The impact of mixed feed of seaweed *Gracilaria* sp. and transfer factor formula on the immune response of Nile tilapia (*Oreochromis niloticus*)

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Abstract. Many efforts are made to improve human health, among which is to pay attention to the content of natural food intake, in order to reduce the intake of chemicals that can damage health. Therefore, it is necessary to provide data about the fish species having a good immune system against bacterial or viral infections, that are either found in their ecosystem or being cultivated. *Gracilaria* sp. is a type of seaweed that contains antioxidant substances, vitamins and proteins. On the other hand, the egg yolk extract and cow milk in nano size powder particles can stimulate the immune system. These nano-sized substances are ingredients of the Transfer Factor Formula (TFF). The mixing of *Gracilaria* sp. and TFF in *Oreochromis niloticus* feed is a breakthrough to determine the immune system in *O. niloticus*. With this mixed feed, it is hoped that the content of the mixed feed has a good effect on the immune system, fish improving the resistance to diseases, in cultured fish. The results showed that *Gracilaria* sp. and the supplement of TFF affected the immune system in the growth of *O. niloticus*, which was indicated by the total feed consumed, Feed Conversion Ratio (FCR), Relative Growth Rate (RGR), and Feed Utilization Efficiency (FUE) values for each treatment. The optimal concentrations combination was observed in the feed mixed with seaweed and TFF at a ratio of 958.8:40:1.2 g, generating a feed consumption ratio of 192.45±25.92 g, a FCR of 11.32±1.61%, a RGR of 2.29±0.04%, and a FUE of 8.97±1.39%.

Key Words: fish, seaweed, mixed feed.

Introduction. Nile tilapia, *Oreochromis niloticus*, is a type of fish that has the ability to grow in low salinity medium (Lima et al 2018). *O. niloticus* is a native organism from the Nile, Uganda, which often migrates to new environments. *O. niloticus* (its commercial name is "Tilapia") is the second most important cultured consumption fish in the world, with an annual production of 3.5 million tons (FAO 2020). Therefore, in 2015-2017 in Thailand, there were mass deaths with a cumulative mortality percentage of 20-100%. This led to the so-called "one-month mortality syndrome". This syndrome is reported to be discovered from infection with a virus from the family Orthomyxoviridae called Tilapia Lake Virus (TiLV). This virus attacks *O. niloticus* seeds or juveniles every month. The clinical signs of infected fish were: eating disorder, poor physical condition, abnormal swimming ability, excessive mucus on the skin, and stomach swelling (Aich et al 2022).

A solution is needed to overcome this issue by increasing the immune system in fish, which contributes to the homeostasis and is a protector against potential dangers from the surrounding environment (Magadan et al 2015). Based on the immune response, a specific and a non-specific immune system can be observed in fish. These two immune systems work together in response to the presence of antigens that penetrate the epithelial membrane (Zerwas et al 2017). One of the strategies deployed for controlling the spread of fish diseases and for improving their immune system is the provision of immunostimulants, through natural feed ingredients, compounds or drugs without side effects and residues expelled in the culture environment.

Seaweed is a potential asset in producing immunostimulant substances (Thépot et al 2021). Seaweed is a marine biological material that contains many bioactive components that can improve health. According to Balaraman & Subramanian (2017), many biological materials have the potential to be immunomodulators, but seaweed has a more complex composition of bioactive compounds. These bioactive compounds are flavonoids, especially fluorotanin, carotenoids, especially fucoxanthin, and sulfated polysaccharides, such as laminaran, carrageenan, agar, fucoidan, ulvan, caulerpin, sulfate galactans and lectins. All of these compounds can be substances that modulate the function and activity of the immune system. According to Michalak & Chojnacka (2015), seaweed also contains many nutrients such as iron and protein, which can increase appetite, so it is important to consume nutritious food to be able to build antibodies. Manufacturers sell transfer factor formula to allow humans or animals to reactivate the immune system in their bodies. However, to identify the benefits and effects of the transfer factor formula mixed with seaweed, separate research was required. Seaweed and transfer factor formula were mixed in fish feed in different ratios.

