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EFFECT OF DIETARY MARINE YEAST AND *SACCHAROMYCES CEREVISIAE* ON MICROBIAL COMPOSITION AND IMMUNE RESPONSE OF *CYPRINUS CARPIO*

Sumbal[1], Shakeela Parveen [1*], Fayyaz Rasool[2], Amina Ayub[3], Muhammad Ahmad[1], Arooj[1], Sadia Parveen[1], Orba[1], Talib Hussain[4]

[1*]Department of Zoology, Wildlife and Fisheries, University of Agriculture Faisalabad, Pakistan
[2]Department of Zoology, Faisalabad Campus, University of Education Lahore, Pakistan
[3]Department of Zoology, Wildlife and Fisheries, Depalpur Okara Campus, University of Agriculture Faisalabad, Pakistan
[4]Department of Zoology, Government College University, Lahore, Pakistan

[*]**Corresponding Author:** Shakeela Parveen
*email: drshakeela.fayyaz@uaf.edu.pk

ABSTRACT
This research was conducted to investigate the effect of dietary marine yeast (DMY) and *Saccharomyces cerevisiae* on microbial composition and immune response of Common Carp (*Cyprinus carpio*). Using natural products like dietary marine yeast was beneficial for Common Carp's immune system and improved microbial community health. In Carp's, *S. cerevisiae* served as a beneficial replacement for the usage of antibiotics and vaccinations. For this experiment, 60 fingerlings were reared under semi-intensive culture condition using earthen ponds and three (T_0, T_1 and T_2) experimental groups were made for this research for 90 days. T_0 was fed with commercial fish feed and considered to be control group while T_1 was fed with dietary marine yeast and T_2 was treated with Baker's yeast to check immune response and microbiota composition. To identify the effect on micro-biotic composition in fish samples were taken from Common Carp intestine, gills and analyses were done in microbiology lab on different culture media like nutrient agar and tryptic soy agar. The statistical design of this experiment was ANOVA and p-value was statistically indicated that the results was observed for intestine, gills microbial composition and immune response highly significant; ($0.0006<0.05$) and ($0.000912<0.05$) indicating that treatment difference was highly significant at ($p<0.05$). Also, Tukey test was used to compare the treatment means, T_0, T_1 and T_2 and the results of all these values were also statistically significant. These results highlight the potential benefits of yeast based diet as a nutritional supplement is might be highly suitable for intestinal health and animal welfare of omnivorous fishes because they contain nucleotide-rich and β-glucan diet which show efficiency in aquaculture systems.

Key words: Aquaculture, Common Carp, dietary marine yeast, Baker's yeast, Micro-biota, Immune response

Introduction
Aquaculture contributes to the economy of many households with an approximation of over hundred million people depending on aquaculture for living (Kobayashi et al., 2015). The development of

aquaculture sector will reduce pressure on the slowly depleting capture fisheries and assist in meeting the expanding demand for fish (Mustapha et al., 2021). Aquaculture represent the fastest-growing animal based primary food producing sectors with 64.6 million tons (MT) production and 8.8% annual growth rate in 2012. The contribution from crustacean aquaculture is 5.7 MT (Gephart et al., 2020). For making feeds, aquaculture sector alone consumed the equivalent of about 23.8 million metric tons (mmt) of fish or 87% of non-food fish (Ruzauskas et al., 2021).

Nutritionally, fish is one of the most affordable and direct sources of protein and micronutrients for millions worldwide, despite the steady decline in capture fisheries (Pauly et al., 2017). Fisheries and aquaculture are essential tools for providing fish consumed globally. Unlike some other animal products, fish is widely accepted across various social, cultural, and religious backgrounds (Thilsted et al., 2016). To maintain current levels of fish consumption, which range from 5 to 45 kg per person per year depending on the country, fish supplies must significantly increase (Egwui et al., 2013).

