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Effect of Cellulolytic gut bacteria as a feed supplement on the growth performance and nutrient digestibility of Asian Seabass (*Lates calcarifer*)

Joydev Maity[1]*, Joydeep Kundu[1], Atanu Pramanik[1] and Bidhan C. Patra[2]

1) Fisheries Research Laboratory, Department of Aquaculture Management & Technology, Vidyasagar University, Midnapore – 721102, West Bengal, India

2) Aquaculture Research Unit, Department of Zoology, Vidyasagar University, Midnapore – 721102, West Bengal, India

Abstract

To study the effect of cellulolytic gut bacteria as a feed supplement on growth performance and nutrient digestibility of Asian seabass (*Lates calcarifer*), a 42 days experiment was done. Group I was fed on control diet (diet A), Group II, III and IV were fed with diet B (*Bacillus subtilis*), C (*Bacillus licheniformis*), and D (*B. subtilis* and *B. licheniformis* in 1:1 ratio) respectively. Higher ($P<0.05$) weight gain percent (248.75 %), specific growth rate (4.80) and apparent fiber digestibility were observed in group IV. Maximum ($P<0.05$) survivability (86.67 %), PER and apparent protein digestibility were significantly ($P<0.05$) higher and FCR (1.24) was significantly ($P<0.05$) lower in animals of group III. Total microbial population and cellulolytic bacterial population in the gut of juvenile seabass of group IV was significantly ($P<0.05$) higher than other groups. Amylolytic bacterial population was in the same range of group III and IV but they were significantly ($P<0.05$) higher than other two groups. Digestive cellulase enzyme activity was significantly ($P<0.05$) higher in group IV, digestive amylase and protease was in the same range of group III and IV. So, these two cellulolytic gut bacteria *B. licheniformis* and *B. subtilis* showed beneficial effect on seabass as feed supplement.

Key words: Asian seabass, cellulolytic gut bacteria, nutrient digestibility, digestive enzyme, feed supplement.

(✉) jmaity2003@yahoo.co.in

Introduction

Asian seabass (*Lates calcarifer,* Bloch) is a highly carnivorous and eurihaline fish. Due to fast growth rate, good taste, flash texture, high demand and high market value, it is considered as candidate species for culture in marine, brackish as well as fresh water environment. In the wild they are diadromous in nature, returning to estuarine or marine water to breed (Greenwood 1976). For successful culture of seabass well formulated feed is an essential prerequisite. Considerable effort has been made in Australia, Thailand, Philippines and more recently in Israel to define the nutritional requirements of this species in order to improve production (Anonymous 1985; Cheong 1989; Boonyaratpalin and Williams 2001). But information on practical diets for seabass is very scanty. It is known that zooplankton is the most suitable food for early stages of larval rearing and micro-crustaceans are the most preferred natural food for fry (Mohanty 2006). It was also reported that relatively high protein is required for seabass growth as they are piscivorous in nature (Davis 1987; Williams *et al.* 2003). A series of studies by Sakaras *et al.* (1988 and 1989) and Boonyaratpalin (1997), reported that protein requirements for juvenile seabass is at around of 45% to 50% level. However, in formulation of seabass diet, it was found that fish meal is the only source of protein. But increasing demand, high cost and uncertain availability of good quality fish meal (Barlow 1989) have resulted in nutritionists searching alternative sources, particularly plant proteins to replace fish meal in the diet of a number of freshwater and marine fish species (Dabrowski *et al.* 1989; Olli *et al.* 1989; Wee and Shu 1989; Lovell 1991; Quartararo *et al.* 1991; Pongmaneerat and Watanabe 1993; Robinson and Li 1994). Use of plant materials as a non-conventional protein source replacing high priced fish meal in formulated fish feed has opened a new area of research to produce cost effective and eco-friendly aquafeed. But being monogastric, fish can't efficiently utilize fibre rich ingredients. Again carnivorous fish like, Seabass (*Lates calcarifer*) can't utilize starch and hence biotechnological approach in terms of supplementation of cellulolytic and amylolytic microbes with feed has to be given priority. Microbial fermentation or supplementation of potential cellulolytic microbes in plant protein based diet for seabass is needed. The bacteria ingested by the fish along with their diet adapt themselves to the environment of the gastrointestinal tract and form a symbiotic association and this kind of bacterial adaptation will help the fish in digestion of fibrous materials in their gut (Chou 1984; Saha *et al.* 2006; Lee *et al.* 1996).

