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Effect of Fermented Silkworm (*Bombyx mori*) Pupae on Growth Performance, Immune Index and Meat Quality in Lingnan Yellow Broiler Chickens

Xiangjie Zhao[1], Zheshi Kuang[2], Jiyuan Yang[2], Muhammad Bilal[1] and Rongling Yang[1]*

[1]*College of Life Science and Food Engineering, Huaiyin Institute of Technology, Huaian 223003, Jiangsu, China*
[2]*Sericultural and Agri-Food Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou 510610, Guangdong, China*

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XZ and ZK conceived and designed the experiment. XZ and JY performed the experiments. XZ, RY and JY analyzed the data. ZK, XZ and RY contributed reagents/ materials/ analysis tools. XZ and MB wrote and revised the paper.

Key words
Silkworm (*Bombyx mori*) pupae, Solid-state fermentation, Fishmeal, Lingnan yellow broiler chicken, Growth performance

ABSTRACT

Silkworm (*Bombyx mori*) pupae (SWP) is a promising feed source with high nutritional value. In present study, the effects of different proportion of solid-state fermented silkworm pupa as a fishmeal substitute were evaluated on the growth performance, immune organ indices, serum biochemical parameters, and meat quality of Lingnan yellow broiler chickens. Lingnan yellow broilers were randomly categorized into 4 groups. The control group was fed with basal diet containing 3% fishmeal, and the treatment group 1, 2, and 3 were fed basal diet with 30%, 60%, and 100% fishmeal substituted with fermented silkworm pupae (FSP), respectively. Broilers were fed the treatment diets in phase 1 (1-21 d) and phase 2 (22-42 d) during the experimental period. Results showed a non-significant difference ($P > 0.05$) between control and treatment groups on the average daily weight gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (feed/gain, F/G) at 21d. At 42 d, the F/G of three treatment groups were all less than the control group. Moreover, the broiler meat quality increased with treatment group compared with the control at 42 d. It also showed that FSP addition could improve the thymic index of broilers at both 21 and 42 d. Among the above treatments, Treatment 3 reached a significant level ($P < 0.05$) as compared to the control in F/G, thymic index and meat quality. All treatment groups had a lower drip loss in meat quality compared to control, and Treatment 1 reached a significant level ($P < 0.05$). In conclusion, the FSP can be employed as a potential economical substitute for the fishmeal to Lingnan yellow broiler chickens.

INTRODUCTION

In recent years, the shortage of feed ingredients, particularly, the protein ingredients has become a global concern (Li *et al*., 2019). The complete and rational utilization of the available protein resources and exploring new protein resources has gradually become an immediate challenge for the feed industry (Carlberg *et al*., 2018). Biotransformation and valorization of a wide range of agricultural processing byproducts by microbial fermentation technology can bring huge economic benefits to feed and farming industry.

Sericulture industry, a traditional characteristic industry originated from the Asian region, has a long history. Among the Asian countries, China has already been recognized as the largest silk producer and exporter in the world (Tuan *et al*., 2019). Silkworm pupae are the main byproduct of the reeling industry. Approximately, China produces 400,000 tons of fresh silkworm pupae annually that account for 70% of the global production (Hu *et al*., 2017). Although silkworm pupae, rich in protein and unsaturated fatty acids, are natural protein resources of great value for development and utilization (Köhler *et al*., 2019; Sadat *et al*., 2022; Wu *et al*., 2021; Akande *et al*., 2020), their easily oxidation, deterioration, and even decaying during the reeling process result in a strong fishy smell, which eventually affects their applications as feed ingredient. The discarding of such unwanted pupae creates a potential environment burden and wasting the

* Corresponding author: yangrongling@163.com
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resources (Hu *et al*., 2017). In recent years, solid-state fermentation (SSF) technology has been used to process silkworm pupae resources, during the SSF stage, not only the fishy smell of silkworm pupae has been removed, and also their fermentation performance has been enhanced, which has substantially increased their value in the feed industry (Yang *et al*., 2021; Zhou *et al*., 2017), and this greatly improves the application prospect of fermented edible insects (Castro-López *et al*., 2020).