Native to Asia and Europe, Common Carp *(Cyprinus carpio*) is right now becoming the most widely cultivated species around the world; especially in Pakistan (Khan et al., 2016). Its nature is omnivorous. It also provides us with a lot of high quality protein content for consumption (Xu et al., 2014). The third-most used to cultivate and significant for economic activity among freshwater species is the *C. carpio*. It has a tendency of adjusting its behavior and feeding niche to the surrounding conditions. Its demand in the market is extremely strong due to its delectable flavor and growing consumer acceptance (Rahman, 2015). Over 22 million metric tons Carp (*cyprinids*) contribute to fish production worldwide and account for approximately 46% of total global aquaculture production and 72% of total freshwater aquatic growth (Sorensen, 2015).

Immunostimulants, such as Baker's yeast and dietary marine yeast, offer a promising and convenient alternative to antibiotics for enhancing disease resistance in aquatic animals (Ruzauskas et al., 2021). The protective effects of yeast on *C. carpio* have been extensively validated (Abdelhamid et al., 2020). The rise of high-density, large-scale aquaculture and increasing stress factors, aquatic animals are becoming more susceptible to diseases (Mes et al., 2023). Traditionally, antibiotics have been widely used to fight bacterial infections in aquaculture (Miranda et al., 2018).

Dietary marine yeast is a non-pathogenic, aerobic and dimorphic yeast separated from marine environments that grows better on a medium constructed by seawater compared to freshwater (Li et al., 2020). It contains high amounts of fatty acids. It has been utilized in aquaculture as a dietary supplement (Mes et al., 2023) On the other hand fish diets boosted immune responses and enhanced microbial health (Jain et al., 2020). Using natural products like dietary marine yeast *C. carpio*'s immune system and microbial community health can be improved. Fish immune systems have been successfully boosted against viral or bacterial infections using yeast (Sanahuja et al., 2023).

Among yeast species, Baker's yeast (*Saccharomyces cerevisiae*) is a prominent. Since ancient times, the species has been extensively used for baking and brewing. It was initially isolated from grape skin (Segner et al., 2012). One of the greatest and most well-liked probiotics is *S. cerevisiae*. By generating some energy for intestinal cells from the yeast cell in aquaculture, it helps to improve gut health (El-Bab et al., 2022). Additionally, they function as immunostimulants. Carp's immunity and macro-biotic composition have been improved by Baker's yeast supplementation orally (Lulijwa et al., 2020). In Carp farming, it serves as a beneficial replacement for the usage of antibiotics and vaccinations (Jain et al., 2022).

Materials and Methods

Study area and duration

The present research was accomplished at Fish Microbiology Laboratory, located at Fish Farm, Department of Zoology, Wildlife life and Fisheries, University of Agriculture, Faisalabad. The experiment was conducted for a period of six month from 1st October to 31 March in three replicates of earthen experimental ponds designated as control, treatment-T_1 and Treatment-T_2.

Investigational Species

Cyprinus carpio is one of the most well-known cyprinid specie and third most widely cultivated and economically significant specie of freshwater fish in the world.

Collection of Experimental Animals

During netting, *C. carpio* of different sizes (2-20g) was taken by netting from freshwater earthen ponds of University of Agriculture, Faisalabad. Fishes of different sizes were found of which required samples were taken. Total 60 fish species were taken, 40 for the experimental group and the other 20 for control group. Fingerlings of *C. carpio* were collected from the research pond and acclimated for 24 hours in lab settings in glass aquaria.

Transfer of Fish in Aquariums

The aquariums were thoroughly rinsed with running water and positioned in their assigned spots. Carefully, fifteen acclimated fish of each species were scooped out from the raceway and placed into the respective aquariums.

Experiment design

For the experiment, three tanks were utilized, with one tank as the control group (consisting of *C. carpio* fed commercial food) and the other two as the experimental groups. The experimental groups were fed on a commercial diet supplemented with Baker's yeast and dietary marine yeast.

Feed Application

Commercial feed was given to the control group, while probiotic dietary marine yeast and Baker's yeast were considered experimental diets for the experimental groups. Initially, fish responded less to the feed due to external low temperature and unfavorable conditions, but they later adjusted to it as shown by the fishes' behavior.

