determined photometrically according to the method described by **Reitman and Frankel, (1957).**

C- Intestinal glucose absorption

An intestinal perfusion technique **(Zarzuelo *et al*., 1990)** was used to study the effects of SB and or probiotic on intestinal glucose absorption in Oreochromis niloticus. Results were expressed as percentage glucose absorption calculated from the amount of glucose in solution before and after perfusion with SB and or probiotic in the perfusion solution.

D- **Proximate analysis of diet and fish**

The tested diets and fish from each treatment were chemically analyzed according to the standard methods described in **AOAC, (2002)** were used to analyze the proximate composition of the tested diets for protein, fat, fibre, ash and moisture while carbohydrate was calculated by subtracting the sum of the values of the other nutrients from 100. Also, analyze the proximate composition of the fish for protein, fat, ash and moisture.

E- Statistical analysis

All data measured in the study were analyzed by comparing means according to least significant difference test, using the general linear model procedure of **SAS (2002).**

RESULTS AND DISCUSSION

A-GROWTH PARAMETERS

As shown in Table 3 growth performance of Nile tilapia was significantly affected by SB supplementation (group 2) only and SB plus probiotics supplementation (group 4). The highest (final weight,

total weight gain and specific growth rate) of Nile tilapia were obtained with feeding diet containing SB plus probiotics (group 4) supplemented diets, followed by fish of group 2 which received SB supplemented diets ($P<0.05$) when compared with those of control group.At the end of experimental period both groups received SB (group 2) only and group received SB plus probiotics supplemented diets(group 4) revealed significant increase in the final body weight, body weight gain, specific growth rate (SGR), when compared with those of control group. The improved growth performance could be associated with the beneficial effect of butyric acid on the proliferation of the intestinal epithelium. Also, probiotics (Protexin) supplementation (group 3) insignificantly increases final body weight, when compared with fish of control group. Total body weight gain (W.G) and the specific growth rate (SGR) significantly increases when compared with those of control group (group 1).

These was insignificant difference in total feed intake between different dietary groups but there were significant improvements in total feed conversion (TFCR) and protein efficiency ratio (PER) in all treated groups when compared with those of control group. The best FCR values observed with group received SB plus probiotics supplemented diets (group 4). These results agreed with (**Zhang et al., 2011**) who found that increase in the weight gain and reducing feed conversion ratio in groups fed diets containing sodium butyrate when compared with the control groups.

The PER results indicated that supplementing diets with SB plus probiotics (group 4) significantly improved protein utilization in tilapia. This contributes to optimizing protein use for growth which is the most expensive feed nutrient. The improvement in the biological value of the supplemented diets in these treatments with high population and low dietary protein demonstrated that the probiotics supplements performed more efficiently in stress situations. This agreed with the results obtained by **Ringo and Gatesoupe. (1998)**.

Table 4. Growth performance parameters of Nile tilapia (*O. niloticus*) as affected by dietary supplementation of sodium butyrate, Protexin and sodium butyrate plus Protexin

	G1	G2	G3	G4
Initial BW	16.7± 0.28a	16.8± 0.28a	16.9± 0.29a	16.8± 0.26a
Final BW	100.4± 4.06b	112.1± 3.94a	106.7± 3.46ab	116.5± 3.78a
WG	83.7 ± 1.76c	95.3± 1.98a	89.8± 0.96b	99.7± 2.78a
SGR	1.08±0.01c	1.14±0.02ab	1.11±0.01b	1.16±0.01a
TFI	165.75±7.25a	175.54±6.41a	168.69±5.45a	178.56±5.27a
T FCR	1.98±0.02a	1.84±0.01c	1.88±0.012b	1.79±0.01c
PER	2.06±0.02c	2.21±0.02ab	2.15±0.02b	2.24±0.02a
Total increase length	2.7±0.18c	3.2±0.05b	3.2±0.16b	3.8±0.04a
Condition factor (K)	3.6±0.14a	3.4±0.02ab	3.4±0.11ab	3.2±0.03b
Survival rate	92.25	95.12	97.20	97.20

