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Evaluation of the prebiotic GroBiotic®-A and brewers yeast as dietary supplements for sub-adult hybrid striped bass (*Morone chrysops*×*M. saxatilis*) challenged in situ with *Mycobacterium marinum*

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Abstract

Dietary supplements such as immunostimulants and prebiotics hold promise as a potential replacement of antibiotics in maintaining fish health. A 21-week feeding trial was conducted to evaluate the commercial prebiotic GroBiotic®-A, a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products, in the diet of hybrid striped bass exposed to chronic mycobacterial infection caused by *Mycobacterium marinum*, as compared to partially autolyzed brewers yeast (Brewtech®). The basal diet was formulated to contain 40% protein, 10% lipid and an estimated digestible energy level of 3.5 kcal/g. Supplements of 1 or 2% brewers yeast and 2% GroBiotic®-A were singularly added to the basal diet and each diet was manufactured by extrusion processing with a twin-screw extruder. Each diet was fed to three replicate groups of small (initially averaging 64.5 g/fish) and one group of large (initially averaging 118 g/fish) hybrid striped bass in 1187-l circular tanks operated as a recirculating system. Fish were fed twice daily to apparent satiation and growth performance monitored for 16 weeks. An in situ infection of *M. marinum* became well established at week 16 such that fish were fed once daily and mortality was monitored for a total of 21 weeks.

Enhanced growth performance was generally observed in fish fed diets supplemented with GroBiotic®-A or brewers yeast compared to fish fed the basal diet throughout the feeding trial with significantly ($P<0.05$) enhanced weight gain observed after 12 weeks of feeding. At the end of the feeding trial, fish fed 2% brewers yeast had significantly higher feed efficiency than fish fed the other diets. The in situ mycobacterial challenge employed in this experiment resulted in overall cumulative mortality of approximately 25%. Fish fed 2% GroBiotic®-A had a significantly ($P<0.05$) enhanced survival (80%) compared to the other treatments (72–73%) at the end of 21 weeks. It is concluded that dietary supplementation of 2% GroBiotic®-A showed moderate but significant ($P<0.05$) protection against mycobacterial infection. Dietary

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supplementation of partially autolyzed brewers yeast also may enhance growth performance under chronic infection of mycobacteria.

Keywords: Prebiotics; Hybrid striped bass; GroBiotic®-A; Brewers yeast; *Saccharomyces cerevisiae*; Immune response; Bacterial resistance; Mycobacteria

1. Introduction

Rapid growth and disease resistance of aquacultured organisms are two of the most important concerns. Traditionally, antibiotics have been supplemented in aquafeeds for prevention and/or treatment of bacterial disease of aquatic animals. It has been reported that antibiotics may enhance growth and feed efficiency by killing intestinal microflora and thus increasing amino acid utilization by the host in some animal species (Rawles et al., 1997). However, the use of antibiotics may pose threats such as development of bacterial strains that are more resistant to antibiotic treatment, or the occurrence of antibiotic residues in cultured organisms for human consumers (FAO, 2002). Increasing concerns of antibiotic use have resulted in a ban on subtherapeutic antibiotic usage in Europe and the potential for a ban in the United States and other countries (Patterson and Burkholder, 2003). These alterations in policy may impact aquaculture and therefore prompt interest in developing alternative strategies for disease control. Beside vaccine development, dietary supplements including probiotics, prebiotics and immunostimulants have received heightened attention. A rapidly expanding body of literature has been established that many intestinal microbial species may have beneficial influences on the performance of fish (reviewed by Irianto and Austin, 2002), and dietary composition is capable of influencing the intestinal microflora of fishes (Ringø et al., 1998; Ringø and Olsen, 1999). However, development of prebiotics, classified as "nondigestible food ingredients that benefically affect the host by stimulating growth and/or activity of a limited number of bacteria in the intestine", is in its infancy with fishes, compared to the progress that has been made in development of prebiotics for poultry (Patterson and Burkholder, 2003). In a previous evaluation of the commercial prebiotic, GroBiotic®-A, a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products, significantly enhanced feed efficiency of juvenile hybrid striped bass was observed (Li and Gatlin, 2004), although the dynamics of the intestinal microflora was not defined in that study. Supplementation of this prebiotic also enhanced respiratory burst of head kidney leucocytes and resistance against *Streptococcus iniae* infection; however, the interpretation of these beneficial influences was complicated by the presence of brewers yeast, which is generally considered to be an immunostimulant for fishes (Siwicki et al., 1994; Ortuño et al., 2002; Li and Gatlin, 2003; Rodríguez et al., 2003).

