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SHORT PAPER

Early Weaning of Sea Bass (*D. labrax*) Larvae: Effects on Growth Performance and Digestive Enzyme Activities

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Abstract

In this study, effects of early weaning of sea bass (*D. labrax*) by microparticulate food on larval development and digestive enzyme activities, acid protease and alkaline protease were investigated until day 40. Larval rearing was conducted in closed sea water systems. Experiments were triplicated on 3 different weaning protocol that introduced on day 15 (MD15), day 20 (MD20) and day 25 (MD25) and also no microparticulate diet (MD) distributed in the control group. At the end of the experiment at 40-day, larval growth was calculated and the best values were obtained from MD25. Statistical differences among groups were not important ($P>0.05$) whereas MD15 was found different than the other groups ($P<0.05$). Also, survival rates were calculated as 3.6%, 14.7%, 43.6%, and 34.1% for the experimental groups, respectively. MD15 and MD20 groups were found different than the other groups ($P<0.05$). Nevertheless, activity of alkaline protease was detected synchronously by mouth opening and exogenous feeding and also increased by larval development. According to histological analysis, pancreas was observed by mouth opening and similarly developed with increasing of larval age. Alkaline protease activity changed by nutritional composition of feed and especially shifting of feed affected the enzymatic activity. There was no differences among the experimental groups ($P>0.05$) while control group was found statistically different ($P<0.05$). According to histological analysis, gastric glands and formation of functional stomach were observed on day 24-25 in all experimental groups. Acid protease activity firstly detected at 25 DAH. Control group was found different than the other groups ($P<0.05$). Finally, in terms of both growth parameters and survival rate and digestive enzyme activities it is clearly concluded that MD supplementation could be started after 25 DAH in *D. labrax* larviculture.

Keywords: Sea bass, microparticulate feed, larval development, early weaning, digestive enzymes.

Levrek (*D. labrax,* L.) Larvalarında Erken Dönem Mikropartikül Yem Girişi: Larval Gelişim ve Sindirim Enzimleri Aktivitesine Olan Etkisi

Özet

Bu çalışmada; levrek (*D. labrax*) balığının larva kültüründe erken dönemde mikro partikül yemler kullanılmasının, larvaların yaşama ve büyüme oranları ile sindirim enzimlerinden asit proteaz ve alkalin proteaz aktivitesi üzerine olan etkileri 40. güne kadar incelenmiştir. Larval yetiştiricilik kapalı devre sistem kullanılarak gerçekleştirilmiştir. 3 tekrarlı olarak yürütülen denemelede larvalara 15. gün (MD15), 20.gün (MD20) ve 25. gün (MD25) olmak üzere üç farklı dönemde mikro partikül yeme geçilmiş, kontrol grubundaki (K) larvalara herhangi bir yem girişi yapılmamıştır. Denemenin bittiği 40. gün sonunda, larvalara ait total boy ve ağırlık gelişimleri incelendiğinde en iyi değerler MD25 grubundan elde edilmiştir. Deneme grupları arasındaki fark önemsizken ($P>0,05$) MD15 grubu diğer gruplar ile arasında önemli farklılıklar bulunmuştur ($P<0,05$). Yaşama oranları ise gruplara göre sırasıyla %3,6, %14,7, %43,6 ve %34,1 olarak saptanmıştır. Bu oranlar karşılaştırıldığında ise MY15 ve MY20 grupları MY25 ve K grubuna göre göreceli olarak farklı bulunmuştur ($P<0,05$). Öte yandan, alkalin proteaz aktivitesi ağız açılımı ve eksojen besin alımı ile tespit edilmiş, larval gelişime bağlı olarak artış göstermiştir. Histolojik kesitler incelendiğinde pankreas ağız açılımı ile birlikte tespit edilmiş, larval yaşa ve gelişime bağlı olarak gelişimini sürdürmüştür. Besin kompozisyonundaki değişimlere bağlı olarak alkalin proteaz aktivitesi değişim göstermiş, özellikle yem değişimleri enzimatik aktiviteyi etkilemiştir. Deneme grupları arasındaki fark önemsiz bulunurken ($P>0,05$) bu grupların kontrol grubu ile arasındaki farklılık önemli bulunmuştur ($P<0,05$). Histolojik kesitler incelendiğinde, gastrik salgı bezleri ve fonksiyonel mide oluşumu bütün deneme gruplarında 24-25. günlerde tespit edilmiştir. Asit proteaz aktivitesi ilk olarak 25. günde tespit edilmiştir. Deneme grupları ile kontrol grubu arasındaki fark önemli bulunmuştur ($P<0,05$). Sonuç olarak, gerek büyüme parametreleri ve yaşama oranı gerekse sindirim enzimleri aktivitesi açısından değerlendirildiğinde levrek larvalarında mikro partikül toz yem girişinin 25. günden sonra yapılabileceği tespit edilmiştir.