Preparation of Microbiological Media

To prepare culture media, distilled water was used and flask contain 28g agar and instruments wrapped in foil paper sterilized in an autoclave for 20 minutes before being cleaned and dried properly to reduce the risk of contamination and chemical reactions.

For culture the bacterial colonies, different media like, Tryptic soy agar (TSA) and Nutrient Agar (NA) used as culture media. Bacterial viable count was obtained using these media.

Nutrient Agar Media Colony

After 24 hours of incubation, the media showed of bacterial colonies of bright yellow transparent color to slightly opalescent gel like *Lactobacillus* spp., *Bifidobacterium*, *Bacillus* spp., was grown on nutrient agar media incubated in petri plates.

Tryptic Soy agar (TSA) Media Colonies

After 18-24 hours of brooding at 30-35 Celsius, cultural characteristics were observed. *Enterococcus, Streptococcus, Bacillus subtilis* were among the bacteria that grew on Tryptic soy agar.

Sample Preparation

The fishes from control and experimental groups were dissected for the preparation of samples. An Eppendorf tube was filled with 0.9% saline solution (9mg of NaCl dissolved in 100ml H2O). Dissected samples of intestine and gills by using sterilized apparatus were placed it in those tubes. Shake well to homogenized the sample solution and labelled accordingly as C for controlled and E for experimental.

Gram Staining

To analyze the characteristic of bacterial species like shape, colony formation, morphology, gram staining technique was done. Bacterial cell's shapes (bacilli, helical, filamentous), arrangements (chains, groups), and other characteristics could be observed under a compound microscope after labeling.

Microscopic Examination

The colonies on agar media after incubation period were observed via naked eye. Microscopically, thin single-celled round and rod-shaped bacteria with curved, convex, opaque and rough populations were discovered.

Immunological Parameters

Microbial Count

A tissue sample was taken by section cut from the intestine of the *C. carpio* then it was kept in 1ml saline solution in an eppendorf tube. Inoculating loops or needles were red hot on the flame and these were used to homogenize the sample. Nutrient agar was added in the petri plates and this was used to grow microbial colonies. Petri plates were wrapped in aluminum foil and placed in Incubator for 24 hours. Then, microbial count was done with the help of colony counter. So the microbial count was done to check the immunological parameters.

White Blood Cells Sample

At the end of trial period, blood sample was obtained from caudal vein of fish via a syringe with ethylene diamine tetra-acetic acid (EDTA) as an anti-coagulant from each sample. Numbers of white blood cells (WBCs) were calculated by using the haemocytometer**.**

The blood samples were collected from fish subjected to euthanasia. Blood samples were obtained from the fish's caudal vein and gill rakers and then they were stored in an antiseptic centrifuge tube at the end of the experiment using a clean syringe.

Statistical Analyses

Statistical analysis was conducted to compare the quantitative results of bacterial population that are present in the intestine of the *C. carpio* by using one-way analysis of variance. Quantitative results obtained from treatments were compared with each other with the help of a single factor (ANOVA) under CRD and Tukey test (Mean Comparison test).

Results

To identify the effect on micro-biotic composition in fish samples were taken from Common Carp intestine, gills and analyses were done in microbiology lab on different culture media like nutrient agar and tryptic soy agar.

Note;

One-way analysis of variance (ANOVA) under CRD and Tukey test for the comparison of total viable count of intestine and gills samples of Common Carp on Nutrient agar media (NA) and Tryptic soy agar (TSA) is shown in table below significant because the value of $P<0.05$ is greater than the values obtained and the difference in the values are shown in the following tables.

* = significant

**= Highly significant

ns = non-significant

Table: 1.1 Analysis of variance (ANOVA) for total microbial count of control (T_0) under experimental groups (T_1 and T_2) from Intestine of Common Carp on NA and TSA media.

