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Dietary brewers yeast and the prebiotic Grobiotic™ AE influence growth performance, immune responses and resistance of hybrid striped bass (*Morone chrysops* × *M. saxatilis*) to *Streptococcus iniae* infection

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Abstract

Use of prebiotics, nondigestible dietary ingredients that beneficially affect the host by selectively stimulating the growth of and/or activating the metabolism of health-promoting bacteria in the intestinal tract, is a novel concept in aquaculture. Two separate feeding trials were conducted to evaluate graded levels of a commercial prebiotic Grobiotic™ AE, a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products, in the diet of hybrid striped bass, as compared to partially autolyzed brewers yeast (Brewtech®). The basal diet in both trials was formulated to contain 40% protein, 10% lipid and an estimated digestible energy level of 3.5 kcal/g. Two levels (1% and 2% of diet) of Grobiotic™ AE and brewers yeast were added to the basal diet with menhaden fish meal and menhaden oil adjusted to provide isonitrogenous and isolipidic diets. Each diet was fed to five (trial 1) or three (trial 2) replicate groups of juvenile hybrid striped bass in 110-l aquaria twice daily at rates approximating apparent satiation for 7 weeks (trial 1) or 4 weeks (trial 2).

Enhanced growth performance was generally observed in fish fed the diets supplemented with Grobiotic™ AE or brewers yeast compared to the basal diet after 7 weeks of feeding in trial 1. Significantly higher ($P < 0.05$) feed efficiency was observed in fish fed diets supplemented with 1% and 2% Grobiotic™ AE. After 4 weeks of feeding in trial 2, growth and feed efficiency were not significantly affected by the various dietary treatments, although some immunological responses were altered. Neutrophil oxidative radical anion production and intracellular superoxide anion

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production of head kidney macrophages tended to be higher in fish fed diets supplemented with brewers yeast or Grobiotic™ AE, while extracellular superoxide anion production of head kidney macrophages from fish fed diets with 1% and 2% brewers yeast and 1% Grobiotic™ AE was significantly ($P < 0.01$) higher than that of fish fed the basal diet. All groups of fish fed brewers yeast and Grobiotic™ AE showed a significantly ($P < 0.01$) enhanced survival (73.3–90%) after bath exposure to *Streptococcus iniae* compared to fish fed the basal diet (53.3%). Based on these data, it is concluded that Grobiotic™ AE and a partially autolyzed brewers yeast can serve as functional feedstuffs in the diets of hybrid striped bass by enhancing growth performance and immunological responses. Further research is needed into the mechanism(s) of action for prebiotics such as Grobiotic™ AE and their application in aquaculture.

Keywords: Prebiotics; Hybrid striped bass; Grobiotic™ AE; Brewers yeast; *Saccharomyces cerevisiae*; Immune response; Bacterial resistance; *Streptococcus iniae*

1. Introduction

Hybrid striped bass production is considered to be the fastest growing segment of the US aquaculture industry over the past decade and has spread to several countries and regions in Europe and Asia (Harrell and Webster, 1997; Hiney et al., 2002). It is known that most of the common disease-causing organisms associated with various aquacultured fish also may affect cultured hybrid striped bass (Plumb, 1997). In recent years, *Streptococcus iniae* has become one of the most threatening pathogenic organisms in hybrid striped bass aquaculture and caused about US$2 million of economic loss to USA hybrid striped bass producers in 2002 (Ostland, 2003). Although positive results have come from experimental treatment of this disease in tilapia with oxytetracycline (Darwish et al., 2002) and in hybrid striped bass with enrofloxacin (Stoffregen et al., 1996), these drugs are still under investigation and currently not approved by the US Food and Drug Administration to treat bacterial diseases of hybrid striped bass.

