

The effect of dietary inactive *Saccharomyces cerevisiae* and complementary commercial feed on growth performance, bacterial population, and blood cells of hybrid grouper (*Epinephelus fuscoguttatus* x *E. polyphkadion*)

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Abstract. The use of immunomodulatory feed additives represents a promising strategy to improve fish health in aquaculture. This study evaluated the effects of dietary inactive yeast (*Saccharomyces cerevisiae*) and complementary commercial feed on growth performance, bacterial population, and hematological parameters of hybrid grouper (*Epinephelus fuscoguttatus* x *E. polyphkadion*). A total of 300 healthy juveniles (22.21 ± 3.83 g) were divided into three groups of 100 fish each. Experimental diets were prepared by separately adding 50 mL of inactive yeast suspension (1.3 × 10⁸ cfu/mL) or 50 mL of dissolved complementary feed solution (10%) to 1 kg of pellets. Fish were fed daily at 3% of biomass for 90 days. Results showed that supplementation with inactive yeast and complementary feed did not significantly affect ($p > 0.05$) bacterial populations, including *Vibrio* spp., in the stomach and intestines. However, both additives increased red and white blood cell concentrations during the first and second months of feeding, indicating improved hematological status. In a challenge test with *Vibrio harveyi*, fish receiving yeast or complementary feed exhibited significantly higher survival rates (86.67 ± 11.55 – 100%) compared to the control group (63.33 ± 5.77%) ($p < 0.05$). These findings suggest that inactive yeast and complementary feed can enhance immune responses and disease resistance in hybrid grouper, supporting their potential as functional feed additives in aquaculture.

Key Words: hematology, immunomodulatory feed, *Vibrio harveyi*.

Introduction. The development of marine fish culture is increasingly being carried out in Indonesia. This is due to the market demand for fish seeds and consumption (Rimmer et al 2013). An increasing incidence of infectious diseases accompanies the development of marine fish culture. Chemicals and antibiotics are often used to prevent and treat this disease. However, antibiotic use is associated with problems such as antibiotic-resistant pathogens (Istiqomah et al 2023).

Yeast and seaweed are probiotics that are used as a substitute for antibiotics (Choudhury et al 2005; Ran et al 2015). Yeast, *Saccharomyces cerevisiae*, produces enzymes, oligosaccharides, amino acids, peptides, organic acids, vitamins, and other soluble components (Gao et al 2008). Due to immunostimulatory or intestinal health-promoting actions, yeast has structural components in the yeast cell wall, such as β -glucan and mannan oligosaccharide (MOS) (Ortuño et al 2002; Dimitroglou et al 2009).

Efforts to improve the body's defense system are critical if the body's defense system is to remain optimal. When the function and number of immune cells are insufficient, efforts to improve them through immunomodulator administration become critical. Immunomodulators are compounds or chemicals that reduce the immune response (immunosuppressant), increase the immunological response (immunostimulant), or enable tissue to become resistant to an antigen (tolerogen) (Baratawidjaja & Rengganis 2010; Cleveland Clinic 2023). Immunostimulants are used as adjunct therapy for diseases caused by pathogenic organisms, assisting in the alleviation of symptoms and accelerating the recovery process. Immunostimulants can also be used as a preventative measure (prevention) to strengthen the body's immune system (Baratawidjaja & Rengganis 2010).

This research aimed to determine the effect of dietary inactive *Saccharomyces cerevisiae* and complementary commercial feed on growth performance, bacterial population, and blood cells of hybrid grouper (*Epinephelus fuscoguttatus* × *E. polyphemadion*), as well as the fish's resistance to *Vibrio harveyi* infection.

Material and Method

Test fish. This study was approved by the Animal Ethics Committees of the Faculty of Veterinary Medicine Udayana University (Approval number B/74/UN 14.2.9/PT.01.04/2024). Hybrid grouper (*Epinephelus fuscoguttatus* × *E. polyphemadion*) juveniles (total length 11.81 ± 0.85 cm; body weight 22.21 ± 3.83 g) were obtained from a private hatchery. Prior to the experiment, 300 fish were acclimated for 5 days in three fiber tanks (1,000 L each; total volume 3,000 L) equipped with a running water system. To minimize external pathogens influence, all fish were treated in freshwater containing 100 ppm formalin for 30 minutes before stocking.