In the present study, we have formulated

plant protein based seabass feed (CP- 36%) supplemented with potential cellulolytic bacteria and fed the seabass fingerlings for 42 days. After this feeding trial, we have analyzed the effect of these cellulolytic bacteria on growth performance, nutrient digestibility and improvement of gut health of those seabass fingerlings.

Materials and methods

The present study on the effect of cellulolytic gut bacteria as a feed supplement on growth performance and apparent nutrient digestibility of Asian seabass, *Lates calcarifer,* was conducted in the Research Laboratory of Latika Sea Food Pvt. Ltd., Contai, Pubra Medinipur, West Bengal, India, an extension Research Station of the Fisheries Research Laboratory, Department of Aquaculture Management and Technology, Vidyasagar University, Midnapore – 721102, West Bengal, India

Weaning of seabass fingerlings

As seabass is a highly carnivorous species and micro-crustaceans are the most preferred natural food for fry (Southgate and Lee 1993; Mohanty 2006; Biswas *et al*. 2010), it is necessary to wean the seabass fingerlings with artificial diet before going to start this experiment. The fingerlings of seabass (average body weight of 4.0 ±0.15 g) were collected from brackishwater ponds of South 24-Parganas, West Bengal, India and acclimatized to the laboratory condition. During fingerling collection the salinity of pond water was 7-10 mg L^{-1} and these fingerlings were then gradually acclimatized to fresh water (0-1 mg L^{-1}). Initially a 20 days weaning of seabass were carried out with the semi moist feed composed of mince meat and wheat flower (8:2). After that control feed was gradually introduced and habituated to accept the feed. When the result of acceptance of feed was satisfactory, the trial started.

Diet preparation

To study the effect of cellulolytic gut bacteria as a feed supplement on growth performance and apparent nutrient digestibility of seabass, one control (A) and three experimental (B, C and D) isonitrogenous diets were prepared (Table 1.). All of these diets were prepared semi moist condition according to seabass feeding habits mentioned earlier. Three experimental diets (B, C and D) were supplemented with 48 hrs culture of two cellulolytic bacterium [*Bacillus subtilis* (B2) and *Bacillus licheniformis* (B6)] isolated and identified from the gut of adult seabass. Dietary ingredients were finely powdered and sieved (pore diameter < 400 µm). Dry ingredients were mixed properly with mixer machine for 30 minutes. After that fish oil was

gradually added and mixing it constantly. Subsequently water was added at rate of 85 ml 100 g^{-1} of feed and was slowly blended into the mixture for making suitable texture, dough for fish food (Lovell 1989). Here wheat flour was act as a binder. A vitamin-mineral mixture (VITAMINETES FORTE, Roche India Ltd., Mumbai, India) and Phosphorylated Vit C were added to the diets after sterilization of the dough. Chromic oxide (0.5 % w/w) was added to each formulated diet as an external digestibility marker. Prepared semi moist feeds were cut in small size and kept into -20^0 C refrigerator for farther use.

2.3. Experimental set up

The feeding trial was conducted under laboratory conditions, in 12 glass aquaria, each containing 90 L of water, for 42 days, with continuous aeration. The fingerlings (mean individual weight of the 120 fingerlings 4.5 ± 0.15 g) were randomly distributed in the glass aquaria at a stocking density of 10 fish per aquarium for four groups with three replicates. Group I, II, III and IV were fed with diet A, B, C, and D respectively. The fishes were fed twice daily: at 07:00 and 16:00 hours, at a feeding rate of 4% (w/w) of the total body weight per day. The daily ration was adjusted every tenth day after weighing the fish from each replicate. The uneaten feed was siphoned off 6 hours after each feeding, and oven dried at 100°C for 24 h to calculate the feed conversion ratio. The faecal samples released by the fish were collected daily from each aquarium by pipetting. The oven dried (60°C) faecal samples were analysed for digestibility estimation. The ranges of water quality parameters were: pH 6.8–7.6; temperature 22–33°C and dissolved oxygen 6.5–7.2 mg L^{-1}. Five fish from each aquarium were sampled at the termination of the feeding experiment; they were taken for study of gut bacterial population and intestinal enzyme study.