Proper nutrition has long been recognized as a critical factor in promoting normal growth and sustaining health of fish. Prepared diets not only provide the essential nutrients that are required for normal physiological functioning but also may serve as the medium by which fish receive other components that may affect their health (Gatlin, 2002). Although the concept of functional feeds is novel to the aquaculture industry, it represents an emerging new paradigm to develop diets that extend beyond satisfying basic nutritional requirements of the cultured organism. For hybrid striped bass, dietary requirements for the most essential nutrients have been established to provide balanced diets than satisfy metabolic needs (Gatlin, 1997; Webster, 2002); however, research on optimization of diets to enhance health is still in its infancy. Probiotics, live microbes that may serve as dietary supplements to improve the intestinal microbial balance, have received some attention in aquaculture (Gatesoupe, 1999; Irianto and Austin, 2002). Evidence of the beneficial effects of probiotics gave birth to the concept of prebiotics (Gibson and Roberfroid, 1995; Teitelbaum and Walker, 2002), which are defined by

Gibson and Roberfroid (1995) as "a nondigestible food ingredient which beneficially affects the host by selectively stimulating the growth of and/or activating the metabolism of one or a limited number of health-promoting bacteria in the intestinal tract, thus improving the host's intestinal balance". Examples of prebiotics include mannan oligosaccharides (White et al., 2002), lactose (Szilagyi, 2002), as well as oligofructose and inulin (Teitelbaum and Walker, 2002). Information pertaining to prebiotics in aquaculture is extremely limited.

Brewers yeast (*Saccharomyces cerevisiae*) is a natural product from the brewing industry that contains various immunostimulating compounds such as β-glucans, nucleic acids as well as mannan oligosaccharides, and has been used as a diet additive for various animals. It has been observed to be capable of enhancing immune responses (Siwicki et al., 1994; Ortuño et al., 2002) as well as growth (Lara-Flores et al., 2002) of various fish species and thus may serve as an excellent health promoter for fish culture. Li and Gatlin (2003) established the beneficial effects of partially autolyzed brewers yeast on immune responses of hybrid striped bass and resistance to *S. iniae* infection. Grobiotic™ AE is a commercial prebiotic mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products. The present study was conducted to determine the effects of graded levels of the prebiotic, Grobiotic™ AE, on hybrid striped bass growth performance, immune responses and resistance to *S. iniae* infection as compared with brewers yeast.

2. Materials and methods

2.1. Experimental diets

The basal diet of Keembiyehetty and Gatlin (1997), which utilized menhaden fish meal as the protein source, was modified to contain 40% protein, 10% lipid and an estimated digestible energy level of 3.5 kcal/kg (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™ AE, a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products, were supplied by International Ingredient (St. Louis, MO, USA). Two incremental levels (1% and 2% of diet) of Grobiotic™ AE and brewers yeast were added to the basal diet and cellulose, menhaden meal and menhaden oil were adjusted to provide isonitrogenous and isolipidic diets (Table 1).

2.2. Feeding trial 1

Juvenile hybrid striped bass (*Morone chrysops* × *M. saxatilis*) were obtained from a commercial supplier (Keo Fish Farm, Keo, AR) and maintained indoors at the Texas A and M University Aquacultural Research and Teaching Facility prior to the feeding trial. Fish were then graded by size and groups of 13 fish with a total weight of 91.4 ± 4.3 g per group were stocked into 110-l aquaria. The basal diet was fed to all fish during a 2-week

Table 1
Composition of experimental diets in feeding trials 1 and 2

Constituent	Basal diet	1% Brewers yeast	2% Brewers yeast	1% Grobiotic™ AE	2% Grobiotic™ AE
Ingredient (% dry weight)					
Menhaden fish meal[a]	59.2	58.2	57.5	58.7	58.3
Dextrin[b]	25.0	25.0	22.5	24.5	23.9
Menhaden oil[a]	4.4	4.5	4.5	4.4	4.4
Mineral premix[c]	4.0	4.0	4.0	4.0	4.0
Vitamin premix[c]	3.0	3.0	3.0	3.0	3.0
Carboxymethyl cellulose[b]	2.0	2.0	2.0	2.0	2.0
Cellulose[b]	2.4	2.3	4.5	2.4	2.4
Brewers yeast[d]	0	1.0	2.0	0	0
Grobiotic™ AE[e]	0	0	0	1.0	2.0
Analyzed proximate composition (% dry matter)[f]					
Dry matter	92.1	92.7	92.6	92.1	90.4
Crude protein ($N \times 6.25$)	40.8	41.9	41.8	39.2	40.5
Crude lipid	8.5	9.9	10.3	8.7	8.6
Ash	14.5	14.4	15.6	14.4	13.9