A mixture of feed with *Gracilaria* sp. seaweed and transfer factor formula is used as an immunomodulator that can stimulate immune enhancement in *O. niloticus*. According to Pangestuti et al (2021), who performed an analysis of the chemical composition of seaweed, *Caulerpa racemose* and *Ulva lactuca* contain many nutritional substances that have the potential to improve the immune system, such as protein, carbohydrates, fats, and minerals. This phenomenon was also found in seaweed *Gracilaria* sp. The content of macro and micro minerals is needed to support the metabolic system, trigger enzyme activity, and support the digestive system (Biesalski & Jana 2018), while seaweeds have the mentioned antimicrobial and fermentative properties (Pringgenies et al 2020). This study aims to determine if additional seaweed *Gracilaria* sp. and transfer factor formula as additional in *O. niloticus* feed can be an immunostimulant mixture, thereby supporting the fish growth.

Based on the above mentioned reasons, the research objectives were to determine the optimal concentration ratios of the fish feed mixture with *Gracilaria* sp. seaweed extract and transfer factor formula supplement, according to the observed effect on the non-specific immune system as well as on the growth of *O. niloticus*, as a bio activator.

Material and Method

Mixed feed making. The test feed included a mixture of commercial pellets with *Glacilaria* sp. seaweed flour and transfer factor formulas (Djunaedi et al 2021). The feed used in the study was a commercial pellet with a diameter of 0.5–0.7 mm. Pellets were mixed with other ingredients with reference to Djunaedi et al (2021) adjusted to different test combinations of commercial feed, seaweed, and transfer factor formula, according to the experimental design of this study. All ingredients were mixed until homogeneity was reached and turned into a paste. After the dough was flat and homogeneous, it was stored in a tray and flattened thin to make it dry quickly. Then the samples were dried under the sun. Dry samples were ground and stored. Next, the feed was repelled and dried in an oven at 35°C until it was tested.

Test fish. The test fish used in this study were *O. niloticus* at a size of 4-6 cm. 4 specimens were stocked in a container, which was brought from Siwarak, Semarang, Central Java. The test fish were adapted for 7 days in a container filled with water and previously aerated and observed for 42 days. The test fish were placed in a plastic bucket filled with saline water and aerated. The feed was given 2 times a day, *at satiation*.

Experimental design. The research design used was a completely randomized design (CRD) with 5 treatments and 3 repetitions for 35 days. Each treatment was a formulation of Nile tilapia commercial feed mixed with seaweed and transfer factor at the following weight (g) ratios: Treatment A988,8:10:1,2, Treatment B978,8:20:1,2, Treatment

C968,8:30:1,2, Treatment D958,8:40:1,2, Treatment E998.8:0:1.2 (control).

Implementation schedule. *O. niloticus* were fed 2 times a day at 09.00 WIB and 16.00 WIB at satiation. The length and weight were measured every 7 days. Water quality management was done by measuring the water temperature every day, while the pH, salinity, and DO measurements every 7 days. Immunological tests were done after the growth period was in accordance with the target in the study by taking blood samples.

Research variable. The response variables measured were the Total Feed Consumed (TFC), the Feed Conversion Ratio (FCR), the Feed Utilization Efficiency (FUE), the Protein Efficiency Ratio (PER), and the Relative Growth Rate (RGR). Feed consumption is the amount of feed consumed by fish. Consumption is an important aspect to evaluate feed quality. TFC can be calculated by reducing the initial weight of the food (g) with final weight of the food (g). While feed conversion is a measure that can be used to assess the efficiency of feed use by calculating the ratio between the amount of feed consumed and body weight gain in a certain period of time. PER is the ability of a protein to generate growth, measures weight gain per gram of protein consumed and used to determine the effectiveness of the protein used in the metabolism (Bake et al 2014).