Feed preparation. The experimental diets were prepared using a commercial pelleted feed (Cargill MarinXcel) containing more than 30% crude protein. Three different treatments were formulated: (1) control feed, (2) feed supplemented with inactive *Saccharomyces cerevisiae*, and (3) feed supplemented with a commercial complementary feed. For each treatment, 1 kg of pellets was placed on a clean tray, and 50 mL of the respective solution was sprayed evenly onto the pellet surface. The solutions consisted of phosphate-buffered saline (PBS, control), inactive *S. cerevisiae* suspension (1.3×10^8 cfu/mL), or dissolved complementary feed 10% (Searup®, Olmix, France). After spraying, the pellets were thoroughly mixed to ensure uniform coating. The treated feed was air-dried at room temperature for 24 hours and subsequently stored frozen until used in the feeding trial.

Feeding. Each treatment feed was delivered to 100 hybrid grouper fish twice a day (3 % of the fish biomass) for 9 weeks.

***Vibrio harveyi* test challenge.** *Vibrio harveyi* stock cultures were grown on tryptic soy agar (TSA) and incubated at 30°C for 24 hours. Colonies were suspended in phosphate-buffered saline (PBS) and adjusted to a concentration of 1×10^8 cfu/mL using the McFarland turbidity standard (McFarland 1907). For the challenge test, 30 fish from each dietary treatment were distributed into nine plastic tanks (150 L each; three tanks per treatment, with 10 fish per tank). Fish were anesthetized with clove oil (0.1 mL/2 L seawater) prior to intramuscular injection of *V. harveyi*. Following injection, fish were maintained in separate tanks and monitored daily for mortality over 14 days. Additionally, 15 fish from each treatment group were injected with the same bacterial

suspension and placed in separate tanks for hematological sampling. Blood samples were collected at 1, 3, 6, and 14 days post-challenge. Fish were anesthetized with clove oil (0.1 mL/2 L seawater) prior to sampling. Approximately 0.5–1.0 mL of blood was drawn from the caudal vein using sterile 1 mL syringes fitted with 24-gauge needles. Red blood cell (RBC) and white blood cell (WBC) counts were determined using a Neubauer hemocytometer under light microscopy. For RBC counts, blood was diluted 1:200 in Hayem’s solution, while WBC counts were performed after dilution in Turk’s solution to lyse erythrocytes and stain leukocyte nuclei.

Test parameters and data analysis. Fish growth is assessed by measuring the overall length and body weight of the fish every two weeks (20 fish/treatment). The absolute length and absolute weight increases are calculated using the formula previously reported by Cokrowati et al (2020). To evaluate bacterial populations, samples were collected monthly from the stomach and intestines of fish in each treatment group. Three to five fish were randomly selected, euthanized, and their gastrointestinal tracts aseptically dissected. Tissue samples were homogenized in sterile phosphate-buffered saline (PBS), serially diluted, and plated on tryptic soy agar (TSA) for enumeration of total bacterial count. For total *Vibrio* count (*Vibrio* spp.), dilutions were plated on thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Plates were incubated at 30°C for 24–48 hours, and colony-forming units (CFU) were counted to determine bacterial load.

Blood samples were collected monthly from 3–5 pooled fish per treatment, as well as at 1, 3, 6, and 14 days following the *Vibrio harveyi* challenge test. Hematological parameters (RBC and WBC) were analyzed as described in the blood sampling protocol. Data on growth performance and survival after the challenge test were analyzed using one-way ANOVA after confirming normality (Kolmogorov–Smirnov test) and homogeneity of variance (Levene’s test). When significant differences were detected, Tukey’s post-hoc test was applied at a significance level of $p < 0.05$.