[a] Omega Protein, Reedville, VA. Menhaden fish meal contained 67.8% protein and 10.7% lipid on a dry-weight basis.
[b] US Biochemical, Cleveland, OH.
[c] Same as Gaylord and Gatlin (2000).
[d] International Ingredient; contained 50.7% crude protein and 2% crude lipid (dry-weight basis).
[e] International Ingredient; contained 30.0% crude protein and 1.2% crude lipid (dry-weight basis).
[f] Means of two analyses.

conditioning period. Water flow rate was maintained at approximately 650 ml/min via a recirculating system which maintained adequate water quality (total ammonia nitrogen ≤ 0.6 mg/l) through biological and mechanical filtration (Li and Gatlin, 2003). Salinity was maintained at 2.5–3.5‰ using well water and synthetic sea salt (Fritz Industries, Dallas, TX). Low-pressure electrical blowers provided aeration via air stones and maintained dissolved oxygen (DO) levels at or near saturation. Water temperature was controlled by ambient air 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 five replicate groups of fish for 7 weeks. All groups were fed their respective diets at the same fixed rate (initially 5% of body weight per day and gradually reduced to 3%). This rate was adjusted each week to maintain a level approaching apparent satiation. Fish were fed in the morning and evening, 7 days each week. Growth and feed efficiency were monitored weekly by collectively weighing each group of fish.

2.3. Feeding trial 2

A second feeding trial was conducted to further evaluate immune responses and disease resistance of hybrid striped bass after feeding experimental diets for 4 weeks. Groups of 17 juvenile hybrid striped bass weighing approximately 19.7 g/fish were stocked into individual aquarium such that initial fish weight averaged 334 ± 3 g/group. These fish were raised in the same culture system as feeding trial 1. Each experimental diet was fed to three replicate groups of fish for 4 weeks. All groups were fed their respective diets at the same fixed rate (initially 4% of body weight per day and gradually reduced to 3.5%). Growth and feed efficiency were monitored weekly by collectively weighing each group of fish.

2.4. Sample collection and analysis

At the end of the second feeding trial, two representative fish from each aquarium were anesthetized with tricaine methane sulfonate (MS-222), and approximately 0.5 ml of blood was collected from the caudal vasculature using a 1-ml syringe and 27-gauge needle. 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. Serum lysozyme activity was determined by turbidimetric assay as described by 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}. Three fish from each aquarium also were euthanized and head kidney samples were pooled for macrophage isolation and assay of extracellular and intracellular superoxide anion. This assay followed the procedure of Secombes (1990), as modified by Sealey and Gatlin (2002a). The amount of extracellular superoxide anion was calculated from the formula of Pick and Mizel (1981).

2.5. Bacterial challenge

At the end of the second trial, an additional 30 fish previously fed each experimental diet were exposed to an estimated LD_{50} dose of *S. iniae*. Before inoculation, an isolate of *S. iniae* originally obtained from tilapia and maintained by the Texas Veterinary Medical Diagnostic Laboratory (TVMDL) was injected into hybrid striped bass to improve virulence, then re-isolated and identified by polymerase chain reaction by TVMDL. This isolate was grown in brain–heart infusion broth (EM Science, Darmstadt, Germany) in a shaking bath at 27 °C overnight as described by Sealey and Gatlin (2002b). The concentration of bacterial suspension was determined by the serial plate count method and diluted to 9.3×10^5 CFU/ml in fresh well water. Thirty fish from each treatment (10 fish from each aquarium of each treatment), pooled separately in mesh baskets, were immersed in the bacterial suspension for 2 h. After bath exposure, 30 fish from each dietary treatment were divided into three groups of 10, and placed into 38-l flow-through aquaria in an isolated culture system. Water temperature was maintained at 27 ± 1 °C with immersion heaters. Fish continued to be fed their respective diets to apparent satiation twice daily, and mortality was monitored for 19 days. The brains of both dead fish and surviving fish were streaked on modified selective agar to determine infection status and confirm death from *S. iniae* (Nguyen and Kanai, 1999).