Sample collection, chemical analysis and data collection

The proximate principles of feed and faecal sample was determined following AOAC (1990) method as follows: moisture content, by oven-drying for 24 h at100°C; crude protein (N x 6.25), by the micro-Kjeldahl digestion and distillation after acid digestion; lipids, by extracting the residue with petroleum ether (40–60°C) for 6 h in a Soxhlet apparatus; crude fibre, as loss on ignition of dried lipid-free residues after digestion with 1.25% H_2SO_4 and 1.25% NaOH (Merck Specialities Pvt. Ltd., Mumbai, India); ash, by ignition of samples at 600°C in a muffle furnace to constant weight. Nitrogen-free extract (NFE) was computed by taking the sum of values for crude protein, crude lipid, ash, crude fibre, and moisture and subtracting this from 100. Chromic oxide

(Merck Specialities Pvt. Ltd., Mumbai, India) levels in the diets and in the faecal samples were estimated by wet digestion method. The water quality parameters were monitored as recommended by the APHA (1989). Average live weight gain (%), specific growth rate (SGR; % day^{-1}), feed conversion ratio (FCR), and protein efficiency ratio (PER) were calculated using following formula.

$$\text{Feed Conversion Ratio (FCR)} = \left[\frac{\text{Dry weight of feed given (g)}}{\text{Increase in wet weight of fish (g)}}\right]$$

$$\text{Protein Efficiency Ratio (PER)} = \left[\frac{\text{Fish weight gain (g)}}{\text{Protein fed (g dry weight basis)}}\right]$$

The apparent digestibility coefficients (ADC) of nutrients were calculated using the following formula:

$$\text{Digestibility coefficient (\%)} = 100 - \frac{\%\ Cr_2O_3 \text{ in diet}}{\%\ Cr_2O_3 \text{ in faeces}} \times \frac{\%\text{ nutrient in faeces}}{\%\text{ nutrient in diet}} \times 100$$

Digestive enzyme assay

The fish from each experimental set were dissected on an ice tray to remove the intestine in order to determine the digestive enzyme activities at the end of the feeding trials. After evisceration, the whole intestine was homogenized with five times (w/v) of ice cold sterile chilled physiological saline (0.9% NaCl in PBS buffer, pH 7.2). Homogenate was centrifuged at 10,000 rpm for 1h at 4ºC and the supernatant was collected and used for enzyme assay. Cellulase activity was assayed (Denison and Koehn 1977) using 1% CMC in citrate buffer (0.1 M, pH 6.75) as substrate. Amylase activity was measured (Bernfield 1955) using 1% soluble starch in phosphate buffer (0.02 M; pH 6.9 containing 0.0067 M NaCl) as substrate. Protease activity was detected by caseinase assay method (Walter 1984). For enzyme activity estimation optical density (OD) was taken in UV-Spectrophotometer (UV-1800, SHIMADJU, Japan).

Microbial culture

The fish from each experimental set were dissected on an ice tray to remove the intestine in order to determine the intestinal microbial population at the end of the feeding trials. The whole intestine was homogenized with five times (w/v) of ice cold sterile chilled physiological saline (0.9% NaCl in PBS buffer, pH 7.2). The homogenate of the intestine of each test fishes was used after 10 serial (1:10) dilutions (Beveridge *et al.* 1991). Samples (0.1 ml) were taken from each dilution and poured

aseptically under laminar flow on sterilized tryptone soya agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India) plates, in duplicate. These culture plates were incubated at 34 °C for 48 h. They were then examined for total bacterial count. To isolate and enumerate cellulase and amylase, producing bacteria, samples (0.1 ml) from diluted gut homogenate was poured on carboxymethylcellulose agar (CMC agar) and starch agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India) media containing plates respectively, in duplicate. These plates were incubated at 34°C for 48 h and then examined for the development of bacterial colonies. By multiplying the number of colonies formed on each plate by the reciprocal of dilution, colony numbers per unit sample volume of gut homogenate were determined (Rahmatullah and Beveridge 1993).