Results

Fish growth. The total length of the fish indicated that feeding with inactive *Saccharomyces cerevisiae* was slightly higher than feeding with complementary feed and the control feed (Figure 1).

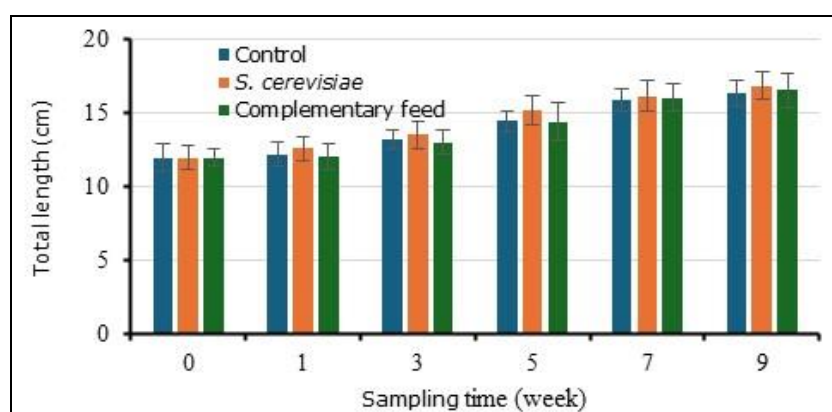


Figure 1. Total length of hybrid grouper improved during 9 weeks of dietary inactive *Saccharomyces cerevisiae* and complementary feed.

The increase in absolute length of fish fed with inactive *S. cerevisiae* was 4.86 ± 0.1 cm, higher ($p < 0.05$) when compared to those fed with complementary feed (4.55 ± 0.65 cm) and control feed (4.39 ± 0.08 cm) (Table 1).

The weekly body weight gain of hybrid grouper given inactive *S. cerevisiae* was greater than the other two treatments (Figure 2). The absolute body weight development of the hybrid grouper, however, was not statistically significantly different ($p > 0.05$) between feeding with inactive *S. cerevisiae* (52.2 ± 11.34 g), complementary feed (46.84 ± 6.82 g), and control (47.42 ± 10.21 g) (Table 1).

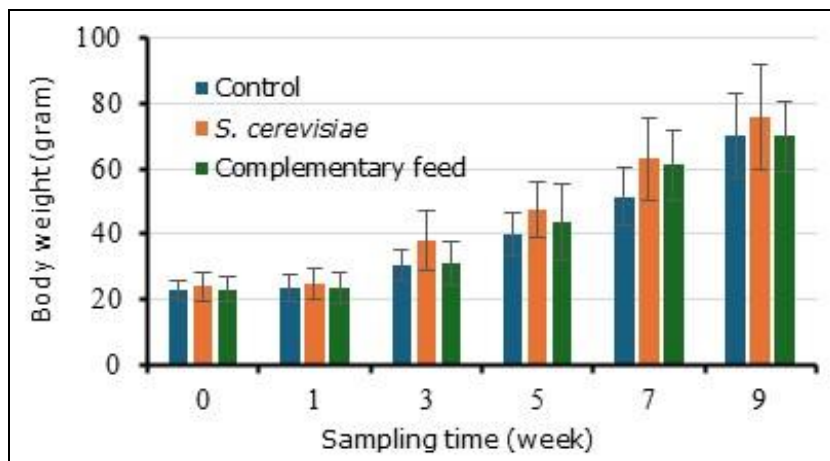


Figure 2. Body weight of hybrid grouper improved during 9 weeks of dietary inactive *Saccharomyces cerevisiae* and complementary feed treatments.