The hybrid striped bass is an important fish for U.S. aquaculture, but it is affected by several pathogenic bacteria such as *S. iniae*, *Aeromonas hydrophila* and *Mycobacterium marinum* (reviewed by Plumb, 1997). With limited availability of approved therapeutic compounds and the inconvenient and costly administration of vaccines, this fish has been used in our laboratory as a model for investigating the interaction between nutrition and disease resistance. Besides *S. iniae,* the prevalence of mycobacteria in wild striped bass and cultured hybrid striped bass has attracted increased attention (Gauthier et al., 2003; Overton et al., 2003). Although mycobacteriosis is generally chronic, this disease may cause severe infection and high cumulative mortality in closed, recirculating systems (Plumb, 1997). In addition, this bacterium is reported to be capable of surviving under many adverse environmental conditions, including low temperature, and may cause infection on the extremities of humans (Plumb, 1997; Mediel et al., 2003). A recent report of the presence of mycobacteria in frozen seafood also raised concern about the safety of such seafood for human consumers (Mediel et al., 2003). Currently, effective treatment of this disease is very limited as reported for other fish species (Colorni et al., 1998). Therefore, the present study was conducted to explore growth performance

and non-specific immune responses of sub-adult hybrid striped bass fed the dietary prebiotic, GroBiotic®-A and brewers yeast under conditions of chronic exposure to *M. marinum*.

2. Materials and methods

2.1. Experimental diets

The basal diet was formulated from practical feedstuffs obtained at a commercial feed mill (Rangen Inc. Angleton, TX) to contain 40% protein, 10% lipid and an estimated digestible energy level of 3.5 kcal/g (Table 1). This diet satisfied and/or exceeded all known nutrient requirements of hybrid striped bass (Gatlin, 1997; Webster, 2002) or other warmwater fishes (National Research Council, 1993). Partially autolyzed brewers yeast (Brewtech®) and GroBiotic®-A, a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products, were supplied by International Ingredient Corporation (St. Louis, MO, USA). Two incremental levels (1% and 2% of diet) of brewers yeast and 2% of GroBiotic®-A were added to the basal diet (Table 1). In addition, 5% menhaden fish oil (Omega Protein Corp., Reedville, VA) was added prior to extrusion processing as described by Rawles and Gatlin (2000) to produce neutrally buoyant 5-mm pellets. Diets were stored in sealed bags in a temperature-controlled room at 22 ± 2 °C until fed.

Table 1
Composition of experimental diets

Constituent	Basal diet	1% Brewers yeast	2% Brewers yeast	2% GroBiotic®-A
Ingredient (% dry weight)				
Menhaden fish meal[a]	34.7	34.4	34	34
Soybean meal[a]	32.6	32.3	31.9	31.9
Wheat flour[a]	31.3	31	30.7	30.7
Menhaden fish oil[b]	5	5	5	5
Salt[a]	1.1	1.1	1.1	1.1
Mineral/vitamin premix[a]	0.3	0.3	0.3	0.3
Brewers yeast[c]	0	1	2	0
GroBiotic®-A[d]	0	0	0	2
Analyzed proximate composition (% dry matter)[e]				
Moisture	4.3	3.5	6.0	4.4
Crude protein (N×6.25)	41.2	38.6	39.1	40.8
Crude lipid	9.0	9.1	9.0	9.1
Ash	9.5	9.7	9.5	9.6

[a] Rangen Inc. Angleton, TX.
[b] Omega Protein Corporation, Reedville, VA.
[c] International Ingredient Corporation, St. Louis, MO. Contained 51.0% crude protein and 1.1% crude lipid (dry-weight basis).
[d] International Ingredient Corporation, St. Louis, MO. Contained 35.2% crude protein and 1.7% crude lipid and ~53% simple and complex carbohydrates including oligosaccharides (dry-weight basis).
[e] Means of two analyses.