Statistical analysis

The experimental data were subjected to analysis two way ANOVA to test the significance among the different types of feed with different said parameters (SPSS Version 11.5, SPSS Inc., Chicago, IL, USA). Where two ways ANOVA should a significant interaction between the two factors used to identify significantly different means using Duncan multiple range test comparison. Differences were considered significant at $p = 0.05$

Results

After 42 days of feeding trial on juvenile seabass fed with four different diets (Table 1), it was found that growth performance (Table 2), apparent nutrient digestibility, digestive enzyme activity and microbial population in gut was significantly ($P<0.05$) better in all groups except group I fed control diet. Weight gain (248.75 %) and specific growth rate (4.80) were significantly ($P<0.05$) higher in animals of group IV, supplemented with both *B. subtilis* and *B. licheniformis* in 1:1 ratio, than other groups. Survivability (86.67 %) and protein efficiency ratio (PER) were significantly ($P<0.05$) higher and FCR (1.24) was significantly ($P<0.05$) lower in animals of group III supplemented with *B. licheniformis* (Table 2).

Apparent protein digestibility was significantly ($P<0.05$) higher in group III followed by group IV, group II and group I but apparent fibre digestibility was significantly ($P<0.05$) higher in group IV as compared to that of other groups. Apparent fat digestibility was not significantly different among all groups (Table 3).

Total microbial population and cellulolytic bacterial population in the gut of juvenile seabass of group IV was significantly ($P<0.05$) higher than other groups. Amylolytic bacterial population was in the same range of group III and IV but they were significantly ($P<0.05$)

higher than other two groups (Fig. 1).

Digestive cellulase activity was significantly (P<0.05) higher in group IV as compared to that of other groups. Digestive amylase and protease was in the same range of group III and IV but they were significantly (P<0.05) higher than other two groups (Fig. 2).

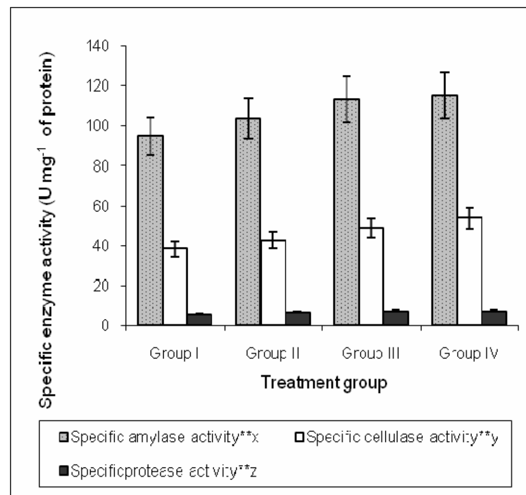

Fig.1 Aerobic bacterial count (CFU g^{-1} of intestinal tissue) in *Lates calcarifer* fingerlings fed different experimental diets for 42 days

Group I fed control diet, group II fed diet B, group III fed diet C, and group IV fed diet D

Data represent the mean ± SE. Bars denoted are significantly different (P<0.05; n=3)

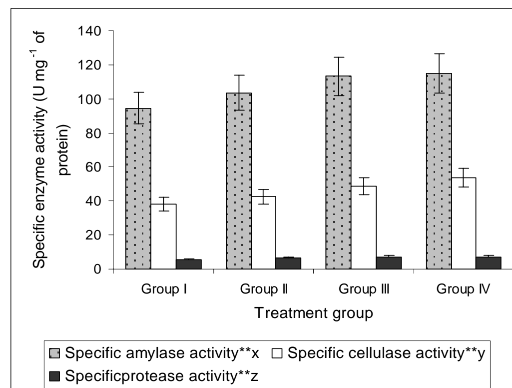

Fig.2 Amylase, cellulase and protease activity in whole intestine of *Lates calcarifer* fingerlings fed different experimental diets for 42 days

Group I fed control diet, group II fed diet B, group III fed diet C, and group IV fed diet D

x **=** µg of maltose liberated mg^{-1} of protein min.$^{-1}$, y **=** µg of D-glucose liberated / mg of protein / min, z **=** µg of L-tyrosine liberated mg^{-1} of protein min.$^{-1}$ Data represent the mean ± SE. Bars denoted are significantly different (P<0.05; n=3)

Table1 Ingredient composition (g 100 g^{-1} of dry weight) and proximate composition of the experimental diets for *Lates calcarifer* fingerlings