Intestinal Microbial Count Data				
Agar	**T_0**	**T_1**	**T_2**	**p-value**
Nutrient agar (NA)	119.8 ± 22.68^{c}	185.1 ± 27.25^{b}	250.3 ± 43.47^{a}	0.000912**
Tryptic soy agar (TSA)	149.5 ± 40.33^{c}	194.1 ± 21.25^{b}	239.1 ± 41.54^{a}	0.0006**

Table: 1.2 Analysis of variance (ANOVA) for total microbial count of control (T_0) under experimental groups (T_1 and T_2) from gills of Common Carp on NA and TSA media.

Gills Microbial Count Data				
Agar	**T_0**	**T_1**	**T_2**	**p-value**
Nutrient agar (NA)	112.2 ± 28.45^{c}	166.2 ± 32.57^{b}	149.5 ± 40.33^{a}	0.000687**
Tryptic soy agar (TSA)	149.5 ± 40.33^{c}	194.1 ± 23.50^{b}	249.4 ± 45.21^{a}	0.0006**

Table: 1.3 Analysis of variance (ANOVA) for total microbial count of control (T_0) under experimental groups (T_1 and T_2) from Intestine of Common Carp on NA media.

Immunological parameters	**T_0**	**T_1**	**T_2**	**P-value**
White blood cells	25.812 ± 3.146^{c}	36.196 ± 4.806^{b}	50.648 ± 6.522^{a}	0.000549**
Neutrophils	22.6 ± 1.75^{c}	33.52 ± 5.91^{b}	47.8 ± 4.52^{a}	0.000136**
Lymphocytes	24.4 ± 3.46^{c}	32.72 ± 4.58^{b}	48.4 ± 5.96^{a}	0.000043**
Monocytes	4.4 ± 0.888^{c}	7.36 ± 1.20^{b}	10.32 ± 1.454^{a}	0.0000617**
Eosinophils	2.38 ± 0.172^{c}	3.52 ± 0.319^{b}	5.28 ± 0.682^{a}	0.00034**

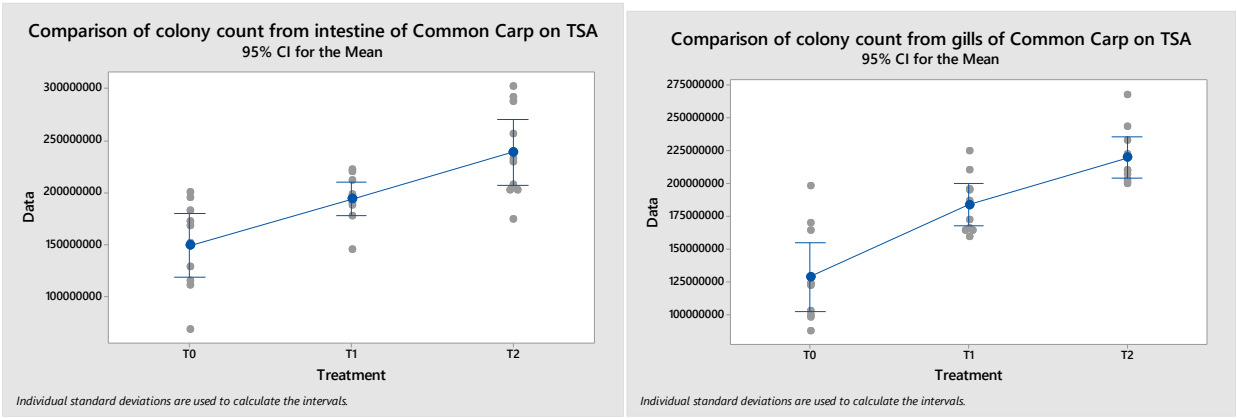

Fig: 1.2 Graphical presentation of comparison of colony count from intestine and gills of Common Carp on TSA.