As an ancient fermentation technique, the SSF owns nearly a thousand years of application history. This technique implicates the microbial metabolic activity to decompose organic matters, improve the protein content, and enhance the biosynthesis of a range of valuable secondary metabolites (Seong and Kim, 2021; Muniz *et al*., 2020; Ferreira *et al*., 2020). In addition, the SSF process also accumulates a variety of enzymes and probiotics (Olukomaiya *et al*., 2019), which significantly improve the digestive performance of fermentation materials. It is reported that application of a mixture of different kinds of microbial strains, such as *Bacillus* sp., *Aspergillus* sp. and *Saccharomyces* sp., in SSF can potentially improve the nutrition value of feedstuff (Olukomaiya *et al*., 2020; Lu *et al*., 2022; Chi and Cho, 2016) and promote the health of animals by these fermented feed (Zhang *et al*., 2022). In recent years, a great progress has been made on the use of SSF technology to harness agricultural byproducts, bringing tremendous application potential to the feed and farming industry (Dou *et al*., 2018; Sugiharto and Ranjitkar, 2019; Olukomaiya *et al*., 2019). Therefore, the present study intends to utilize SSF technology to treat silkworm pupae and to evaluate their fermentation performance. The growth performance, slaughter performance, and blood biochemical parameters of broilers fed with fermented pupa feed were investigated. In addition, its performance as a replacement of fishmeal was also evaluated.

MATERIALS AND METHODS

Fermentation strains and culture conditions

The SWP were purchased from market and stored at room temperature until further use. The composition of the dry pupa powder was as follows: crude protein 52.9±1.6%, crude fat 21.9±2.2%, crude ash 5.9±0.3%, and crude fiber 5.4±0.6%. Three bacterial and fungal strains were used in fermentation in this study. A specific pupa-protein degradation strain, *Bacillus amyloliquefaciens* CY21, was earlier isolated and screened in our group (Zhao *et al*., 2013). *Aspergillus oryzae* CICC40188 was procured from the China Center of Industrial Culture Collection (Beijing, China). Whereas *Aspergillus sojae* GDMCC3.33 was provided by the Guangdong Microbial Culture Collection Center (Guangdong, China). *B. amyloliquefaciens* was cultivated in a flask with medium (natural pH) containing 0.5% peptone, 0.3% yeast extract, and 0.5% NaCl, at 180 rpm, 37 °C, for 24 h. The bacterial density was then adjusted to 10^8 CFU/mL for further use. *A. oryzae* and *A. sojae* were cultured in a flask with medium containing 3% sucrose, 0.2% $NaNO_3$, 0.05% $MgSO_4$, 0.05% KCl, 0.1% K_2HPO_4, and 0.001% $FeSO_4$, at 250 rpm and 28 ℃ for 48 h. The cultures with spores were preserved kept for subsequent use. The regents of NaCl, KCl, K_2HPO_4, and $FeSO_4$ were all analytical grades.

Preparation of fermented silkworm pupae

The fermentation of SWP was developed in a framed wooden case with a 20 kg scale per batch. Molasses was added up to 3% (w/w) of the silkworm pupae weight. The ratio of silkworm to water was 1:0.3 and the inoculation amount was 7% (w/w) of fermentation materials weight. The inocula were composed of *B. amyloliquefaciens, A. oryzae* and *A. sojae* with a ratio of 1:1:1 (v:v:v). The materials were mixed well, sealed with gauze, and incubated at 30 °C in an incubator. After 72 h of the fermentation, the fermentation product was placed on a china platter, dried in a drum wind drying oven (firstly at 80 °C for 1 h and then at 60 °C until full dryness), and ground into powder sample.