The previous results of growth parameters showed that Short chain fatty acids (SCFA) play a key role as an energy source, butyric acid being the most readily oxidized to CO2 among all the other SCFA in the intestine (**Fleming and Gill, 1997**).Butyric acid was also shown to induce cell differentiation and to regulate the growth and proliferation of normal colonic and ileal mucosa (**Treem et al., 1994**), whereas it can actively reduce the growth rate of cells in colorectal cancer (**Berry and Paraskeva, 1988**). A deeper understanding of the role of butyric acid in the intestinal metabolism of food animals is needed in order to guarantee safe and efficient meat production. Changes in gut morphology are important as they can affect growth rate. Short chain fatty acids (SCFA) produced by microbial fermentation from dietary fiber stimulate epithelial cell proliferation resulting in a larger absorptive surface (**Sakata, 1988**). Moreover, the fact that normal colonic epithelia derive 60 to 70 % of their energy supply from SCFA, particularly from butyric acid (**Scheppach et al., 1992**) must be considered. The latter induces cell differentiation and regulates the growth and the proliferation of normal colonic mucosa (**Treem et al., 1994**) The improved growth performance could be associated with the beneficial effect of butyric acid on the proliferation of the intestinal epithelium.

The previous results of growth parameters, the results indicated a positive acceptable effect of the used probiotic The obtained results could be attributed to the ability of probiotic to adhere to the intestinal mucosa of O. niloticus producing a wide range of relevant digestive enzymes (amylase, lipase and protease) which have the ability to denaturate the indigestible components in the diets, the ability to detoxify the potentially harmful components of feed and the ability to produce a lot of essential vitamin B. complex members particularly Biotin and vitamin B_{12}, the matter of which resulted in high food utilization and an increase in digestibility of different diet components. These results supported those of **Marzouk et al. (2008)** who used probiotic (*Bacillus subtilis* and *Saccharomyces cerevisae*) in the food of tilapia and found that these probiotic bacteria increased the food absorption by enhancing the protease level and consequently gave a better growth.

Table 5. Proximate chemical analysis (%; on dry matter basis) of Nile tilapia (*O. niloticus*) as affected by dietary supplementation of sodium butyrate, Protexin and sodium butyrate plus Protexin

Item	G1	G2	G3	G4
moisture	73.7±0.7a	72.3± 0.3a	73.3± 0.3a	72.7± 0.3a
DM	26.3± 0.7a	27.7± 0.3a	26.7± 0.3a	27.7± 0.3a
Cp	62.3± 0.3c	66.5±0.3a	65.4± 0.3b	68.0± 0.6a
Ether extract	21.9± 0.2a	19.6±0.2b	19.8± 0.2b	19.0± 0.3b
Ash	15.8±0.2a	13.9±0.3c	14.8±0.3b	13.0±0.3c

Table (5) showed that there was insignificant difference in dry matter content between different treated groups. Concerning body crude protein all treated groups showed significant increase when compared with the control groups. Body lipid content demonstrated an opposite trend to body protein content. Sodium butyrate and or protexin supplementation significant improved body protein percentage. While body ether extract and ash was significant decreased in groups fed SB and or protexin when compared with those of control group.

These results in agree with those of **Ye et al. (2011)** who analyzed body composition in Japanese flounder. There was an increase in body protein content in fish fed prebiotic and or probiotic containing diet compared to the control. Fish fed diets *B. clausii* + *fructooligosaccharide* and *B. clausii* + *fructooligosaccharide* + *Mannan oligosaccharide* also exhibited significantly higher body protein content than fish fed the control diet. There was decrease in body fat content in all treated fish when compared with the control one. Higher body protein content in the treatment groups implies on this fact that by application of probitic, the ingested food was converted more effectively into the structural protein and subsequently resulted more muscle as it is a desirable aspect in fish farming.

Concerning the effect of sodium butyrate and Probiotic either alone or in combination on blood glucose level and intestinal glucose absorption, table (7). We found that, the supplementation of ration with probiotic caused non significant increase in both blood glucose level and intestinal glucose absorption while sodium butyrate either alone or in combination with probiotic significantly increased both blood glucose level and intestinal glucose absorption. This concurs with **Zhanguo et al., (2009)** who reported that, supplementation of butyrate improved serum glucose level in C57BL/6 mice. The same authors revealed that, the mechanism of butyrate action is related to promotion of energy expenditure and induction of mitochondria function.