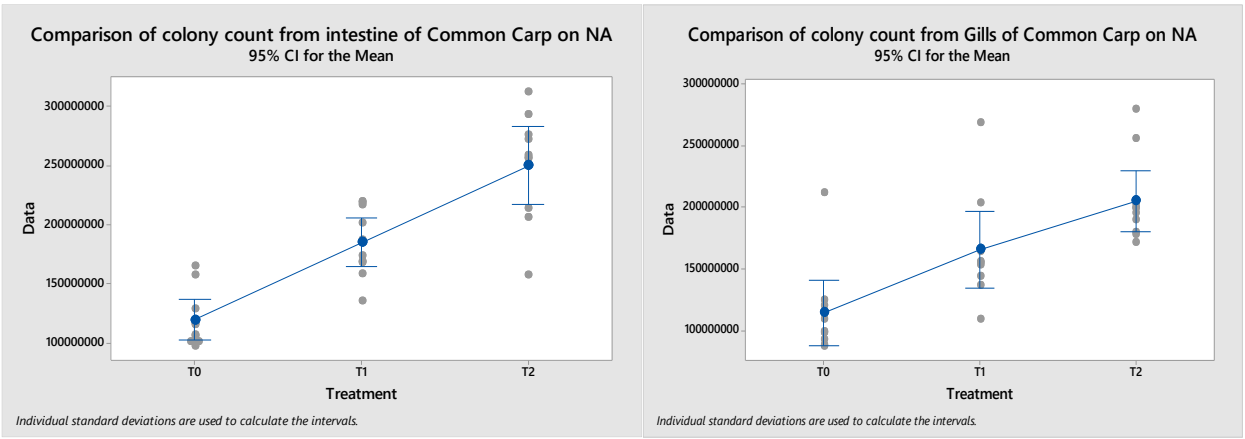

Fig: 1.1Graphical presentation of comparison of colony count from intestine and gills of Common Carp on NA.

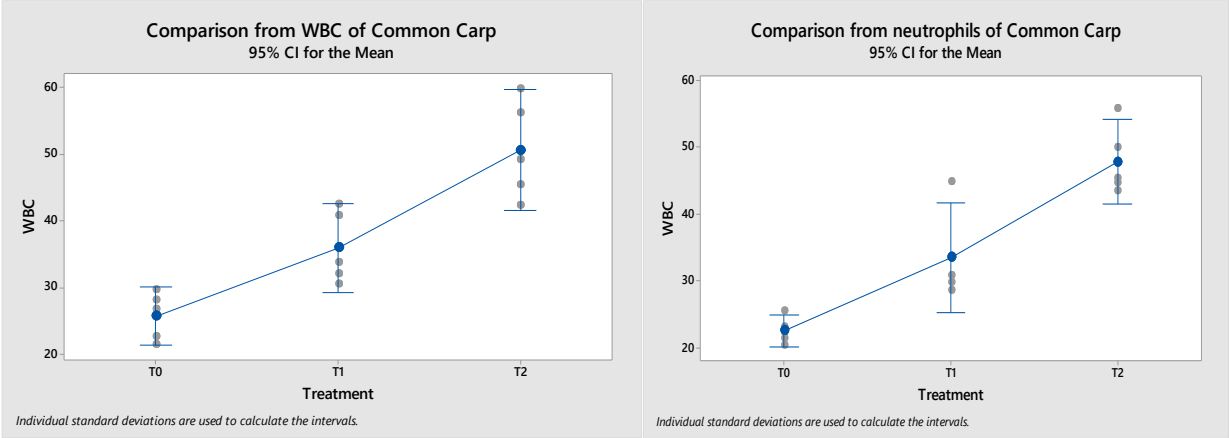

Fig: 4.13 Graphical presentation of comparison from WBC and Neutrophils of Common Carp.

