



## ROLE OF SERICITE IN MODULATING GUT MICROBES AND HAEMATOLOGICAL PROFILES OF SILVER CARP

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### ABSTRACT

Researchers explored sericite's impact on Silver carp's health and growth, conducting a feeding trial with 2.5% and 5% sericite levels. The study examined intestinal tissues and gut microbiota, revealing reduced microorganisms, especially *E.coli* and Enterobacteriaceae. Sericite may possess antibacterial properties or promote healthier gut microbiota. Hematological parameters improved with sericite inclusion, including increased Hb, Htc, and RBCs, enhancing oxygen-carrying capacity and blood health. Immune responses also strengthened, with elevated WBCs, monocytes, and lymphocytes. These findings suggest sericite's potential benefits in sustainable aquaculture, reducing disease susceptibility and promoting fish well-being. Overall, sericite shows promise for enhancing Silver carp's health and well-being, making it a valuable additive for fish feed and a potential tool for promoting sustainable aquaculture practices.

## INTRODUCTION

Fish, crustaceans, aquatic plants, and molluscs can all be raised through a practice known as aquaculture. The most affordable and highly nutritious protein sources are aquatic animals, which are also great food supplements for poor people by supplying vital vitamins, proteins, micronutrients, and minerals (Pradeepkiran, 2019). According to the Global Aquaculture Alliance, by 2030, aquaculture will produce 62% of the fish used as food. These five nations collectively provided 82.2% of the global output in 2016 (Galappaththi *et al.*, 2020).

Fish meal contains a rich source of many trace elements and essential minerals as compared to animal meat (including Iron, Magnesium, Phosphorus, Calcium, Potassium, Copper, Zinc, Sodium, Trivalent Chromium, Iodine, Selenium, and Fluorine) as well as contain many indispensable vitamins (Vitamin A, Vitamin B12, choline, folic acid, etc.) (Tacon, 2022). In addition, several substances with medical and commercial value have been found in aquatic environments, making them an essential resource in and of themselves (Tahseen, 2022).

One of the Carp species, the Silver carp (*Hypophthalmichthys molitrix*), has a more significant potential for aquaculture in underdeveloped nations due to its faster development rate and less expensive feeding costs than other fish species. Many countries worldwide have imported Silver carp for aquaculture and biological control of algae blooms (Behera *et al.*, 2018). A benthopelagic fish, or fish that swims near the water's surface, this species is well-known for its behavior of leaping out of the water when disturbed. They graze on phytoplankton, zooplankton, and detritus, but phytoplankton makes up the bulk of their diet (Ahmed *et al.*, 2019).

Silver carp production is expanding yearly, but there is still a restriction on how much of this fish can be processed. The breeding of

Silver carp spread to numerous ponds near farmland (Hedayati and Hassan Nataj Niazie, 2015). Due to differences in the physiology of various portions of the digestive system, the variety of bacteria differs across different areas of the intestine (Khurana *et al.*, 2021). The microbiota impacts the host's growth, digestion, nutrition, resistance to disease and immunity. In fish, the gut microbiota enhances their immune system and protects them from diseases (Wang *et al.*, 2015). Silver carp productivity and health depend on gut microbiota understanding. *Bacillus*, *Acinetobacter*, *Aeromonas*, and *Microbacterium*, which release extracellular enzymes for digestion, have been found in the Silver carp gut microbiota using standard culture-dependent and advanced molecular approaches. Most gut microbiota microorganisms cannot be grown, so these groups make up a minor amount in the gut of Silver carp (Luo *et al.*, 2017).

Comparatively, *Pseudomonas*, *Bacteroides* type A, and *Aeromonas* predominate in freshwater fish species' intestinal microbiota. In contrast, *Micrococcus*, *Enterobacteriaceae*, *Plesiomonas*, *Clostridium*, *Bacteroides* type B, *Fusarium*, and *Acinetobacter* are much less common (Wang *et al.*, 2018). For decades, scientists in the lab and field have taken blood samples from fish to evaluate their hormonal, reproductive, immune, nutritional, and health status and for use in genetic research. Nonetheless, shockingly, hematological studies employing biological techniques/tools or even more advanced PCR methodologies need to be addressed in fish physiology (Seibel *et al.*, 2021).

Hematological indicators can therefore offer crucial insights into the current health status of fish. For sustainable aquaculture, fish health and growth conditions must be perfectly balanced. Artificial feed that falls short of all essential nutrients affects growth and feed efficiency and causes pathological alterations and other deficiency symptoms in

fish (Ayoola, 2016). Many studies have examined the advantages of several aqua feed additives, such as organic acids, nucleotides, synbiotic, and clay minerals, to enhance fish health (Na-Phatthalung *et al.*, 2018; Hassaan *et al.*, 2020). One of these strategies involves adding environmentally-safe inert clays to fishmeal to increase productivity (Ghasemi *et al.*, 2018). The fundamental distinction between mica and illite is that illite has more water and silica, while micas include more significant amounts of potassium as interlayer cations. When heated, sericite clays transform into a highly reactive glassy phase. Their ideal firing temperature range is extensive, and they can produce mullite-rich ceramic bodies at temperatures as low as about 1000 °C (Gonzalez-Miranda *et al.*, 2018).