The Total Feed Consumed (TFC) was estimated using the following formula (Pereira et al 2007):

$$TFC = F1 - F2$$

Where:
TFC - total feed consumed;
F1 - initial weight of the food (g);
F2 - final weight of the food (g).

The Feed Conversion Rate (FCR) was calculated using the following formula (Prakoso et al 2020):

$$FCR = F / [(Wt + d) - Wo]$$

Where:
F - the amount of feed consumed;
Wt - fish biomass at the end of the study (g);
Wo - fish biomass at the beginning of the study (g);
d - biomass of fish which died during the study (g).

The Feed Utilization Efficiency (FUE) was calculated using the following formula (Tacon 1987):

$$FUE = [(Wt - Wo) / F) \times 100]$$

Where:
FUE – feed utilization efficiency (%);
Wt – total weight at the end of the experiment (g);
Wo – total fish weight at the beginning of the experiment (g);
F - the amount of feed consumed (g).

The Protein Efficiency Ratio (PER) was calculated using the following formula (Bake et al 2014):

$$PER = [(Wt - Wo) / Pi] \times 100$$

Where:
Wt - fish biomass at the end of the study (g);

Wo - fish biomass at the beginning of the study (g);
P - the protein weight of the feed consumed (g).
The Relative Growth Rate (RGR) was calculated using the following formula (De Silva & Anderson 1995):

$$RGR = [(Wt - Wo) / (Wo \times t)] \times 100$$

Where:
Wt - biomass at the end of the study (g);
Wo - biomass at the beginning of the study (g);
t - length of study (days).

Water quality. The water quality parameters observed in this study were physicochemical parameters of water including temperature, pH, salinity, and dissolved oxygen. Measurement of water temperature was carried out every afternoon before feeding, and measurements of pH, salinity and dissolved oxygen were carried out once every 7 days. Observations on the number and diversity of plankton and of the TSS were done in the laboratory.

Results

Total Feed Consumed (TFC). The following are the results of the feed consumption ratio obtained from the five treatments that have been given (Table 1). According to their feed consumption ratio values' decrease, the order of the five treatments was: D (192.45±25.92 g), E (170.60±15.17 g), C (169.27±20.83 g), B (155.56±20.67 g), A (138.55±14.76 g). The results showed that the highest feed consumption ratio was shown in treatment D (feed, seaweed and transfer factor formula in a weight ratio of 958.8:40:1.2), with an average feed consumption ratio of 192.45±25.92 g. Meanwhile, the lowest feed consumption ratio value was found in treatment A (feed, seaweed and transfer factor formula in a weight ratio of 988.8:10:1.2), with an average feed consumption ratio of 138.55±14.76 g. Treatments A, B, and C had an average feed consumption ratio value below the average value determined in treatment E, which was the control treatment.

Table 1

Results of the total feed consumed in the five treatments

Repetition	*Treatment*				
	A	*B*	*C*	*D*	*E (Control)*
1	132.51	169.58	179.70	162.58	186.07
2	127.76	165.28	182.82	205.77	169.97
3	155.37	131.82	145.29	209.00	155.76
∑x	415.64	466.68	507.81	577.35	511.80
Average	138.55	155.56	169.27	192.45	170.60
SD	14.76	20.67	20.83	25.92	15.17

Feed Conversion Ratio (FCR). Results of the study on the impact of adding *Gracilaria* seaweed, based on the transfer factor formula of the FCR can be seen in Table 2. According to the feed conversion ratio values' decrease, the five treatments were: A (49.31±8.20%), B (40.23±5.18%), C (18.03±3.29%), E (11.64±1.24%), and D (11.32±1.61%). The highest FCR value was found in treatment A (feed, seaweed and transfer factor formula in a weight ratio of 988.8:10:1.2), while the lowest value was found in treatment D (feed, seaweed and transfer factor formula in a weight ratio of 958.8:40:1.2). The only treatment that had an FCR rate below the control treatment was treatment D, while the other treatments (treatments A, B, and C) had a higher FCR value than in treatment E.