Anahtar Kelimeler: Levrek, mikropartikül yem, larval gelişim, erken toz yem girişi, sindirim enzimleri.

Introduction

Larval rearing stage in marine fish propagation under culture conditions mainly still depends on supply of live prey such as rotifer (*Brachionus plicatilis* and *Brachionus rotundiformis*) and *Artemia* sp. Nevertheless, the supply of *Artemia* cycts in large amounts during the larval rearing period involves large investments aimed at establishing uninterrupted food chain and maintenance of the nutritional quality by enrichment. This is especially, relevant in sea bass larval rearing, where live food accounts for 79% of the production cost for juveniles up to 45 days old. In the first 3 months of life, live food represents 50% of the feed cost even though they constitute only 1.6% of the total dry weight of food required (Person-Le Ruyet *et al*., 1993). Nowadays, more studies are focused on early weaning and its effects on digestive physiology related to studies on the development of the digestive system and gastrointestinal physiology of marine fish larvae. Additionally, intensive research has been conducted to find full or partial replacements for live food organisms such as rotifers (Kolkovski and Tandler,. 1996; Fernandez−Diaz and Yufera, 1997; Rosenlund *et al*., 1997; Cahu *et al*., 1998; Southgate and Partridge, 1998; Yufera *et al*., 1999) and in that in intensive marine fish hatcheries initial feeding is done with acquired *Artemia* nauplii is substituting live rotifer culture.

Marine fish larvae, including sea bass, undergo major developmental changes in their digestive functions during the first month of life until the acquisition of an adult mode of digestion (Cahu and Zambonino Infante, 1995; Zambonino Infante and Cahu, 2001). Recent studies have more particularly focused on the functional changes in the digestive tract during larval development by studying the onset and the variation of pancreatic and intestinal digestive enzymes and the response of these enzymes to diet concentration and composition. In this respect, early weaning by MD in marine fish larvae is remarkable and is accepted as a new physical and financial approach aimed at substituting live food production (Baskerville-Bridges and Kling, 2000; Hamlin and Kling, 2001; Alves *et al*., 2005; Curnow *et al*., 2006). Furthermore, substitution of live food by formulated diets and co-feeding of sea bass larvae is closely related with digestive enzyme activities. This situation indicates that although weaning can be achieved at metamorphosis and/or formation of functional stomach, earlier introduction of MD can be resulted poor growth performance and enzymatic activities (Cahu and Zambonino Infante, 1995, Cahu and Zambonino Infante, 2001).

There are some literatures on early weaning of sea bass by MD (Cahu and Zambonino Infante, 1994; Cahu *et al*., 1999; Suzer *et al*., 2007), however, no study measured enzymes (alkaline and acid proteases) during weaning time. The aim of this study is to determine the earliest point at which the larvae can be successfully weaned onto MD and also investigate the effects on some digestive enzyme activities (alkaline and acid proteases) in delayed initial feeding (without need rotifer) sea bass larvae and to measure some additional different digestive enzymes with respect to previous studies.

Materials and Methods

Larval Rearing

Larval rearing was carried out in 3 closed sea water systems that included 3 cylinder−conical shape 3 m^3 tanks per system. The colour of the tanks was dark-grey and larvae were stocked at a density of 100 individuals L^{-1}. Water temperature, dissolved oxygen, salinity, pH, ammonia and nitrite levels were monitored daily. Water temperature was maintained between 15 and 20°C (temperature increased day by day from 15 to 16°C between 0 and 7^{th} days, 16 to 19°C between day 8 and 21, and from 19 to 20°C between day 22 and 40). During the larval culture period, oxygen, salinity and pH were maintained at >85%, 38‰ and 7.8, respectively. Ammonia and nitrite were kept constant and below 0.01 mg L^{-1}. Water exchange rate increased gradually with the age of the larvae. Light was supplied by fluorescent tubes, with a power of 50-100 lux at water surface. Photoperiod was set on light cycle daily until the end of larval rearing period (16 h light: 8 h dark).