2.2. Feeding trial

Juvenile hybrid striped bass (*Morone chrysops*×*M. saxatilis*) were obtained from a commercial supplier (Keo Fish Farm, Keo, AR) and maintained in an earthen pond at the Texas A&M University Aquacultural Research and Teaching Facility prior to the feeding trial. Three fish were randomly obtained from this population and analyzed by the Texas Veterinary Medical Diagnostic Laboratory (TVMDL) using routine culture and histopathology to confirm they were pathogen-free. The culture system consisted of sixteen 1134-l round fiberglass tanks connected as a closed recirculating system to a settling chamber and a biological filter. This system had held hybrid striped bass with a severe mycobacteria infection shortly before the present feeding trial. Fish were seined from the pond, transported to the culture system and fed the basal diet to apparent satiation twice per day for a 2-week conditioning period. Fish were then graded by size and 12 groups of 50 fish averaging 64.5 g each with a total weight of 3225 ± 142 g (mean ± S.D.) per group were stocked into individual tanks and four groups of 50 fish averaging 118 g with a total weight of 5900 ± 81 g (mean ± S.D.) per group were stocked in the remaining tanks, according to a randomized complete block design. Water flow rate was maintained at approximately 2 l/min via a recirculating system which maintained adequate water quality through biological and mechanical filtration. Salinity was maintained at 2–3% using well water and synthetic sea salt (Fritz Industries Inc., Dallas, TX). Low pressure electrical blowers provided aeration via air stones and maintained dissolved oxygen (DO) levels between 4 and 6 mg/l. Water temperature was controlled by conditioning

the ambient air in the building and remained at 26 ± 1 °C throughout the trial. A 12 h light:12 h dark photoperiod was maintained with fluorescent lights controlled by timers.

Each experimental diet was fed to three groups of small fish and one group of large fish for 16 weeks. All groups were fed to apparent satiation in the morning and evening, 7 days each week. Growth and feed efficiency were monitored monthly by collectively weighing each group of fish. Weight gain was expressed as the increase in total cumulative biomass per tank. After week 16, a chronic mycobacterial infection became severe and was confirmed by the TVMDL to be caused by *M. marinum* based on biochemical tests. At that juncture, fish were fed to apparent satiation once per day for additional 5 weeks. Mortality was monitored twice daily during this period. All procedures were approved by the Animal Care and Use Committee of Texas A&M University.

2.3. Sample collection and analysis

At the end of the 16-week feeding trial, three apparently healthy fish (no obvious skin lesions and visceral granulomas) from each tank (12 fish per treatment) were anesthetized with tricaine methane sulfonate (MS-222), and approximately 2 ml of blood was collected from the caudal vasculature using a 3-ml syringe and 23-gauge needle. These representative fish were euthanized and head kidney samples were pooled for macrophage isolation and assay of respiratory burst of head kidney leukocytes. The assay of extracellular and intracellular superoxide anion followed the procedure of Secombes (1990), as modified by Sealey and Gatlin (2002a). The amount of extracellular superoxide anion was calculated by the formula of Pick and Mizel (1981). Whole blood neutrophil oxidative radical production was determined as described by Siwicki et al. (1994). Absorbance was converted to nitro blue tetrazolium (NBT) units based on a standard curve of NBT diformazan/ml blood. Plasma was separated as previously described (Li and Gatlin, 2003) and lysozyme activity was determined by a turbidimetric assay (Jørgensen et al., 1993). A lysozyme activity unit was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min^{-1} at pH 6.1. Serum peroxidase was analyzed as described by Rodríguez et al. (2003).

2.4. Statistics

Data from the feeding trial, immune response assays and the bacterial challenge were subjected to analysis of variance according to a randomized complete block design using a significant level of $P \leq 0.05$. If a significantly main effect was observed, treatment means were separated using the LSD comparison test. All statistical analysis was conducted with Statistix® Analytical Software (Tallahassee, FL).