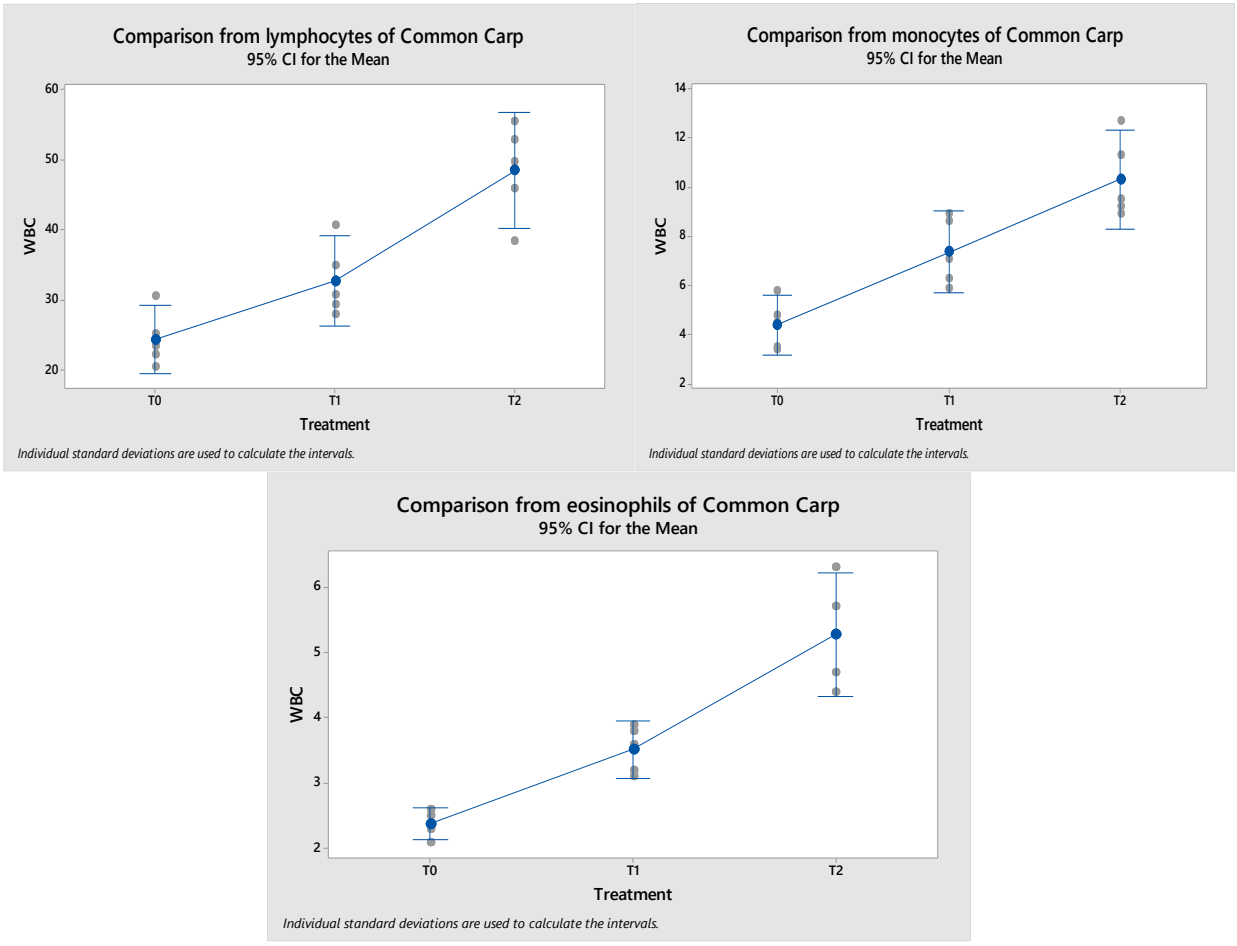

Fig: 1.3 Graphical presentation of comparison from Lymphocytes, Monocytes and Eosinophils of Common Carp.

Discussion

Baker's yeast (*Saccharomyces cerevisiae*) is abundant in immune-stimulating compounds like β-glucans, nucleotides, oligosaccharides, selenium and various other microbial nutrients, including vitamins, enzymes and amino acids. These elements have been shown to positively affect the microbiota and immune responses of *C. carpio*. These results align with the findings of Licona-Jain et al. (2022) in *C. carpio* fingerlings.