Table 2

Results of the feed conversion ratio

Repetition	Treatment				
	A	*B*	*C*	*D*	*E*
1	41.15	43.76	21.02	9.46	12.81
2	49.23	42.65	18.57	12.14	11.78
3	57.54	34.28	14.51	12.35	10.34
∑x	147.93	120.70	54.09	33.96	34.92
Average	49.31	40.23	18.03	11.32	11.64
SD	8.20	5.18	3.29	1.61	1.24

Protein Efficiency Ratio (PER). PER value of treatment D, which is 0.224, was the highest compared to the other treatments, while treatment E was lower than treatment D, namely 0.216. This shows that the quality of the protein contained in treatment D is better than treatment E, treatment A, B and C (Table 3).

Table 3

Result of protein efficiency ratio

Repetition	Treatment				
	A	*B*	*C*	*D*	*E*
1	0.061	0.057	0.119	0.264	0.195
2	0.051	0.058	0.135	0.206	0.212
3	0.043	0.073	0.172	0.202	0.242
Σ x	0.155	0.188	0.426	0.672	0.649
Average	0.052	0.063	0.142	0.224	0.216
SD	0.009	0.009	0.027	0.035	0.024

Feed Utilization Efficiency (FUE). Results on the impact of adding seaweed, based on the transfer factor formula on the FUE can be seen in Table 4. According to the feed utilization efficiency ratio values' decrease, the order of the five treatments was: D (8.97±1.39%), E (8.66±0.94%), C (5.68±1.10%), B (2.52±0.35%), and A (2.07±0.35%).

Table 4

Feed utilization efficiency

Repetition	Treatment				
	A	*B*	*C*	*D*	*E*
1	2.43	2.29	4.76	10.57	7.81
2	2.03	2.34	5.39	8.24	8.49
3	1.74	2.92	6.89	8.10	9.68
∑x	6.20	7.55	17.04	26.90	25.97
Average	2.07	2.52	5.68	8.97	8.66
SD	0.35	0.35	1.10	1.39	0.94

The results showed that the highest feed utilization efficiency rate was shown in treatment D (feed seaweed and transfer factor formula in a weight ratio of 958.8:40:1.2), while the lowest value was shown in treatment A (feed, seaweed and transfer factor formula in a weight ratio of 988.8:10:1.2). The efficiency value obtained can be categorized as low because all treatments showed an efficiency result below 50%.

Relative Growth Rate (RGR). The growth rate results of the five treatments are shown in the Table 5. According to their RGR values' decrease, the order of the five treatments was: D (2.29±0.04%), E (1.98±0.06%), C (1.29±0.11%), B (0.50±0.01%), and A (0.37±0.04%). The results showed that the highest relative growth rate was shown in treatment D (feed, seaweed and transfer factor formula in a weight ratio of 958.8:40:1.2), while the lowest value was found in treatment A (feed, seaweed and transfer factor formula in a weight ratio of 988.8:10:1.2).

Table 5

Relative growth rate results

Repetition	*Treatment (RGR)*				
	A	*B*	*C*	*D*	*E*
1	0.42	0.50	1.14	2.33	1.98
2	0.35	0.51	1.32	2.31	1.91
3	0.36	0.50	1.35	2.25	2.04
∑x	1.12	1.51	3.80	6.88	5.93
Average	0.37	0.50	1.27	2.29	1.98
SD	0.04	0.01	0.11	0.04	0.06

The relative growth rates in treatments A, B, and C were lower than those in treatment E, which served as control. Only treatment D had a higher relative growth rate than the control. Results of the weekly relative growth rate of *O. niloticus* can be seen in Table 6.