Feed formulation and feeding plan

The Animal Experiments Committee of the Sericultural and Agri-Food Research Institute has approved all animal experiments. The feed formulation and feeding plan were according to Stefanello *et al*. (2017) and made modifications (Stefanello *et al*., 2017). A total of 480 healthy 1-day-old Lingnan yellow broilers (fast-growing yellow broilers) with similar body weight (37.07±0.19) were randomly divided into 4 groups with 4 replicates in each group and 30 animals in each repeat. The treatment was divided into 2 stages (total of 42 d), with the first stage from 1 d to 21 d and the second stage from 22 d to 42 d. In each stage, the broilers were fed with experimental diets at different nutrient levels (Control group and T1, T2 and T3 group). The control group was the basic diet for broilers, and in group T1, T2 and T3, 30%, 60% and 100% of fish meal were replaced by FSP on the basis of basic diets for broilers both in 1-21d and 22-42d stage. The isonitrogenous, isocaloric diet (Table I) was formulated according to the yellow broiler nutrition standards listed in the Agricultural Industry Standard of China (NY/T33-2004). The experimental broiler chickens were given feed and water *ad libitum* and managed according to the conventional management procedures. A 24-h non-stop lighting was provided. The room was controlled at 25-32℃, with regular cleaning and disinfection.

Table I. The formulation and nutrient levels of experimental diet (1-42 d).

Item	1-21 d [c]				22-42 d [c]			
	Control	T1	T2	T3	Control	T1	T2	T3
Corn (%)	58.50	58.25	57.95	57.67	62.00	61.72	61.70	61.5
Bean cake (%)	29.50	29.80	30.15	30.15	24.30	24.60	24.65	24.9
Bran (%)	3.00	3.00	3.00	3.00	2.00	2.00	2.00	2.00
Fish meal (%)	3.00	2.10	1.20	-	1.50	1.05	0.60	-
FSP (%)	-	0.90	1.80	3.00	-	0.45	0.90	1.50
Cotton seed meal (%)	-	-	-	-	3.00	3.00	3.00	3.00
Rapeseed oil (%)	2.00	1.95	1.90	1.82	3.20	3.18	3.15	3.10
Premix [a] (%)	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
CP (%)	21.05	21.04	21.04	21.00	19.03	19.06	19.04	19.04
Calorie (MJ/kg)	12.12	12.12	12.12	12.12	12.54	12.54	12.54	12.54
Lysine [b] (%)	0.78	0.78	0.77	0.77	0.83	0.82	0.82	0.82
Methionine [b] (%)	0.25	0.25	0.25	0.25	0.27	0.27	0.27	0.27
Threonine [b] (%)	0.48	0.47	0.47	0.47	0.50	0.50	0.50	0.50

[a] the premix provides vitamins and minerals as follows (per kg of diet): VA15000 IU; VD_3 3900 IU; VE 30 IU; VK_3 3 mg; VB_1 2.4 mg; VB_2 9 mg; VB_6 4.5mg; VB_{12} 0.02mg; pantothenic acid 30 mg; niacin 45 mg; folic acid 1.2 mg; biotin 0.18 mg; choline 700 mg; $CuSO_4 \cdot 5H_2O$ 8 mg; $ZnSO_4 \cdot 7H_2O$ 40 mg; $FeSO_4 \cdot 7H_2O$ 80 mg; $MnSO_4 \cdot 5H_2O$ 100 mg; KI 0135 mg; Na_2SeO_3 0.15 mg. [b]Metabolizable energy was based on calculated values. [c] Growth period.

Measurement of the indices

All the broilers were repeatedly weighed at 1, 21, and 42 d. The feed was withdrawn 12 h before weighing, while the water was given continuously, and the fasting body weight was measured at 8:00 in the next morning (12 h after the feed withdrawal). The average daily weight gain (ADG), average final body weight (BW), average daily feed intake (ADFI), and feed conversion ratio (feed/gain, F/G) were then calculated.

After the measurement of fasting body weight at 21 and 42 d, four birds were randomly selected from each repeat, numbered, and weighed for live weight. Blood samples were immediately collected from the jugular or wing vein, and the birds were then killed by bleeding. The coagulated blood samples (10 mL) were centrifuged at 3,000 rpm for 15 min to prepare for sera, which were then stored at -20 °C for further analysis. The serum samples were analyzed on Hitachi automatic biochemical analyzer (Hitachi Company, Tokyo, Japan) for biochemical indices, including total protein (TP), albumin (ALB), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total cholesterol (T-CHO), glucose (GLU), immunoglobulin G (IgG), uric acid (UA), and total triglyceride (TG). After collecting the blood, the birds were killed, and the feathers were removed by wet plucking. The thymus, spleen, and bursa of Fabricius were then dissected. After removing the adipose tissue, the carcass was weighed for fresh weight. The absolute weight was used to calculate immune organ indices (Wang *et al*., 2018). At 42 d, four birds were randomly selected from each repeat and weighed individually. The pectoral muscle was then dissected to measure for crude protein, crude fat, moisture, drip loss, and tenderness.