Also, short-chain fatty acids has been shown to be effective in the treatment of ulcerative colitis in which sodium butyrate decreased reactive oxygen species generation by neutrophils, which are responsible for mucosal injury and subsequently increased absorption **(Liu et al., 2001).** Sodium butyrate is a preferred fuel source by colonocytes even when glucose is available **(MacFarlane and MacFarlane 2003).** SB not only improved animal growth but also increased the length of the ileal microvilli and depth of the cecal crypts on intestinal mucosa **(Kotunia et al., 2004 and Maurizio et al., 2008). Böcker et al. (2003)** reported a favorable effect of Na-butyrate on the expression of genes regulating the development of intestinal mucosa. Increased in serum glucose concentrations indicate that glucose

oxidation decreased in ruminal mucosa cells, with increases in the use (by mitochondria of mucosal cells) of volatile fatty acids from microbiological fermentation, and of Na-butyrate supplementation **(Ślusarczyk et al., 2010).** This increase in intestinal glucose absorption may also be discussed as follows. The jejunum is the main site of glucose absorption. The sugar is transported across the mucosal membrane by a sodium-dependent secondary active process that relies on the sodium gradient established by the Na^+-K^+ ATPase also Known as Na^+-K^+ pump **(Schultz & Curran, 1970).**

The Na^+-K^+ ATPase couples the exchange of three cytoplasmic Na^+ ions with two extracellular K^+ ions to the hydrolysis of one molecule of ATP resulting in the establishment of an electrochemical gradient across the plasma membrane **(Berribi-Bertran *et al.*, 2001).** The sodium gradient created by the pump provides the driving force for several secondary active transport process like that responsible for the absorption of glucose from the lumen. Any change in the activity of Na^+-K^+ ATPase is expected to increase or decrease the sodium gradient and consequently affect glucose absorption. Therefore, the increase in the intestinal glucose absorption seen in the present study may be ascribed to the activation of Na^+-K^+ ATPase activity. In the contrary, Dietary SB did not influence the absorptive function of jejunum **(Zhonghong and Yuming, 2007).** These controversial data may be due to using different species as study subjects or different experimental treatment. Concerning the effect of sodium butyrate and Probiotic either alone or in combination on total protein, albumin, globulin, GPT, GOT and ALP, table (7).

The results revealed that, SB and probiotic either alone or in combination have no adverse effects on liver reflected in increased of serum protein pattern and normal values of enzymatic activity. **Bakr and Haggag, (2005)** reported that, sodium butyrate supplementation resulted in increased total protein, albumin, globulin, and total leucocytic count. The same authors revealed that, SB stimulate the local; immune response, gut associated lymphoid tissues, modulation of blood immune parameters.

Table 6. Total protein, albumin, globulin, blood glucose level , intestinal glucose absorption, GPT, GOT and ALP of Nile tilapia (*O. niloticus*) as affected by dietary supplementation of sodium butyrate, Protexin and sodium butyrate plus Protexin

item	G1	G2	G3	G4
Total protein	3.92±0.18b	4.56±0.16a	4.32±0.20ab	4.62±0.16a
Albumin	2.87±0.09a	3.02±0.14a	2.99±0.16a	2.76±0.12a
Globulin	1.05±0.15c	1.54±0.12ab	1.33±0.09b	1.86±0.10a
Glucose	86.75±0.80b	97.50±1.83a	88.00±0.90b	98.25±1.26a
Absorption	27.25±0.65b	44.00±0.84a	27.50±0.95b	44.50±0.56a
GPT	38.30±0.51a	32.60±0.97b	33.48±1.12b	32.20±0.83b
GOT	106.00±7.65a	90.40±2.82b	90.80±7.65a	82.60±1.40b
ALP	73.82±0.89a	57.18±2.48b	57.00±2.55b	53.82±2.14b

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