2.6. Statistics

Data from the feeding trials, immune response assays and the bacterial challenge were subjected to analysis of variance and Duncan's multiple-range test using the Statistical Analysis System. Differences in treatment means were considered significant at $P<0.05$.

3. Results

3.1. Feeding trial 1

After the 7-week feeding period, enhanced weight gain was generally observed in fish fed diets supplemented with 1% and 2% Grobiotic™ AE compared to those fed the basal diet, although only feed efficiency was significantly ($P<0.05$) improved (Table 2). Survival during feeding trial 1 was high and no significant differences were observed among treatments.

3.2. Feeding trial 2

No significant differences in growth, feed efficiency and survival were observed in fish fed experimental diets after 4 weeks. Neutrophil oxidative radical production of fish fed brewers yeast and Grobiotic™ AE tended ($P=0.15$) to be higher than that of fish fed the basal diet (Table 3). Serum lysozyme was highly variable, with no significant differences observed among the various dietary treatments (Table 3). Intracellular superoxide anion production of head kidney macrophages was not affected by the dietary factors in this experiment; however, extracellular superoxide anion production was observed to be significantly higher in fish fed diets with brewers yeast and 1% Grobiotic™ AE (Table 3).

Table 2
Performance of hybrid striped bass fed diets containing various amounts of dried brewers yeast and Grobiotic™ AE for 7 weeks in feeding trial 1[a]

Diet	Weight gain (% of initial wt[b])	Feed efficiency (g gain/g feed)	Survival (%)
Basal	388	0.93[b]	92.3
1% Brewers yeast	388	0.95[a,b]	100
2% Brewers yeast	404	0.95[a,b]	93.8
1% Grobiotic™ AE	420	1.0[a]	95.4
2% Grobiotic™ AE	405	1.0[a]	96.9
Pr>F[c]	0.45	0.05	0.28
Pooled se	5.97	0.21	1.20

[a] Values represent means of five replicate groups. Values in a column that do not have the same superscript are significantly different at $P\leq0.05$ based on Duncan's multiple range test.

[b] Fish initially weighed 7.0±0.3 g each.

[c] Significance probability associated with the F statistic.

Table 3
Neutrophil oxidative production (NBT test), serum lysozyme, extracellular and intracellular superoxide anion production of head kidney macrophages of hybrid striped bass fed experimental diets in feeding trial 2[a]

Diet	NBT test ($mg\ ml^{-1}$)[b]	Lysozyme (10^3 Units/l)[b]	Extracellular superoxide anion (nmol O_2^-)[c]	Intracellular superoxide anion (O.D. at 620 nm)[c]
Basal	1.20	473	7.21[c]	1.28
1% Brewers yeast	1.40	515	11.22[a]	1.55
2% Brewers yeast	1.58	472	10.55[a]	1.66
1% Grobiotic™ AE	1.28	448	9.71[a,b]	1.79
2% Grobiotic™ AE	1.31	640	8.34[b,c]	1.59
Pr > F[d]	0.15	0.728	0.0008	0.67
Pooled se	0.045	43.778	0.274	0.163

[a] Values in a column that do not have the same superscript are significantly different at $P \leq 0.05$ based on Duncan's multiple range test.
[b] Means of six fish (two from each of three replicate groups).
[c] Means of three composite samples of head kidney cells from three fish in each replicate group.
[d] Significance probability associated with the F statistic.