Statistical analysis

All the data were presented as mean ± standard error and analyzed by One-Way analysis of variance (ANOVA) using the SPSS14.0 software. Multiple comparisons were conducted using Duncan's new multiple range test. The level of significant difference was set at $P<0.05$.

RESULT

Growth performance

The growth performances of addition of FSP as a substitute for fish meal in Lingnan yellow broiler chickens are reported in Table II. At 21 and 42 d, the ADFI in treatment groups was decreased compared with control, though the differences were not significant ($P>0.05$). The ADG in each treatment group showed no significant ($P>0.05$) difference compared with that in the control group both at 21 and 42 d. Results also showed that at 21 d, the F/G in each treatment group was close to that in the control group; however, at 42 d, the F/G in treatment groups was lower than that in the control group. The difference increased with the added amount of fermented pupae; especially, the difference in F/G between T3 and

control was statistically significant ($P < 0.05$). At 21 d, replacement of the fishmeal with fermented pupae not only achieved the same ADG and F/G but also displayed no adverse effects on the growth performance of birds.

Table II. The effect of supplementing FSP into basal diet on the growth performance of Lingnan yellow broilers.

Groups	ADG (g)	ADFI (g)	F/G
1-21 d			
Control	18.87±0.34	33.62±0.13	1.78±0.29
T1	18.91±0.13	33.38±0.30	1.76±0.13
T2	18.86±0.37	33.57±0.68	1.78±0.11
T3	18.64±0.14	33.28±0.12	1.78±0.01
22-42 d			
Control	41.54±0.70	106.45±1.24	2.56±0.07a
T1	43.89±1.27	105.86±0.39	2.41±0.06ab
T2	42.75±0.86	106.40±4.0	2.48±0.06ab
T3	44.46±1.04	105.87±0.45	2.38±0.05b

The data were presented as mean±standard error (n=4). In the same column, same superscript letter indicates significant difference between groups (P<0.05). The same applies hereinafter.

Immune organs

The effects of supplementing FSP into basal diet on the immune organs of Lingnan yellow broilers are reported in Table III. At 21 and 42 d, the thymic index in each treatment group was increased compared with the control group, and the increase in T3 was statistically significant ($P<0.05$). However, no statistically significant ($P>0.05$) differences were found on the indices of spleen and bursa of Fabricius in each treatment group between two growth stages.

Table III. The effect of supplementing fermented silkworm pupae on the immune organs of Lingnan yellow broilers.

Groups		Thymic index * (mg/g)	Spleen index * (mg/g)	The index of bursa of fabricius * (mg/g)
1-21 d	Control	3.08±0.78a	0.82±0.14a	2.10±0.79
	T1	3.84±0.25ab	1.21±1.31ab	2.97±0.94
	T2	3.87±0.41ab	1.36±0.20b	2.67±0.62
	T3	4.97±0.59b	1.11±0.53ab	2.83±0.66
22-42 d	Control	2.97±0.88a	1.23±0.09	0.92±0.30a
	T1	3.67±0.82ab	1.61±0.21	1.79±0.45b
	T2	4.34±0.61ab	1.75±0.57	1.66±0.53b
	T3	4.85±0.82b	1.30±0.09	1.60±0.30b

* Organ weight to live weight ratio.

Blood biochemical indices

The effects of supplementing FSP on the blood biochemical indices of Lingnan yellow broilers are reported in Table IV. There were no significant ($P > 0.05$) differences in the level of ALT, ALB, GLU, IGG, T-CHO, TP and TG of blood indices in all treatment groups compared with the control groups in both two growth stages. At 21 d, the UA level in T3 was significantly ($P < 0.05$) higher than that in the control group. However, at 42 d, there were no significant differences between treatment and control groups.