Table 6

Weekly relative growth rate results

Repetition	*Treatment (RGR)*				
	A	*B*	*C*	*D*	*E (Control)*
1	0.01	0.64	1.45	2.68	2.50
2	0.38	0.65	1.35	2.82	2.39
3	0.5	0.58	1.73	2.55	2.01
∑x	0.88	1.87	4.53	8.05	6.90
Average	0.44	0.62	1.51	2.68	2.30
SD	0.08	0.04	0.20	0.14	0.26

The rate of the weekly relative growth results has the same value as the relative rate for 35 days, which were from the highest to the lowest, respectively D (2.68±0.14%), E (2.30±0.26%), C (1.51±0.20%), B (0.62±0.01%), and A (0.44±0.08%). The results showed that the highest relative growth rate was shown in treatment D (feed, seaweed and transfer factor formula in a weight ratio of 958.8:40:1.2), while the lowest value was indicated by treatment A (feed, seaweed and transfer factor formula in a weight ratio of 988.8:10:1.2). This condition is suspected because the preference for feed has decreased, so that consumption became lower.

Water quality. Water quality during the research period can be seen in the following Table 7. DO values obtained ranged from 4.58-4.70 mg L^{-1}, pH values ranged from 7.02-7.12, salinity was 5 psu, water temperature ranged from 25.85-26.04°C, and the air temperature was 28.3°C. The water quality parameter was considered appropriate for the Nile tilapia cultivation (in a fresh water medium).

Table 7

Water quality parameter value range

Water quality parameter value range	
DO (mg L^{-1})	4.58-4.70
pH	7.02-7.12
Salinity (ppt)	5
Water temperature (°C)	25.85-26.04
Air temperature (°C)	28.3

Discussion. In fish conservancy, the feed given must accommodate the nutritional needs of the fish for its growth and must be adapted to its eating habits and behavior (Subandiyono et al 2018). The feed nutrition consists of protein, fat and carbohydrates, vitamins, and minerals. The balance between energy and protein is very important in determining the value of fish energy retention because if the energy needs are high, the protein will be broken down and used as an energy source for activities instead of. The minimum protein requirement in feed for *O. niloticus* growth is 30% (Siddiqui et al 1988). Feed is one of the important factors that affect the growth and survival of fish to be cultivated. Carbohydrates are a source of energy and generally come from plants. The function of carbohydrates themselves is to meet energy needs and food supplies in the body. The impact of carbohydrates on growth is caused by several factors, which are carbohydrate levels in feed, carbohydrate digestibility, intake of food levels, environmental conditions and species. Carbohydrate requirements for each fish are different. The optimum carbohydrate content in omnivorous fish is 0-50% (Boonanuntanasarn et al 2018).

The test results of feeding combined in several treatments showed that the best results were shown in a mixture of seaweed feed with the highest transfer factor formula. A transfer factor are immune messenger molecules which are active in the cell-mediated immunity, transferring the ability of immunity from the donor to the recipient. According to El-Sayed (2006), the optimal protein content required to support the growth of Nile tilapia ranges 30-40% for juveniles 20-30% for adults; this value will be lower if the maintenance is done in the pond by adding a natural feed, which can also contribute with a certain amount of protein. According to Eid et al (2019), a fat content of 4% in feed is sufficient for *O. niloticus*, but a fat content of 12% in feed will result in maximum development. Vitamins play a role in metabolism. Vitamins act as cofactors for the hydroxylation reactions of amino acids, which enhance the protein efficiency for growth. The correlation of vitamins with the efficiency of fatty acid transport to be oxidized into energy will also support growth. In addition, vitamins also serve as antioxidants in reducing peroxidation of fat. Vitamin C in increases the body's resistance to stress in fish fry, enhancing their survival rate (Herrera et al 2019), which occurred in all treatments: all test animals survived which is correlated with the feed content of seaweed, in particular to its high vitamins and nutrients concentration (Rosemary et al 2019).