Table 1
The absolute length and absolute body weight of hybrid grouper fed a diet supplemented with inactive *Saccharomyces cerevisiae* and complementary feed

Parameters	Treatment		
	Control	<i>S. cerevisiae</i>	Complementary feed
Average initial total length (cm)	11.96 ± 0.96	11.98 ± 0.86	11.99 ± 0.53
Average final total length (cm)	16.35 ± 0.88	16.84 ± 0.95	16.53 ± 1.17
Absolute total length increase (cm)	4.39 ± 0.08 ^a	4.86 ± 0.1 ^b	4.55 ± 0.65 ^a
Average initial body weight (g)	22.91 ± 2.76	23.95 ± 4.65	23.23 ± 3.79
Average final body weight (g)	70.33 ± 12.96	76.15 ± 15.99	70.07 ± 10.62
Absolute body weight gain (g)	47.42 ± 10.21 ^a	52.2 ± 11.34 ^a	46.84 ± 6.82 ^a

Note: Values within the same row that share identical superscript letters are not significantly different ($p > 0.05$). Values with different superscript letters indicate statistically significant differences among treatment groups.

Bacterial load. Bacterial enumeration in the stomach organs of hybrid grouper fish in the first and second months revealed nearly identical quantities in all treatments (Figure 3). Total bacteria and *Vibrio* spp. were lower in the first month (log 6.06 ± 4.4 - 6.22 ± 4.81 and log 3.93 ± 3.83 - 4.26 ± 3.79) than in the second month (log 8.52 ± 7.51 - 8.87 ± 8.0 and log 4.0 ± 3.0 - 5.43 ± 5.18) after treatment.

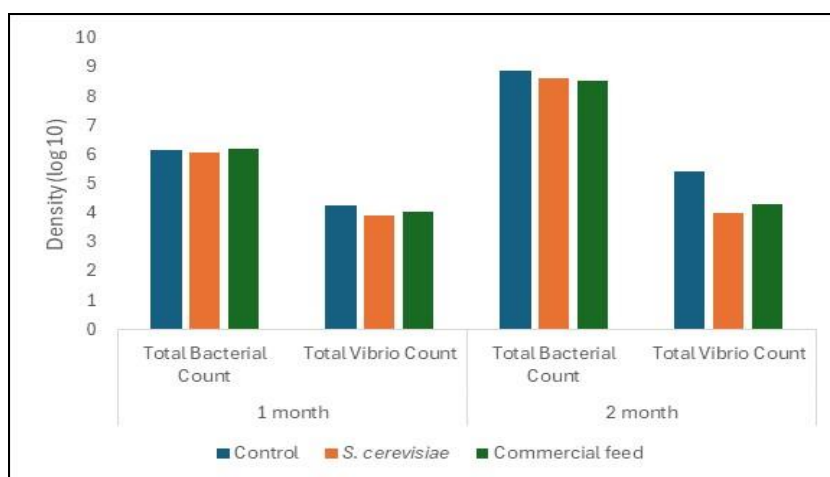


Figure 3. Bacterial populations and *Vibrio* spp. in the stomach of hybrid grouper fed supplemented diets with inactive *Saccharomyces cerevisiae* and complementary feed.

Total bacteria and *Vibrio* spp. levels increased in the intestines of hybrid grouper in the second month following treatment (Figure 4). In the first month, total gut bacteria ranged from $\log 6.01 \pm 4.73$ to $\log 7.0 \pm 4.73$. Meanwhile, it increased to $\log 9.0 \pm 7.02$ to 9.89 ± 9.18 in all treatments in the second month. In the first month, *Vibrio* spp. in the treatment with the addition of complementary feed was slightly lower ($\log 3.74 \pm 3.52$) compared to the control and the addition of inactive *S. cerevisiae* ($\log 5.0 \pm 4.71$ and $\log 5.0 \pm 4.73$). However, in the second month, *Vibrio* spp. was reduced ($\log 4.3 \pm 3.05$) in the treatment with the addition of inactive *S. cerevisiae* compared to the control and addition of complementary feed ($\log 7.26 \pm 7.02$ and $\log 7.32 \pm 6.55$).