Meat quality

As seen in Table V, the meet quality test at 42 d showed that the pectoral muscle protein content in each treatment group increased compared with the control group, and T3 displayed the highest protein content, which was significantly higher than that in control group ($P <0.05$). The difference in water and fat contents of pectoral muscle between the treatment and control groups was not significant ($P > 0.05$). The drip loss in each treatment group was lower than that in control group, with T1 showing the smallest drip loss. The differences were significant between treatment and control groups ($P< 0.05$), but insignificant among the treatment groups ($P > 0.05$). The sheer force of pectoral muscle in each treatment group was significantly lower than that in control group ($P < 0.05$).

DISCUSSION

The amount of supplemented FSP in this study was low. During both stages (1-21 and 22-42 d), the feed intake in treatment groups was slightly lower than that in the control group, and there was no significant difference. The same effect of silkworm pupa powder supplementation to 50% in carp that did not affect the growth rate of fish has already been reported (Nandeesha *et al*., 2000). This is possibly because that the fat content in fermented pupae is higher than that in fishmeal, and animals firstly use fat as energy. After fermentation, the macromolecular proteins in pupae were degraded into small molecules, which were easily digested, absorbed, and thus utilized more efficiently by the animals. And a significant lower F/G in T3 than the control at 22-42 d demonstrated that Rangacharyulu *et al.* (2003) used fermented silkworm pupae and natural pupa powder, respectively, to completely replace the fishmeal in pond polyculture of *Catla catla*, *Cirrhinus mrigala*, and *Labeo rohita.* Above results showed that among the natural silkworm pupa powder, fermented silkworm pupae and fish meal groups, the fermented silkworm pupae group had a lower F/G value and higher growth rate, while the difference between the latter 2 groups was not significant,

Table IV. The effect of supplementing FSP into basal diet on the blood biochemical indices of Lingnan yellow broilers.

Growth period	1-21 d				22-42 d			
	Control	T1	T2	T3	Control	T1	T2	T3
ALT (U/L)	5.03±1.03	4.20±0.70	3.83±0.43	3.80±0.55	4.1±0.35	3.9±0.45	4.1±0.30	4.1±0.20
ALB (g/L)	29.23±0.78	29.97±2.64	30.37±1.13	27.40±1.95	16.00±0.56	17.17±0.74	14.93±0.15	16.17±1.23
GLU (g/L)	12.77±0.26	13.20±0.35	13.17±0.26	13.97±0.74	12.27±0.30	12.27±0.31	11.28±1.09	12.50±0.40
IGG (g/L)	0.24±0.02	0.24 ±0.06	0.24±0.04	0.25±0.01	0.23±0.01	0.24 ±0.08	0.23±0.12	0.24±0.01
T-CHO (mmol/L)	3.43±0.17	3.83 ±0.26	3.86±0.05	3.14±0.15	3.56±0.17	3.80±0.22	3.86±0.26	3.47±0.20
TP (g/L)	29.23±0.78	29.97±0.64	30.37±1.13	27.40±1.95	33.33±1.99	35.20±1.40	30.30±0.49	32.60±2.30
UA (μmol/L)	173.33± 13.97[a]	205.33± 42.83[ab]	172.00± 18.58[a]	309.33± 63.26[b]	174.67± 31.96	219.67± 29.25	198.00± 15.01	176.21± 26.58
TG (mmol/L)	0.23±0.02	0.25±0.01	0.27±0.06	0.21±0.03	0.22±0.01	0.24±0.01	0.26±0.08	0.19±0.02

Table V. The effect of supplementing FSP into basal diet on the meat quality of Lingnan yellow broilers (22-42 d).