Ingredients	Diet			
	A[a]	B	C	D
Fish meal	20.00	20.00	20.00	20.00
Poultry offal	20.00	20.00	20.00	20.00
MOC	14.00	14.00	14.00	14.00
Soybean meal	12.00	12.00	12.00	12.00
Groundnut cake	10.00	10.00	10.00	10.00
Wheat flour	17.00	17.00	17.00	17.00
Fish oil	2.50	2.50	2.50	2.50
Lecithin	3.00	3.00	3.00	3.00
Mineral and Vitamin premix[b]	0.90	0.90	0.90	0.90
Phosphorylated Vit C	0.10	0.10	0.10	0.10
Cr_2O_3	0.50	0.50	0.50	0.50
Culture of *Bacillus subtilis* (B2) (v/w)[c]	-	1.00	-	-
Culture of *Bacillus licheniformis* (B6) (v/w)[c]	-	-	1.00	-
Mixture of B2 and B6 in 1:1 ratio(v/w)[c]	-	-	-	1.00
Proximate composition (g 100 g^{-1} of dry matter basis)[d]				
Crude protein	36.64	36.98	36.24	36.73
Crude lipid	15.83	15.76	15.81	15.97
Crude fibre	3.66	3.61	3.57	3.59
Ash	12.71	12.76	12.68	12.65
Moisture	8.46	9.21	8.79	8.85
Nitrogen-free extract	22.70	21.68	22.91	22.21

[a]Control diet; [b]mineral and Vitamin premix (Vitaminetes Forte, Roche India Ltd., India); each 800 mg tablet contains: vitamin A IP (as acetate) 2500 IU; thiamine mononitrate IP (vitamin B1) 2 mg; riboflavine IP (vitamin B2) 3 mg; nicotinamide IP 25 mg; pyridoxine hydrochloride IP (vitamin B6) 1.5 mg; calcium pantothenate IP 5 mg; cyanocobalamin IP (vitamin B12) 1 mcg; ascorbic acid IP (vitamin C) 50 mg; cholecalciferol IP (vitamin D3) 200 IU; ·-tocopherol acetate IP (vitamin E acetate) 10 mg; biotin USP (vitamin H) 0.05 vmg; calcium phosphate IP 208 mg; dried ferrous sulphate IP 10.62 mg; magnesium phosphate, dibasic 48 mg; manganese hypophosphate 0.6 mg; total phosphorus 44.60 mg; [c]all cultures were mixed at 1% v/w basis; bacterial concentration in feed was, B2 - 3.14×10^8 CFU 100 g^{-1} of feed and B6 - 2.98×10^8 CFU 100 g^{-1} of feed; [d] number of samples for each determination = 3

Table 2 Growth performances of *Lates calcarifer* fingerlings fed different experimental diets for 42 days

Parameter	Group I (control diet, A)	Group II (diet B)	Group III (diet C)	Group IV (diet D)	SEM
Initial body wt. (g)	4.40±0.06	4.34±0.01	4.42±0.01	4.32±0.01	0.03
Final body wt. (g)**	11.43±0.10^{a}	13.16±0.11^{b}	14.82±0.21^{c}	16.07±0.07^{d}	0.13
Total wt. gain (g)**	7.02±0.01^{a}	8.82±0.11^{b}	10.40±0.21^{c}	11.75±0.09^{d}	0.14
ADG (mg d^{-1})**	167.22±2.42^{a}	210.00±2.68^{b}	247.62±4.92^{c}	279.84±2.16^{d}	5.23
Weight gain** percent	159.57±3.58^{a}	203.39±2.86^{b}	235.30±4.72^{c}	271.88±2.96^{d}	3.53
DM intake (g d^{-1})**	0.43±0.01^{a}	0.44±0.03^{a}	0.45±0.01^{a}	0.60±0.02^{b}	0.02
FCR **	2.55±0.07^{c}	2.09±0.13^{b}	1.80±0.03^{a}	2.14±0.06^{b}	0.05
SGR (%)	2.27±0.03^{a}	2.64±0.02^{b}	2.88±0.03^{c}	3.13±0.02^{d}	0.04
PER**	1.07±0.03^{a}	1.29±0.08^{b}	1.54±0.02^{c}	1.27±0.03^{b}	0.02
Survivability (%)	73.33±3.33^{a}	80.00±5.77^{b}	86.67±3.33^{d}	83.33±6.66^{c}	5.00