3. Results

3.1. Growth performance

Generally, fish fed the diets supplemented with brewers yeast and GroBiotic®-A had better growth performance during the 16-week feeding trial (Table 2). After 4 weeks of feeding, fish fed 2% brewers yeast had a significantly ($P < 0.05$) higher weight gain than fish fed the basal diet and the diet supplemented with 2% GroBiotic®-A. After 12 weeks, fish fed 1% and 2% brewers yeast and 2% GroBiotic®-A had significantly higher weight gain than fish fed the basal diet, and feed efficiency showed a similar trend. At the end of the 16-week period, fish fed 2% brewers yeast and GroBiotic®-A had the highest biomass, which tended to be significant ($P = 0.11$). Feed efficiency of fish fed 2% brewers yeast was significantly greater than the other treatments.

3.2. Immune response assays

No significant effects of the various diets were observed on neutrophil oxidative radical production, serum lysozyme and intracellular superoxide anion production by head kidney macrophages of sub-adult hybrid striped bass after the 16-week period (Table 3). However, fish fed 1% and 2% brewers yeast had a significantly ($P < 0.01$) higher serum peroxidase level than fish fed the basal diet and the diet supplemented with 2% GroBiotic®-A diet. The extracellular superoxide anion production of head kidney macrophages

Table 2
Weight gain (g gain/tank) and feed efficiency (g gain/g feed) of hybrid striped bass fed diets containing various amounts of dried brewers yeast and GroBiotic®-A*

Diet	4 weeks		8 weeks		12 weeks		16 weeks	
	Weight gain	Feed efficiency	Weight gain	Feed efficiency	Weight gain	Feed efficiency	Weight gain	Feed efficiency
Basal	3298[a]	1.11	6257	1.02	8168[a]	0.89	9237	0.78[a]
1% Brewers yeast	3550[ab]	1.24	6734	1.04	9052[b]	0.93	9349	0.76[a]
2% Brewers yeast	3574[b]	1.21	6880	0.95	9385[b]	0.97	9876	0.82[b]
2% GroBiotic®-A	3242[a]	1.12	6693	1.03	9322[b]	0.96	9878	0.78[a]
ANOVA, Pr≥F**	0.04	0.33	0.19	0.87	0.05	0.13	0.11	0.02
Pooled S.E.	160.3	0.08	348.8	0.07	413.2	0.04	307.5	0.02

* Values represent means of four replicate groups with three replicates of small fish initially averaging 64.5 g/fish and one replicate of large fish averaging 118 g/fish. Values in a column that do not have the same superscript are significantly different at $P \leq 0.05$ based on LSD comparison test.
** Significance probability associated with the F statistic.

of fish fed the basal diet was significantly higher compared to fish fed diets supplemented with 1% brewers yeast and 2% GroBiotic®-A (Table 3).

3.3. Bacterial challenge

The survival of fish in the feeding trial decreased over time as the severity of the mycobacterial infection increased. This in situ mycobacterial challenge resulted in approximately 25% mortality over the 21-week period. Fish fed the diets with 1% brewers yeast and 2% GroBiotic®-A had enhanced survival ($P<0.05$) at the 12- and 16-week intervals (Fig. 1). At the end of the 21-week period, survival of fish fed 2% GroBiotic®-A was significantly higher than fish fed the basal diet and brewers yeast supplemented diets (Fig. 1). Typical signs of mycobacterial infection including granulomas in the spleen, head kidney and a pale granulomatous liver with rough granular surface after necropsy as well as ulcerations and hemorrhaging in the skin were observed in moribund fish. The cause of mycobacteria was confirmed by the TVMDL via bacterial isolation and histopathology.

4. Discussion

Mycobacterial species have been known for years to be capable of infecting many fish species in both fresh water and seawater (Frerichs, 1993; Plumb, 1997; dos Santos et al., 2002). Striped bass and hybrid

Table 3
Neutrophil oxidative production (NBT test), serum lysozyme and extracellular and intracellular superoxide anion production of head kidney macrophages of hybrid striped bass fed experimental diets for 16 weeks*