3.3. Bacterial challenge

The disease challenge with live *S. iniae* resulted in approximately 50% mortality of fish fed the basal diet after 19 days. Survival of fish fed diets containing Grobiotic™ AE or brewers yeast was significantly ($P < 0.01$) higher than fish fed the basal diet after the same period (Fig. 1). The moribund fish showed typical symptoms of *S. iniae* including extremely erratic swimming, cloudiness of eyes, hemorrhages around mouth and base of fins, dark pigmentation and slow acceptance or refusal of

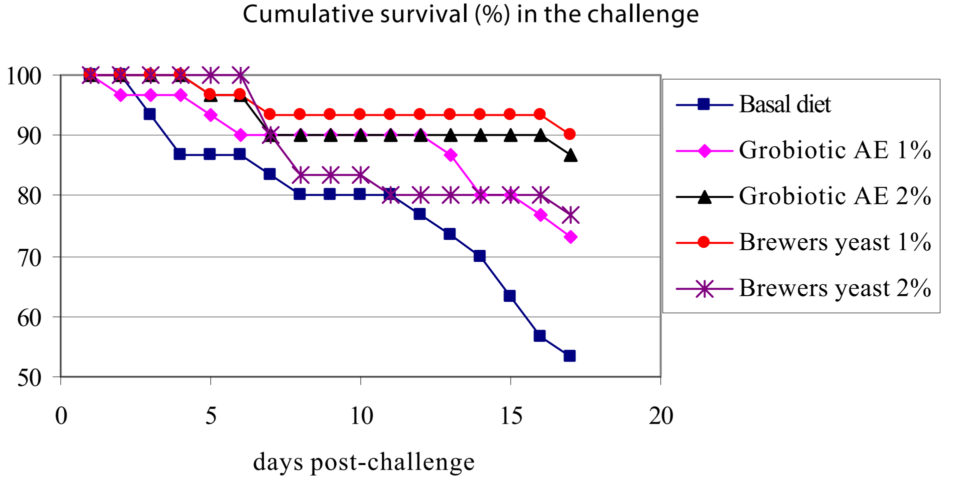

Fig. 1. Percent cumulative survival rate of hybrid striped bass fed incremental levels of Grobiotic™ AE and brewers yeast (1% and 2%) for 4 weeks and subsequently exposed to *S. iniae* bath ($P < 0.01$). Symbols represent means of three replicate tanks per treatment. Pooled S.E. was 3.10.

feed. No *S.iniae* was isolated from the brain of surviving fish, while colonies of *S. iniae* were isolated from dead fish.

4. Discussion

The concept of prebiotics arose from the observation that inulin and oligofructose selectively stimulate the growth of bifidobacteria, which are considered to be beneficial to human health (reviewed by Blaut, 2002; Teitelbaum and Walker, 2002). Other substances including mannan oligosaccharides (White et al., 2002) and lactose (Szilagyi, 2002) also have been reported to possess prebiotic functions in humans and/or terrestrial animals. Such information on prebiotics in aquatic organism is very limited. Olsen et al. (2001) observed that a diet supplemented with 15% inulin caused harmful effects to Arctic charr. However, their previous studies (Ringø et al., 1998; Ringø and Olsen, 1999) showed that dietary fatty acids and carbohydrates altered the bacterial flora of the gastrointestinal tract of fish. In this present study, the commercial prebiotic, Grobiotic™AE, significantly enhanced feed efficiency, immunological responses and resistance of hybrid striped bass to *S. iniae* infection. The beneficial influence of Grobiotic™AE on growth was possibly due to alteration of the intestinal microflora by mannan oligofructose, lactose or other carbonhydrates from the dairy ingredient, partially autolyzed yeast and/or dried fermentation products; however, a detailed study of intestinal microbiology is needed. Lactic acid bacteria have been considered beneficial residents of the fish's intestinal ecosystem by producing bacteriocins, which inhibit growth of certain fish pathogens and thus positively affect the host's microflora (Ringø et al., 1998). Some reports have shown that lactose, as well as other prebiotic ingredients, may promote the maintenance of lactic acid-producing bacteria and prevent the expansion of potential pathogens in certain human diseases (Szilagyi, 2002). However, the hypothesis needs to be tested by further studies in fish.