Feeding Trials

Newly hatched larvae fed from day 8 with *Artemia* nauplii grade (AF 430, INVE Aquaculture, Ghent, Belgium) at 1−2 individuals.ml^{-1}, and from day 12 with *Artemia* nauplii grade (AF 480, INVE Aquaculture, Ghent, Belgium) at 1−2 individuals.ml^{-1} from day 15 until day 40, *Artemia* metanauplii at 2−4 individuals ml^{-1} (EG, Artemia Systems SA), both enriched with Protein Selco (Artemia Systems SA, Ghent, Belgium). Nevertheless, as experimental design in 3 groups of larvae, MD (Gemma, Skretting, Stavanger, Norway) were introduced on day 15, 20 and 25, named as group MD15, MD20 and MD25. Also, control group was fed only *Artemia* metanauplii until end of the experiment (Figure 1).

Sampling and Enzymatic Assays

Growth rate was monitored by sampling groups of larvae from each tank a 5 days interval (30 larvae sample $group^{-1}$). Specific growth rate was calculated by formulae SGR = 100 (Ln FBW −Ln IBW)/Δt, with IBW, FBW: initial, final body weight of fish (mg), Δt: time interval (day). At the end of the experiment, larval survival was determined. Pooled samples of larvae (50-250 individuals, depending on age and

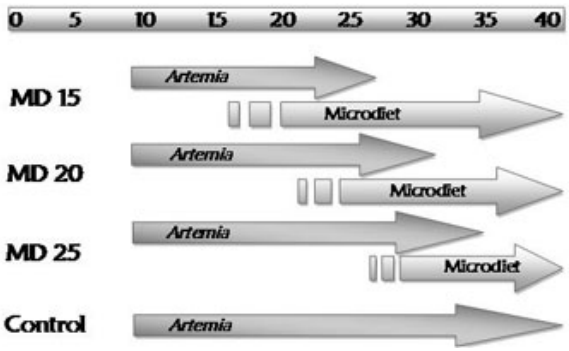

Figure 1. Early weaning protocol during the experiment.

size) were collected for enzyme analysis at 8, 11, 15, 17, 20, 22, 25, 27, 30, 35, and 40 DAH. Whole body homogenates were used for enzymatic assays and samples were taken at the same hour, before food distribution.

Samples were collected and homogenized in 5 volumes v/w of ice-cold distilled water. Extracts utilized for enzyme assays were obtained after homogenization of larvae (35 mg ml^{-1}) in cold 50 mM Tris−HCl buffer, pH 8.0, followed by centrifugation (13.500x*g*; 30 min at 4°C). Alkaline and acid protease activities in the supernatant were measured using the methods detailed by Alarcón *et al*. (1998) and Anson (1938), respectively. One unit of activity was defined as 1 µg of tyrosine released per min. Enzymatic activities were expressed as specific activity (mU/mg $protein^{-1}$). Protein was determined by the Bradford method (Bradford, 1976). All spectrophotometric analyses were performed by Jenway 6300 UV−visible Spectrophotometer.

Histological Analyses

For the histological study, 10 larvae were fixed in neutral formalin solution, dehydrated in different alcohol series, embedded in a parafin vax and cut in 3-5 µm-thick sagittal sections using a Leica RM 2125 rotary microtome. The Haematoxylin/Eosin (HE) stain was used for general histological observations to describe the development of the gastrointestinal system. Then, slides with sections were mounted permanently using Entellan. The sections of fish were randomly examined under an Olympus CX31 microscope. Photographs were taken with an Olympus DP20 digital photomicrographic attachment.

Statistical Analysis

All measurements were carried out in triplicate. Results are given as mean ±SD. The variance homogeneity of the data were analyzed using Levene test. Survival data were compared by Fischer's chi−square test and enzymatic activity data were compared by one−way ANOVA, followed by Newman−Keul's multiple range test, when significant differences were found at the 0.05 level. Statistical analyses were performed by SPSS 15.0 software.