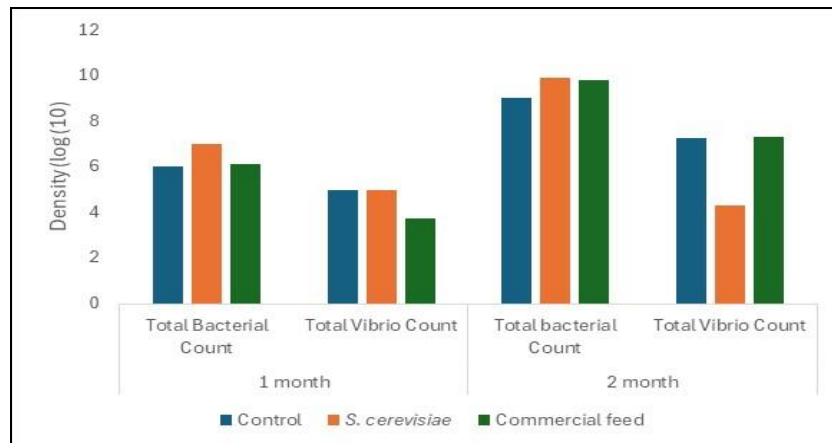


Figure 4. Bacterial populations and *Vibrio* spp. in the intestine of hybrid grouper fed supplemented diets with inactive *Saccharomyces cerevisiae* and complementary feed.

Red blood cells and white blood cells count. Analysis of red blood cells (erythrocytes) from hybrid grouper fed inactive *S. cerevisiae* and complementary feed revealed higher numbers in the first month (1,865,000 and 1,760,000 cells/mm³) and second month (1,955,000 and 2,025,000 cells/mm³) after treatment compared to the control treatment (1,485,000 and 1,710,000 cells/mm³) (Figure 5).

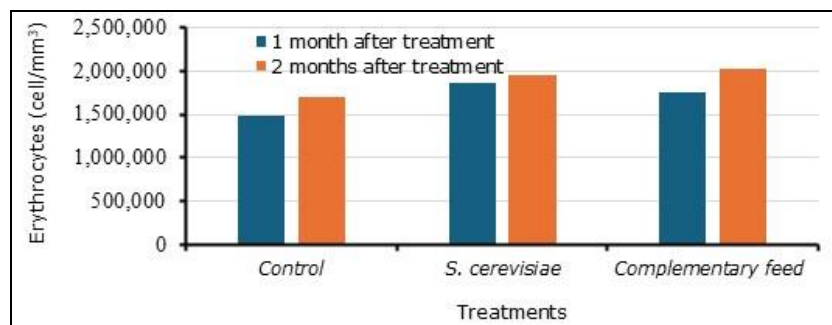


Figure 5. The number of red blood cells (erythrocytes) in hybrid grouper given inactive *Saccharomyces cerevisiae* and complementary feed for up to 2 months.

White blood cells (leukocytes) increased in hybrid grouper fed inactive *S. cerevisiae* and complementary feed after 1 month (325 and 375 cells/mm³) and 2 months (387 and 350 cells/mm³) of administration compared to the control treatment (125 and 135 cells/mm³) (Figure 6).

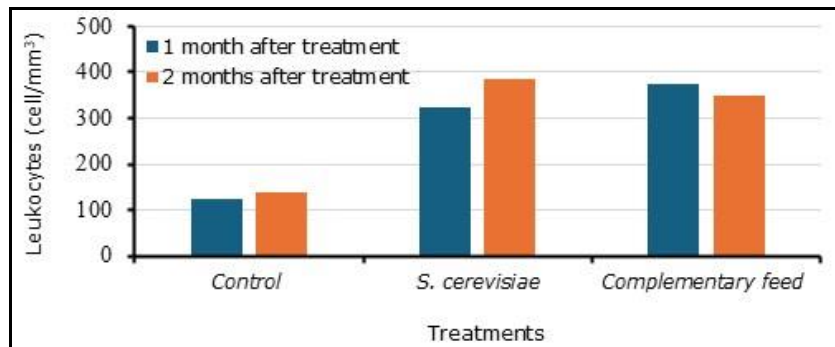


Figure 6. The number of white blood cells (leukocytes) in hybrid grouper fish given inactive *Saccharomyces cerevisiae* and complementary feed for up to 2 months.