Dietary marine yeast is non-pathogenic, aerobic and dimorphic yeast separated from marine environments that grow better on a medium constructed by seawater compared to freshwater.

Considering of its composition in significant fatty acids. It has been utilized in aquaculture as a dietary supplement. On the other hand, fish diets boosted immune responses and enhanced health. Using natural products like dietary marine yeast Carp's immune system and microbial community health can be improved. Fish immune systems have been successfully boosted against viral or bacterial infections using yeast. These findings were in similar with the outcomes of Zheng et al. (2017) in fingerlings of *C. carpio*.

Yeast-based supplementation increased the fraction of *Bacillus* spp., while decreasing Vibrio spp., levels in *C. carpio*. Yousefi et al. (2020) reported a similar observation with Silver Carp fingerlings. It is worth noting that the usage of Baker's yeast supplements resulted in a considerable improvement in healthy gut bacteria growth performance. The use of dietary marine yeast improved intestinal health and boosted the immunological response in *C. carpio*. Our findings also revealed that the microbiota had a favorable impact on gut and gill health. These findings are similar with those of Hoseinifar et al. (2015) in Rohu fingerlings.

In accordance to our findings, using probiotics or dietary marine yeast and Baker's yeast diets to modify the microbiota and prevent pathogen colonization also improves animal health. According to our findings, the presence of yeast *Saccharomyces cerevisiae* in diet confirms that fish intestine inhibits dangerous microbes and promotes the growth of good bacteria. It should be noted that for *Lactobacillus spp*., was present in all intestinal samples, independent of yeast administration. Opiyo et al. (2019) reported a similar discovery in Rohu fingerlings. However, *Lactobacilli* have also been identified as part of the natural gut flora yeast. Zhou et al. (2022) reported similar findings in Grass Carp.

It was discovered that fish given Baker's yeast diets had a greater quantity and variety of bacteria in their guts than those provided nutritional marine yeast diets. The intestinal micro-biota guards against infection's and actively exchange regulatory signals with host to stimulate mucosal immunity. A recent study on *C. carpio* found that a microbial addition improved the impact of reducing intestinal inflammation and establishing intestinal homeostasis (Abdel-Tawwab et al., 2021).

Unexpectedly, when dietary marine yeast and Baker's yeast were considered, *C. carpio* fed yeast-supplemented diets exhibited a stronger immune response. In addition to their well-known involvement in reverse cholesterol transport and lipid metabolism, apolipoproteins have anti-inflammatory, antibacterial and antioxidant properties. Similar findings were reported by Reyes-Becerril et al. (2021) who found that immune response in *C. carpio* is not regulated by dietary protein levels, and our data suggest that the use of different yeast sources, such as dietary marine yeast and Baker's yeast, may affect immunity and microbiome composition. Carballo et al. (2019) reported a similar discovery in Nile Tilapia fingerlings. Some probiotics have been shown to enhance fish's natural complement function.

Monitoring white blood cell parameters in fish can be useful in aquaculture settings for assessing fish population health, diagnosing illnesses or infections and evaluating the success of management and disease prevention efforts. At the end of experiment, the activity of white blood cells(WBC) was detected. In the *C. carpio* control group (T_0), white blood cell count was 29.72 ×103µL. The Baker's yeast (T_1) experimental group had a value of 42.50×103µL. The value for the dietary marine yeast (T_2) experimental group was 59.78×103µL, compared to the treatment group. These findings are statistically significant. These results were identical to the outcomes of Sanahuja et al. (2023) in *C. carpio* and Nile Tilapia fingerlings.

Conclusion

These results highlight the intestinal micro-biota and immune response of *C. carpio* was influenced by dietary marine yeast and Baker's yeast. The results suggest that a yeast-based diet can enhance immune function of the omnivorous carp's intestine health. This implies that a yeast-supplemented diet, rich in nucleotides and β-glucans, may be highly beneficial for the intestinal health and overall health of omnivorous fish.

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