Results and Discussion

The data presented in this paper complement previous information on larval digestive enzymes from the same experimental fish but different enzymes and not included histological analysis (Suzer *et al*., 2007). During the last decade more studies have been focused on substitution of live food by MD as early weaning in order to remove of disadvantageous of live food production. Although relatively limited successful studies has been reported related with using MD in early weaning (Cahu and Zambonino Infante, 1994; Cahu *et al*., 1999; Suzer *et al*., 2007), in the recent years, improvement of extruder feed technology and exploring the new food formulae increased larval survival rate and quality (Cahu and Zambonino Infante, 2001; Kolkovski, 2001). Since, Applebaum (1989) recommended for the successful rearing of marine larvae using inert diets, in which he suggested that raising the temperature of the water increases larval metabolism and activity level, potentially improving their readiness to accept inert diets. Growth of *D. labrax* larvae in groups during the 40 day period of study is described in Table 1. In all experimental groups except MD15, larvae multiplied their weight by a factor approximately 100−fold from day 0 to day 40. The best growth parameters were measured in the MD25 groups and also followed by MD20 and control group. Growth parameters and survival rates of groups were summarized in Table 1.

The weaning of sea bass larvae before 20 DAH did not induced larval growth and larval development in all groups was satisfactory except for group MD15. Similar results were reported in our previous study (Suzer *et al*., 2007) and by Cahu and Zambonino Infante (1994). Also Person Le Ruyet *et al*. (1993) indicated that no successful weaning prior to day 20 was recorded for sea bass larvae. Besides, as described by Cahu and Zambonino Infante (2001), formulation of compound diet adequate for fish larvae is not easy to achieve, because the estimation of nutritional requirements of fish larvae cannot be

Table 1. Growth parameters

	MD15	MD20	MD25	Control
Total length (mm)	18.45±1.9	20.86±2.1	22.12±3.6	20.76±4.3
Weight (mg)	34.21±4.1	45.56±3.7	48.78±5.3	44.34±3.6
SGR (%/day)	4.92	9.02	9.17	8.95
Survival rate (%)	3.6	14.7	43.6	34.1

conducted by traditional nutritional approaches; since, for the moment, commercially formulated diets do not support the growth of larvae.

In all groups alkaline protease activity was firstly measured at 8 DAH and also specific activity of this enzyme gradually increased to 15 DAH. After this date, it sharply decreased concurrently with supplementation of MD in other experimental groups except MD 25. From 25 DAH, alkaline protease activity slowly declined in all experimental groups until end of the experiment (Figure 2). Although no differences were found between experimental groups ($P>0.05$), control group was significantly different then these groups ($P<0.05$).

Substitution of live food by formulated diets and co-feeding of sea bass larvae is closely related with digestive enzyme activities. This situation indicates that although weaning can be achieved at metamorphosis and/or formation of functional stomach, earlier introduction of MD can result relatively lower growth performance and enzymatic activities as described for sea bass *Lates calcarifer*, (Walford and Lam, 1993), Japanese flounder *Paralichthyis olivaceus* (Kanazawa *et al*., 1989), Atlantic cod *Gadus morhua* (Baskerville-Bridges and Kling, 2000) and haddock *Melanogrammus aeglefinus* (Hamlin and Kling, 2001).