Survival rate after *Vibrio harveyi* challenge. The results of the challenge test with *V. harveyi* revealed that the addition of inactive *S. cerevisiae* and complementary feed in the feed reduced hybrid grouper mortality ($p < 0.05$) (Table 2).

Table 2

Survival rate of hybrid grouper (%) after challenge with *Vibrio harveyi*

Parameter	Treatment		
	Control	<i>S. cerevisiae</i>	Complementary feed
Survival rate	63.33 ± 5.77 ^a	100 ^c	86.67 ± 11.55 ^b

Note: Values within the same row that share identical superscript letters are not significantly different ($p > 0.05$). Values with different superscript letters indicate statistically significant differences among treatment groups.

Table 2 also demonstrates that the inclusion of inactive *S. cerevisiae* and complementary feed in the fish feed was able to boost the immunity of hybrid grouper, as evidenced by higher fish survival (86.67 ± 11.55 to 100 %) compared to the control treatment (63.33 ± 5.77 %) after 2 weeks post-challenge with *V. harveyi*. The ability to combat antigen was demonstrated by an increase in white blood cells of hybrid grouper fed with inactive *S. cerevisiae* and complementary feed that was greater than the control diet (Figure 7B) after 3 and 6 days of infection.

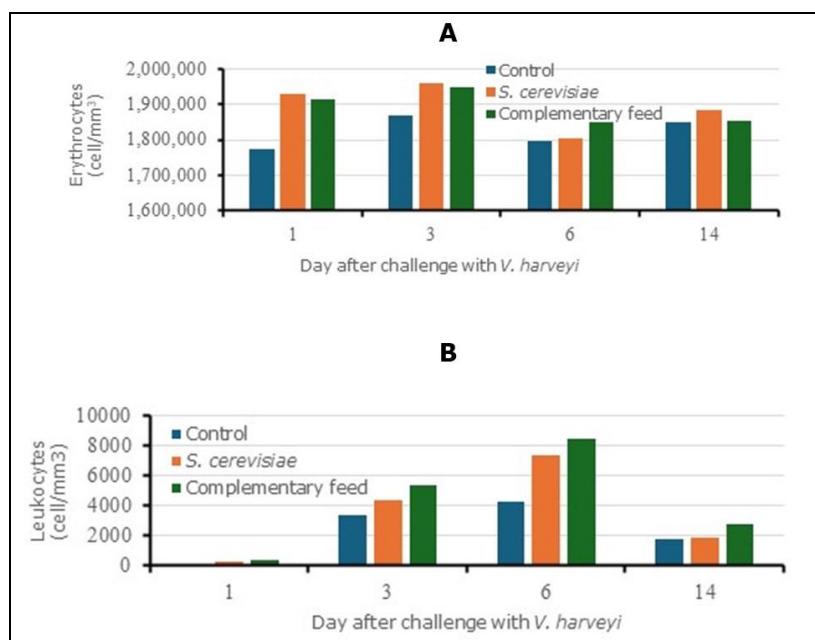


Figure 7. A - number of red blood cells (erythrocytes). B - number of white blood cells (leukocytes) in hybrid grouper fed inactive *Saccharomyces cerevisiae* and complementary feed after a *Vibrio harveyi* challenge test.

Discussion. Baker's yeast (*Saccharomyces cerevisiae*) affects the growth of Asian sea bass (*Lates calcarifer*), according to Cokrowati et al (2020). Giving *S. cerevisiae* at a dose of 0.20 % of the feed weight resulted in the optimum growth of sea bass. The addition of 1.5 % baker's yeast/kg commercial artificial feed has also been found to have an influence on the efficiency of feed consumption, growth, and survival of Sangkuriang catfish seeds (*Clarias gariepinus* var. Sangkuriang) (Rachmawati et al 2020).