Diet	NBT test (mg ml^{-1})**	Lysozyme (10^3 units/l)**	Peroxidase (O.D. at 450 nm)**	Extracellular superoxide anion (nmol O_2^-)***	Intracellular superoxide anion (O.D. at 620 nm)***
Basal	2.50	2323	0.428[a]	4.50[a]	0.597
1% Brewers yeast	2.40	2224	0.729[b]	3.77[b]	0.529
2% Brewers yeast	2.48	2752	0.706[b]	4.31[ab]	0.706
2% GroBiotic®-A	2.41	2124	0.497[a]	3.89[b]	0.742
ANOVA, Pr≥F****	0.49	0.13	0.01	0.05	0.85
Pooled S.E.	0.10	186	0.059	0.34	0.193

* Values in a column that do not have the same superscript are significantly different at $P \leq 0.05$ based on LSD comparison test.
** Means of three individual fish from each of four replicate tanks.
*** Means of composite samples of head kidney cells from three fish in each of four replicate tanks.
**** Significance probability associated with the F statistic.

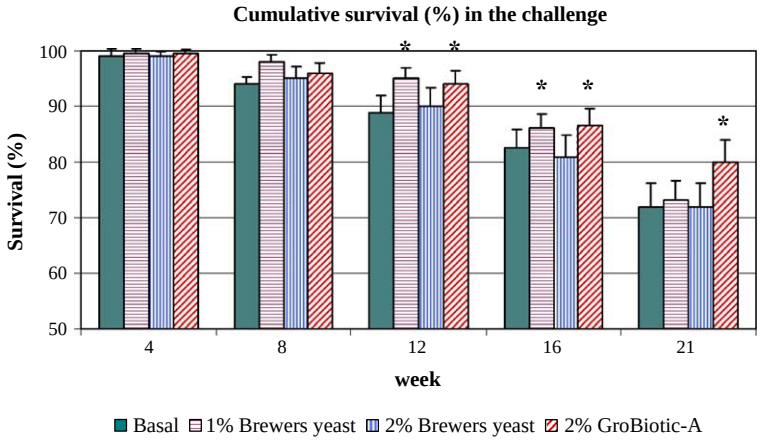

Fig. 1. Percent cumulative survival of hybrid striped bass fed 2% of GroBiotic®-A and brewers yeast (1% and 2%) for 4, 8, 12, 16 and 21 weeks and exposed to *M. marinum*. Bars represent means of four replicate tanks per treatment. Treatments with an asterisk (*) had a significantly ($P<0.05$) higher survival than treatments without an asterisk at each specific time interval.

striped bass may be one of the most susceptible species based on increasing case reports in recent years (Gauthier et al., 2003; Harms et al., 2003; Overton et al., 2003). Although the presence of mycobacteria is rather common in wild fish populations, it has become one of the most serious infections in intensive, recirculating culture systems (Plumb, 1997). Besides the economic need for developing anti-mycobacteriosis strategies, the chronic progression of this disease provided an opportunity to explore growth and immune responses of hybrid striped bass fed different immunostimulants. Because water has long been recognized as a primary source for mycobacterial transmission (Goslee and Wolinsky, 1976; dos Santos et al., 2002; Gauthier et al., 2003), chronic exposure to mycobacteria-contaminated water was employed in this trial to mimic a natural infection and to prevent possible excessive mortality from acute inflammation, although experimental mycobacterial infections traditionally have been conducted by intramuscular (Wolf and Smith, 1999) or intraperitoneal injections (Colorni et al., 1998; Gauthier et al., 2003), which have been used for research on pathology and immunology associated with this disease.

Increased weight gain and feed efficiency were generally observed in hybrid striped bass fed diets supplemented with partially autolyzed brewers yeast and GroBiotic®-A at each sampling time (4, 8, 12 and 16 weeks). However, variation within treatments complicated the interpretation of these data. Fish fed the diet supplemented with 2% brewers yeast had consistently and significantly better growth performance throughout the feeding trial compared to fish fed the basal diet. To the best of our knowledge, this is the first time to report that dietary supplementation of inactivated or autolyzed brewers yeast could serve as a growth enhance under certain conditions such as chronic infection, although biologically-active brewers yeast had been reported to serve as a probiotic and enhance growth of tilapia (Lara-Flores et al., 2002). Feed efficiency of every dietary treatment decreased over time in the present study. It is speculated that this reduction was correlated with the severity of the mycobacterial infection. With the progression of the mycobacterial infection, inflammation was induced and immune responses were upregulated accordingly. Increased expense for growth would be expected as a consequence of dramatically increased requirement for amino acids to synthesize differentiated proteins for immune functions, substrates for nitric oxide production (arginine) and energy for macrophages and lymphocytes (glutatmine). It also was noted that weight gain of fish fed all experimental diets decreased between weeks 12 and 16. Although progress of mycobacteriosis could possibly suppress feed intake and increase the cost of growth, over-accumulation of metabolic wastes such as ammonia (up to 10.6 mg/l) and nitrite (up to 1.2 mg/l) may have contributed more to the undermined feed