Some researchers pointed out that digestive enzyme activity can be used as an indicator of larval food acceptance and to some extent serve as an indicator for the digestive capacity in relation to the type of feed offered. It is well known that trypsin and chymotrypsin are specific of pancreatic protein hydrolysis (Nolting *et al*., 1999; Zambonino Infante and Cahu, 2001). Some of the main factors effecting that alkaline protease (trypsin and trypsin-like) activities in fish larvae are larval rearing techniques (clear and/or green water) age, feeding protocol and food characteristics (Zambonino Infante and Cahu, 2001; Cahu and Zambonino Infante, 2001). In this study, specific activity of alkaline protease was higher before MD introduction during the *Artemia* feeding stage. Also, a significant decrease was measured in this activity in all groups after starting MD ($p>0.05$). Similar results were reported in our previous study that trypsin and chymotrypsin activities demonstrated almost the same profile in the same species. Additionally, these results were paralleled with study by Nolting *et al*. (1999). And also these researchers pointed out that protein content of larvae fed *Artemia* nauplii was higher and the growth of larvae fed the MD was reduced. Besides, it is reported that the trypsin activity contribution of *Artemia* as a maximum 5% of the total assayed activity in 20-day-old sea bass larvae (Cahu and Zambonino Infante, 1995). On the other hand, histological observations supported these findings that before formation of functional stomach exocrine pancreas have a major role both for the larval digestion and also secretion of pancreatic enzymes such as trypsin, chymotrypsin and other alkaline proteases. Acid protease activity was firstly detected at 25 DAH in all groups. After this date, it fluctuated in experimental groups while this activity gradually increased in control group (Figure 3). In similar, there was no differences among experimental groups ($P>0.05$), however control group was found significantly different than the experimental groups ($P<0.05$).

It is well reported that acid protease (pepsin and pepsin-like) activity usually appears in relation with the formation of a functional stomach and also determination of starting time for weaning in the digestive physiology of fish larvae (Cahu and Zambonino Infante, 2001; Kolkovski, 2001; Suzer *et al*. 2007). Also, it is recorded that pepsin was firstly detected on day 24 and/or 25 in sea bass larvae (Zambonino Infante and Cahu, 1994; Suzer *et al*, 2007). On the other hand, this result was supported by histological observations whereas gastric glands were observed on day 24 in this experiment. Similarly, this activity was measured for the first time at 25 DAH and it is indicated that lower survival rates in MD15 and MD20 groups were related detection of acid protease and functional stomach because MD was introduced on day 15 and 20 to these groups respectively. Especially, the lowest survival rate was calculated in group MD15 ($P>0.05$) and it is thought that MD was not digested in digestive tract and introduced *Artemia* metanauplii was insufficient for larvae. However survival rate was lower than MD25 and control groups, this difference was not significant ($P<0.05$) and it could be that relatively lower survival rate resulted from starting of MD 5 days before formation of functional stomach. Additionally, in our previous study, similar findings were noted and also we detected pepsin activity on day 25 in sea bass larvae concurrently with starting of weaning (Suzer *et al*., 2007). Besides, functional stomach and pepsin was reported on day 17 DAH for sea bass *Lates calcarifer* (Walford and Lam, 1993) 40 DAH for gilthead sea bream *S. aurata* (Moyano *et al*., 1996), 25 DAH for common pandora *Pagellus erythrinus.*

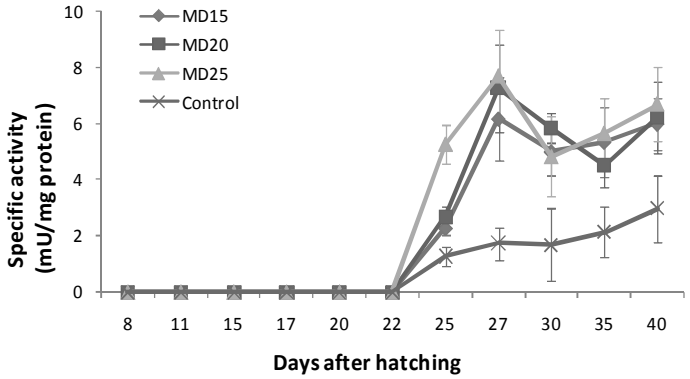

Figure 3. The specific activities of acid protease assayed whole larvae homogenates in groups MD15 (◆), MD20 (■), MD25 (▲) and Control (✖). Results are expressed as means ±SD (n=5).

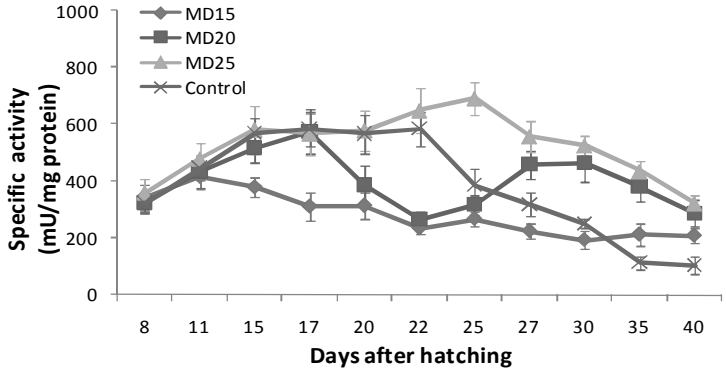

Figure 2. The specific activities of alkaline protease assayed whole larvae homogenates in groups MD15 (◆), MD20 (■), MD25 (▲) and Control (✖). Results are expressed as means ±SD (n=5).