**$P<0.05$; a, b, c, d: values bearing different superscript in a row differ significantly.

Table 3 Apparent nutrient digestibility of *Lates calcarifer* fingerlings fed different experimental diets for 42 days

Digestibility (%)	Group I (control diet)	Group II (diet B)	Group III (diet C)	Group IV (diet D)
DM**	82.25±0.17^{a}	87.63±0.26^{b}	92.34±0.36^{c}	92.81±0.14^{c}
OM**	85.63±0.24^{a}	89.90±0.45^{b}	94.65±0.26^{c}	95.08±0.11^{c}
CP**	84.21±0.18^{a}	89.93±0.39^{b}	95.61±0.26^{d}	92.61±0.21^{c}
CF**	79.05±0.46^{a}	82.64±0.14^{b}	85.48±0.17^{c}	91.02±0.67^{d}
EE**	83.70±0.21	84.00±0.18	84.32±0.43	83.60±0.27

**$P<0.05$; a, b, c, d: values bearing different superscript in a row differ significantly.

Discussion

Due to increasing scarcity of good quality fish meal, aquaculture has to depend on feed of plant origin which are rich in cellulose and starch and not easily digested by fish. Cellulose, a polymer of glucose, being the primary structural material of plant cell wall, is the most abundant carbohydrate in nature (Spano *et al.* 1975). Very few animals are able to utilize these resources efficiently (Goodenough and Goodenough 1993; Lovell 1989). Utilization of cellulose as a nutrient source requires the enzyme cellulase that cleaves β-1, 4-glycosidic bonds in the polymer to release β-D-glucose units (Barr *et al.* 1996). Biodegradation of cellulose and hemicellulose by the cellulolytic activities of endosymbionts in the digestive tract of termites, sea turtles, shiponds, ruminants and reptiles has been well studied (Hungate 1975), but a few reports are available regarding microbial cellulase production in the gastrointestinal tract of fish (Saha and Ray 1998). Use of plant materials as a non-conventional protein source replacing high priced fish meal in formulated fish feed has opened a new area of research to produce cost effective aquafeed. But plant materials contain high amount of cellulosic substances and fishes cannot utilize them because they unable to produce cellulase endogenously. The crude fibre, cellulose and hemicellulose contents and the antinutritional factors, tannin, phytic acid and mimosine in the *Leucaena* leaf meal decreased due to inoculation (for 15 days at 37°C) of fish intestinal bacteria, *Bacillus subtilis* (isolated from *Cyprinus carpio*) and *B. circulans* (isolated from *Oreochromis mossambicus*) having extra cellular cellulolytic and amylolytic activities (Bairagi *et al.* 2004; Harpaz, *et al.* 2005).

Carnivorous species e.g. Seabass cannot efficiently utilize cellulose due to lack of enzyme cellulase. As cellulolytic microbes are capable of breaking complex ligno-cellulosic bond present in cell wall of plant based feed ingredients and amylolytic microbes are capable of degrading starch to a simple sugar, their supplementation with the feed to the fishes will help to utilize the nutrient more efficiently. In the present study two potential cellulase producing bacteria, *B. licheniformis* and *B. subtilis*, isolated from the gut of adult seabass were supplemented with plant protein based diet and fed to the seabass fingerlings for 42 days. *B. licheniformis* supplementation in the diet of group III animals showed better survivability, protein efficiency ratio, FCR and protein digestibility where as joint supplementation of *B. licheniformis* and *B. subtilis* (1:1 ratio) in the diet of group IV showed better weight gain, specific growth rate, fibre digestibility, digestive cellulase activity and cellulolytic bacterial population in their gut.

The outcomes of the present study indicated that these two cellulolytic gut bacteria *B. licheniformis* and *B. subtilis* showed beneficial effects on seabass fingerlings as feed supplement for better growth, nutrient digestibility, digestive enzyme activity and gut microbial population. So, low cost fibrous feed ingredients can be included at a desirable inclusion level in seabass feed formulations, supplemented with cellulolytic bacteria and the feed cost, the major component of aquaculture, can be minimized. This intern will help the farmers to make the seabass culture more profitable.

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