The inclusion of 2% inactive *Saccharomyces cerevisiae* in the diet has been reported to significantly enhance weight gain and specific growth rate in rabbitfish (*Siganus guttatus*), compared to diets without baker's yeast supplementation (Kamaruddin et al 2019). Giving baker's yeast at a dose of 3 g/kg of feed resulted in a feed conversion ratio (FCR) of 1.28 ± 0.03 , specific growth rate (SGR) of 5.90 ± 0.09 %, and survival rate of *Puntius javanicus* of 95.00 ± 8.66 % (Sumardiyani et al 2020).

Aside from baker's yeast, which is available in powder form, commercial brewer's yeast, which is a by-product of the brewing process in the form of inactive yeast (dead yeast cells), has been shown to have high immunostimulant characteristics and can be used as a source of nucleotides. Brewer's yeast was able to enhance non-specific cellular and humoral immunity in zander (*Sander lucioperca*) at levels of 40 and 60 g/kg of feed. Although the addition of brewer's yeast had no substantial effect on the fish's growth (Jarmołowicz et al 2012).

The bacterial population in the maintenance water may influence it. According to Kim and Lee (2017), the population level of total bacteria and *Vibrio* spp. in fish is inversely related to the population level of total bacteria and *Vibrio* spp. in rearing water.

The increasing of total bacteria and *Vibrio* spp. demonstrates that the hybrid grouper's digestive tract contains the bacteria *Vibrio* spp. These bacteria are most likely part of the natural flora of grouper fish. Healthy rainbow trout (*Oncorhynchus mykiss*) seeds contain approximately 10^2 - 10^7 cfu/g of bacteria, which includes gram-positive cocci, *Enterobacteriaceae* spp., *Coryneforms* spp., *Vibrio* spp., and *Aeromonas* spp. (Diler et al 2000). The addition of inactive *S. cerevisiae* and complementary feed had no influence on the bacterial population and *Vibrio* spp. in the stomach and intestines of hybrid grouper, according to these findings.

The addition of baker's yeast to diet has a considerable effect on the amount of red blood cells in Asian sea bass, according to Cokrowati et al (2020). The amount of red blood cells grew in proportion to the number of *S. cerevisiae* in the feed. An increase in the amount of red blood cells was also observed in rabbitfish fed additional *S. cerevisiae* feed (Kamaruddin et al 2019).

After 12 weeks of administration, providing supplementary feed such as methionine was found to be able to improve the percentage of white blood cells (neutrophils) and inhibit the expression of apoptotic genes in European sea bass (*Dicentrarchus labrax*) (Machado et al 2020). *S. cerevisiae* in feed has also been shown to increase blood protein, globulin, red blood cells, and white blood cells in tilapia (*Oreochromis niloticus*) (Dawood et al 2020).

These findings support the use of inactive *S. cerevisiae* and complementary feed as immunomodulators in grouper. Complementary feed, such as Searup immune system booster, which is manufactured from algae extract and contains polysaccharides, vitamins, amino acids, and natural minerals, can assist groupers to enhance their immunity. These findings support the use of inactive *Saccharomyces cerevisiae* and complementary feed as immunomodulators in grouper. One example of such complementary feed is Searup, an immune system booster manufactured from algae extract. It contains polysaccharides, vitamins, amino acids, and natural minerals that can enhance the immune response of groupers. Importantly, Searup is reported to have a composition similar to yeast products, consisting of approximately 85 – 90% polysaccharides and 10 – 15% protein along with other bioactive components beneficial for fish growth and development (Nguyen et al 1998; Øverland et al 2013). Yeast-derived products in aquaculture feeds are widely recognized as nutritional and functional additives, contributing to improved immune function and intestinal health in fish (Yilmaz et al 2007).

Conclusions. According to the findings of the study, the addition of inactive *Saccharomyces cerevisiae* and complementary feed has no statistically significant effect on the growth of hybrid grouper, but these two ingredients can increase the number of red blood cells and white blood cells, as well as the survival rate of hybrid grouper after infection with *Vibrio harveyi*.

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Conflict of interest. The authors declare that there is no conflict of interest.

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