(Suzer *et al*., 2006), 30 DAH for red porgy *Pagrus pagrus* (Darias *et al*., 2005).

When larvae began to feeding with *Artemia* nauplii, stomach developing started with presence of gastric glands. Gastric glands were first appeared on 24 DAH and these glands were determined as strongly and mucusal acinar cell accumulates at 30 DAH. Then, increasing number of gastric glands observed with larval development and fully developed glandular stomach could be defined at 35 DAH. The gastric region occurred of a anterior and posterior part with mucosal folds, respectively, that were separated by the stricture that appeared during the first feeding stages. During this phase (20-40 DAH), the mucosa folds increased in length and number in the anterior gastric region, while the caudal gastric region grew gradually (Figures 4 and 5). In the intestine, the underlying connective tissue entered the thickened points of the epithelium seen in the first feeding to form factual mucosa folds, which grew in size and enlarged during this period. In some larvae, large lipid droplets were found in the epithelial cells, especially in the posterior part of the intestine (Figures 4 and 5).

In conclusion, the present study demonstrated that whatever developmental stage, larvae were able to modulate their digestive enzyme activities in response to shifting diet (Cahu and Zambonino Infante, 1994). Also, growth and survival rate were found relatively lower than the other groups due to not formation of stomach and gastric glands in group MD15. This finding indicated that gastric activity and pepsin secretion should occur for MD digestion. Also, for the successful rearing of marine larvae using MD, in which Applebaum (1989) suggested that raising the temperature of the water increases larval metabolism and activity level, potentially improving their readiness to accept inert diets. It is clearly investigated that supplementation of MD and digestion of all its ingredients could be resulted enhanced growth and survival rate after formation of stomach and activity of gastric glands then 25 DAH for sea bass larvae. Also, further studies should be focused on early weaning and digestive enzyme expression with introduction of MD in other cultured species.

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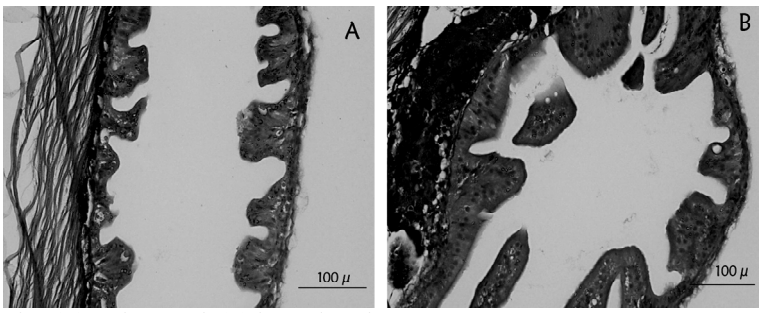

Figure 4. Incipient intestine (A) and stomach (B) in sea bass larvae at 20 DAH.

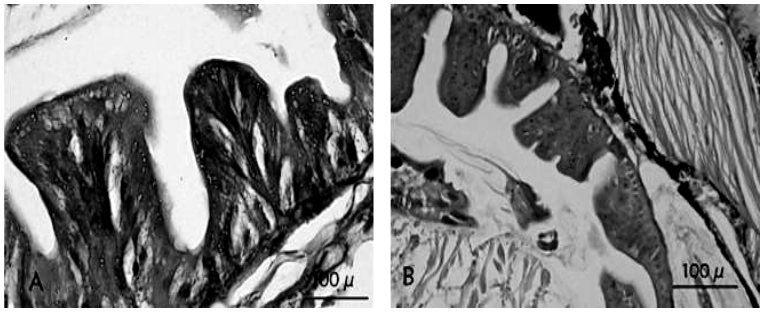

Figure 5. Fully developed intestine (A) and stomach (B) in sea bass larvae at 30 DAH.

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