22-42 d	Moisture (%)	CP (%)	Intermuscular fat	Drip loss	Shear force (kg/cm^2)
Control	72.10±0.35	88.08±0.64a	3.24±0.07	7.43±0.78b	3.27±0.39a
T1	72.24±0.25	89.23±0.27ab	3.12±0.11	3.80±0.64a	2.75±0.24b
T2	72.74±0.60	88.98±0.32a	3.30±0.06	4.53±1.40ab	2.84±0.17b
T3	72.64±0.35	90.21±0.16b	3.28±0.28	5.11±0.90ab	2.94±0.44b

which suggesting that the feeding performance of pupae was improved by fermentation, which is consistent with our findings that the effect of fermented pupae on the production performance of broilers is better than that of fish meal. And also, only the F/G in T3 was significantly lower than those in control group at 42 d, indicating that in the growth during 22-42 d, replacement of partial fishmeal with fermented pupae could not influence on average daily gain or average daily food intake, but exhibited a better feeding effect

Thymus, spleen, and bursa of Fabricius are the major immune organs of birds. The increase in their absolute and relative weights indicates an enhanced cellular and humoral immune function of the body (Shokri *et al*., 2021; Ma *et al*., 2022). In this study, only the thymus indices showed a significant difference in T3 compared with the control that indicates a positive effect of fermented pupae on the development of immune organs of broiler chickens. This could increase the ability of broilers to combat against a range of infections caused by various pathogenic microorganisms and to resist various stresses.

Blood is the carrier transporting nutrients and metabolic wastes in the body. Serum biochemical parameters can be used as an important indicator to reflect the changes of *in vivo* metabolism and function of certain tissues and organs in animals under irregular diet resources (Rafiullah *et al*., 2020). To date, there is no report on the effect of feed supplementation of fermented pupae on the blood parameters of broilers. Results revealed that the UA level in treatment groups in the feeding stage of 1-21 d was significantly different from that in the control group. However, other blood parameters exhibited no significant difference among the groups. Blood UA is an important product of protein catabolism in birds, directly reflecting the levels of body's protein catabolism and nutritional status. A slightly higher UA level might indicate a poorer digestion and absorption and ineffective utilization of the fermented pupa protein (Aberra *et al*., 2013). In both feeding stages (1-21 and 22-42 d), there is no significant difference in the growth performance of broilers between the treatment and control groups, demonstrating that supplementation of fermented pupae did not affect the healthy growth of broiler chickens. Besides microorganism contamination, chitin in insects' pupae has also been reported to play an important role in animal nutrition (Moniello *et al*., 2019). A higher level of chitin in feed may influence the nutrition digestion and further interference the animal immune system which induce physiological stress at the same time, as observed i.e., in fish and hens (Zarantoniello *et al*., 2018; Cardinaletti *et al*., 2019). Therefore, the solid-state fermentation process of insect pupae powder can decompose chitin to some extent certain and the fermented

insect pupae powder will further enhance nutrition digestion in feed.

The meat quality of broiler chickens is an important evaluation criterion for their commodity (Pietras *et al*., 2021). In addition to a small amount of fat and minerals, protein forms the majority of the dry matter of pectoral muscle. The higher the protein content, the higher the nutritional value of muscle. Although water is not a nutrient of the meat, its content directly associates with the status, quality, and even flavor of the meat and meat products. Moreover, the increase of the intramuscular fat content improves the tenderness, juiciness, and flavor of the meat. The present study demonstrated that significant differences were noted in meat protein, drip loss and shear force in treatment groups compared with the controls, and these indices are a reflection of meat quality improvement.

CONCLUSION

Comparative study of the effects of fermented silkworm pupae and fish meal on the growth of yellow broiler chickens indicated that supplementation of FSP into the basal daily diet of yellow broilers did not affect their growth performance and blood biochemistry. A notable positive effect of FSP supplementation was observed on the immune organs of Lingnan yellow broiler chickens. Among FSP supplementation group, thymic index, spleen index and the index of bursa of Fabricius were all substantially improved compared with the fishmeal groups. In addition, indexes of meat quality in FSP groups were superior to fishmeal groups, and the drip loss of meat in FSP groups was significantly lower than that in fishmeal groups. Considering the lower price of the FSP than fishmeal, the FSP is an attractive alternative and economical choice in Lingnan yellow broiler chicken's diet. Therefore, with comprehensive consideration of the effects of adding FSP feed compared with fishmeal on the growth performance, health status, meat quality and economic principles of broilers breeding, the T2 in 1-21 days and T1 in 22-42 days were recommended, while FSP can replace 60% and 30% fishmeal at 1-21 days and at 22-42 days, respectively when compared to the control group.

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Statement of conflict of interest

The authors have declared no conflict of interests.

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