intake and subsequently reduced growth, because the concern of spreading this bacterium through effluent restricted the exchange of water between system and environment.

A previous study in our laboratory showed that feeding a diet supplemented with brewers yeast for 16 weeks could increase blood neutrophil oxidative radical production and extracellular superoxide anion production of head kidney leucocytes of juvenile hybrid striped bass (Li and Gatlin, 2003). This present study failed to support our previous observation, possibly due to the masking effect of inflammation induced by mycobacterial infection, if no difference in age/size responses to immunostimulation by dietary supplements was involved. At the end of 16 weeks, over 50% of hybrid striped bass in the trial were observed to have skin ulcerations and hemorrhages, suggesting a high prevalence of mycobacterium in the present study. Although only apparently healthy fish were sampled for immune response assays, mycobacteria-infected fish sometimes showed rather normal appearances (Gauthier et al., 2003). This uncertainty in infection status compromised the conclusiveness of data from the immune response assays. Fish fed diets supplemented with 1% or 2% brewers yeast for 16 weeks had a significantly higher serum proxidase level compared to fish fed the basal diet and diet supplemented with GroBiotic®-A. Rodríguez et al. (2003) observed that 6 weeks, but not 2 or 4 weeks of feeding a cell-wall modified yeast (33% glucan, 56% mannoproteins and 11% chitin) decreased serum proxidase level of gilthead sea bream. The effect of dietary brewers yeast on serum peroxidase warrants further investigation. Intracellular superoxide anion production of head kidney leucocytes and serum lysozyme were not affected by dietary treatments in the present study. However, it is noted that the serum lysozyme measured in the present study was dramatically higher than all the values previously published for hybrid striped bass (Sealey and Gatlin, 2002b; Li and Gatlin, 2003, 2004; Jaramillo and Gatlin, 2004; Li et al., 2004). Although serum lysozyme level of hybrid striped bass is influenced by genetic polymorphisms (Wang and Gatlin, unpublished data) and variation in genetic makeup of fish may contribute to the phenomenon, it is speculated that mycobacteriosis stimulated serum lysozyme level of hybrid striped bass in the present study. Chen et al. (1998) reported that injection of the extracellular product of *Mycobacterium* sp. induced an elevation in serum lysozyme level. Although pathology of mycobacteriosis is well defined, the immunological responses associated with mycobacterial infection are still limited. Bartos and Sommer (1981) and Harms et al. (2003) reported responses of cellular immunity and transforming growth factor-β associated with mycobacteriosis, but further investigation of potential interactions among dietary strategies, immune responses and mycobacterial infection is warranted.

In the present study, dietary supplementation of the prebiotic GroBiotic®-A significantly enhanced survival of hybrid striped bass during the in situ mycobacterial challenge, suggesting a potential use of this prebiotic in aquaculture. Based on knowledge acquired from human and terrestrial animals, prebiotics are usually most effective against enteric diseases. It is known that ingestion of feed also is a port of entry for mycobacteria in some fish species including snakehead (Chinabut et al., 1990). This could possibly be a factor contributing to the positive response associated with the GroBiotic®-A supplement; however, efforts to characterize intestinal microbiology of fish in the present study failed. Research on prebiotics for human use since 1995 has established that some bacterial species are health-promoting and are capable of selectively utilizing non-digestible dietary components in the colon such as inulin and lactose (Manning and Gibson, 2004). Lactobacilli also have been reported to be probiotics for fishes (reviewed by Irianto and Austin, 2002). Positive influences of GroBiotic®-A on growth performance and disease resistance of hybrid striped bass in the present study showed desirable influences of prebiotics for aquaculture.

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