Minerals consist of calcium, phosphorus, and iron. Minerals are needed by fish in small amounts and they are also important for other animals. Iron is required for the blood and plays a role in the respiratory function. Calcium and phosphorus play a role in forming the bone structure, teeth, scales, and other metabolic functions. Calcium is an abundant mineral in the body: 1.5-2% of the body weight, with more than 99% found in the bones. Growth is the process of increasing the length, weight and volume of an organism over a certain period of time, caused by tissue changes due to the division of muscle and bone cells which are the largest part of the fish body. Growth is influenced by several factors, which are internal and external factors. Internal factors include heredity, disease resistance, and the ability to utilize food. Meanwhile, external factors include the physical, chemical, and biological characteristics of the waters. All physical, chemical, biological, and ecological factors should be controlled for optimal growth (Sinada & Abdel Karim 1984). The value of PER describes the feed protein utilization rate for fish growth. The interaction between dietary protein level and E/P ratio influenced the efficiency of dietary protein utilization (Nisrinah & Subandiyono 2013).

Fish growth is influenced by several factors including feed, cultivation containers, temperature, salinity, season, and physical activity (Jun et al 2012). As fish are poikilothermal and live in water, they are greatly influenced by the cultivation media. Changes in media conditions, such as salinity, will affect the body of the fish. Nile tilapia cultivation in different salinity conditions (from fresh to brackish water ponds) will affect the ratio of feed consumption and growth. *O. niloticus* grow well in saline media, as well as in freshwater with a salinity level of less than 19‰, so *O. niloticus* cultivated up to 15‰ salinity can still grow well in their natural habitat, because salinity will increase the feed consumption in order to maintain the osmoregulation process in the fish. Salinity increase plays a role in the utilization of feed energy because more protein is stored and only a little is broken down or used for energy in maintaining the body's alkali balance (Gan et al 2016). Salinity that is too high can affect growth, which is observed in the treatments, due to its effect on the metabolism, through alterations of the Cl^-, Na^+ and K^+ -ATPases activity in the gill epithelial cells. Changes in metabolic processes absorb the energy that should be used for (Aboseif et al 2022). This causes fish growth to be sub-optimal.

The survival rate is influenced by internal factors including sex, heredity, age, reproduction, disease resistance, and external factors including water quality, stocking density, number, and composition of complete amino acids in feed. In addition, factors that affect survival are biotic and abiotic. Biotic factors are competitors, parasites, age, predation, population density, adaptability of animals, and human handling. Meanwhile, the abiotic factors are the physical and chemical characteristics of an aquatic environment (Aboseif et al 2022). However, the immune system is very helpful in the process of growth and survival of fish which has been proven by the feeding test results using a mixture with seaweed and transfer factor formula.

The results showed that *Gracilaria* sp. and transfer factor formula supplementation had an effect on the immune system in the cultured Nile tilapia. Results of the feed consumption ratio, FCR, RGR, and FUE tests were different for each treatment given. Treatment D gave the best results. *Glacilaria* sp. seaweed is known as a highly nutritious food (Francavilla et al 2013). This can be seen in some information about the seaweed *Glacilaria* sp. The food supplementation with *Gracilaria* sp. methanol extract is indicated to have an effect on the innate immune system and skin color in the test specimens (Peixoto et al 2019; Fajardo et al 2022).

Conclusions. The addition of a mixture of *Gracilaria* sp. and transfer factor formula supplements in feed stimulates the increase of non-specific immunity in *O. niloticus*, characterized by the varying effect of feed concentration on FCR, RGR, FUE, and feed consumption ratio values. The optimal concentration in the feed mixture of *Gracilaria* sp. and transfer factor formula supplementation was found in the treatment D (*O. niloticus* feed mixed with seaweed and transfer factor formula with a weight ratio of 958.8:40:1.2), demonstrated by a feed consumption ratio value of 192.45±25.92 g, a FCR value of 11.32±1.61%, a RGR value of 2.29±0.04%, and a FUE value of 8.97±1.39%. The mixture of fish feed with *Gracilaria* sp. and transfer factor formula supplements has a potential as a bio-activator for Nile tilapia growth because it can reduce the FCR value of the feed and increase the growth rate.

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