Brewers yeast has been recognized to have potential as a substitute for live food in the production of certain fish (Nayar et al., 1998) or as a potential replacement for fish meal (Rumsey et al., 1991; Oliva-Teles and Goncalves, 2001). As a protein feedstuff, brewers yeast has been included in commercial diet formulations for several fish species, including salmonids (National Research Council, 1993). The cell walls of yeast also may provide very important non-nutritive compounds that may benefit fish health, including mannose polymers covalently linked to peptides (mannoprotein), glucose polymers (glucans), chitin in minor amounts (Cabib et al., 1982) as well as nucleic acids (Rumsey et al., 1992). The beneficial influence of glucans has been demonstrated with various fish species (Verlhac et al., 1998; Sahoo and Mukherjee, 2002; Couso et al., 2003). Enhanced immunological responses including respiratory burst also have been reported for dietary chitin (Esteban et al., 2001). According to Rumsey et al. (1992) and Cabib et al. (1982), yeast cells provide about 7.7% crude glucan and 1% chitin. It is known that glucan is capable of enhancing innate immune responses, including respiratory burst of head kidney macrophages, serum complement activity and serum lysozyme (Engstad et al., 1992; Jørgensen et al., 1993) when administered by injection. However, the increase of serum lysozyme was not observed in fish orally administered glucan or chitin

(Verlhac et al., 1998; Esteban et al., 2001; Ortuño et al., 2002), which agrees with the results of the present study. Serum lysozyme activity of hybrid striped bass in the present study varied greatly among individual fish. A similar observation was reported in a previous study with hybrid striped bass after 8 and 16 weeks of feeding with diets supplemented with brewers yeast (Li and Gatlin, 2003). Jørgensen et al. (1993) observed rainbow trout injected with 1% β-1,3-glucan showed significantly enhanced extracellular superoxide anion production of head kidney cells. Results of the present study showed that extracellular superoxide anion production of head kidney macrophages of hybrid striped bass also could be activated by oral administration of glucan and/or chitin from brewers yeast.

Nucleic acids, accounting for 12–20% of the total nitrogen of brewers yeast, are found primarily in the purine and pyrimidine bases of nucleoproteins (Rumsey et al., 1992). Rumsey et al. (1992) observed that supplementation of RNA extract from brewers yeast elevated the level of hepatic nucleic acids in rainbow trout and assumed that dietary nucleic acids could be incorporated into the tissue pool. However, detailed information on digestion and absorption of nucleic acids by fish is still limited. In recent years, a growing number of reports have indicated that oral administration of nucleotides is capable of enhancing immune responses and/or diseases resistance in several fish species, including hybrid striped bass (Burrells et al., 2001; Sakai et al., 2001). The extent to which hybrid striped bass are capable of utilizing nucleic acids from brewers yeast and their contribution to enhancement of immune responses and disease resistance need to be investigated further. In the present study, Grobiotic™ AE and brewers yeast influenced immune response in very similar ways. The specific mechanism(s) by which these compounds exert their beneficial influence remains unknown, although intestinal microflora is recognized as a major determinant for the development of the immune system (Blaut, 2002).

Disease associated with *S. iniae* represents a serious health and economic problem in cultured fish species, with an annual economic loss estimated at greater than US$150 million worldwide (Buchanan et al., 2003). In North America, the hybrid striped bass industry has been particularly impacted (Buchanan et al., 2003). In the present study, survival of hybrid striped bass fed brewers yeast and Grobiotic™ AE after *S. iniae* challenge was significantly higher than fish fed the basal diet. This demonstrates the potential of dietary strategies in reducing the risk of disease, which is generally associated with aquaculture. Recent progress in identification of virulence genes of *S. iniae* and development of gene vaccines against this pathogenic bacterium for hybrid striped bass would contribute greatly to reducing its impact in aquaculture (Buchanan et al., 2003). Grobiotic™ AE and brewers yeast also hold promise of promoting fish health and resistance to infection from other pathogenic organism. Fattal-German and Bizzini (1992) observed the antiviral property of oral administration of live yeast cells in mice. This phenomenon is also worth exploring in fish as well as challenging with other pathogens in future. With the increasing concerns about use of antibiotics in aquaculture, various pre- and probiotics that have shown potential as alternative treatments (Nikoskelainen et al., 2001; reviewed by Irianto and Austin, 2002) should receive further consideration. How to utilize dietary strategies and prebiotics to maximize the efficiency of probiotics is a promising subject and more research is warranted.

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