## **MATERIALS AND METHODS**

The research was carried out to assess the impact of dietary sericite on the gut microbiota and haematological parameters of Silver carp. The study was conducted for two months at the Department of Zoology, Wildlife, and Fisheries, University of Agriculture, Faisalabad.

### **Pre-stock Management**

Warm water was used to clean the aquarium. Water chemistry was monitored regularly using a test kit, and the fish were added slowly after several weeks of cycling and ensuring stable water chemistry. The aquarium was periodically maintained by performing water changes, cleaning the filtration system, and removing any uneaten food or debris from the tank. Environmental adjustments were made to ensure the fish thrived in the experimental aquarium.

### **Experimental Design**

The fish were randomly distributed into two treatment groups, the control group and the experimental group. The control group was fed only commercial feed, while the experimental groups were fed 2.5% and 5% sericite-supplemented commercial feed.

The feeding trial lasted for eight weeks. The final length and weight of the fish were noted.

### **Treatments**

Two treatments were considered: T<sub>0</sub> Control group with commercial feed. T<sub>1</sub> Treatment group with 2.5g sericite as supplementary feed. T<sub>2</sub> Treatment group with 5g sericite as supplementary feed.

### **Fish and Feed Management**

Healthy Silver carp with an average weight of 90-110g were obtained from freshwater earthen ponds of the University of Agriculture, Faisalabad, and acclimatized for 12 days. During the trial, the fish were kept in 120-litre aquaria with three replicates at a density of 10 fish per aquarium. Water was continuously aerated and maintained at 25 ± 1°C, pH 7.5 ± 0.5, and dissolved oxygen levels of 5-7 mg/L. The fish were fed twice a day with commercial feed and sericite-supplemented commercial feed.

### **Sericite Preparation**

Sericite obtained from a local source in Pakistan was analyzed, autoclaved at 121°C for 20 minutes, and added to commercial feed at a final concentration of 3%.

### **Apparatus and Chemicals**

The following apparatus were required for microbiological research work. Glass slides, glass rod, spreader, cover slip, oven, distilled water, 100ml flask, matchbox, cotton plugs, NA (nutrient agar) media, ethanol, measuring cylinders, Eppendorf tubes, saline solution, micropipette tips of 100-1000µm, pH meter, DO (dissolved oxygen) and salinity meter, syringes, hematocytometer, centrifuge tube.

### **Decontamination**

All the apparatus, working place, and sampling bags were decontaminated for microbiological work. The surface was cleaned with distilled water and then disinfected with 70% ethanol.

### **Sterilization**

The process of killing microbial life in a clean environment is sterilization. Sterilization was done through moist or dry sterilization.

Culturing media and all glassware were kept in an autoclave at 200lb, 121°C for 15-20 minutes, wrapped in aluminum foil during moist sterilization. The inoculation needles were sterilized on a red-hot flame. The equipment was kept in the oven at 7°C for 120 minutes in dry sterilization.

### Collecting Samples

Ten samples of Silver carp were collected in clear and sterilized polythene bags, immediately transported to the laboratory, and refrigerated for further analysis. The sample collection was done with complete care and safety precautions. These samples were dissected to collect the gut of the sample fish.

### Preparation of Gut Microbial Media

A solution of culture media was used to prepare culture media and kept in an autoclave at 15°C for around 2 hours after making a solution with distilled water. The chemicals used in the laboratory were of high-quality measures and taken in average values. All the materials were clean-dried to avoid any contamination. Bacterial identification in the gut of fishes was made using media like Eosin Methylene Blue (EDTA), Nutrient Agar, Tryptic Soy Agar, and RCV-sucrose. These media can be used to determine the total viable bacterial count.

### Nutrient agar

Nutrient agar can support the successful cultivation of various types of bacteria. It contains essential minerals and nutrients that are necessary for the growth of most bacteria, fungi, and yeasts. Agar is incorporated into the medium as a solidifying agent, allowing it to be poured onto Petri plates, creating a suitable surface for microbial growth. The pH of the nutrient agar is approximately 7.0.

### Composition of Nutrient Agar

Peptone	5.0 g/l
Yeast	2.0 g/l,
Sodium Chloride	5.0 g/l
Agar	15.0 g/l
Lemon Powder	1.0 g/l
Distilled Water	1000 ml

### Preparation of Nutrient Agar

Dissolve 18.75g of agar in distilled water and adjust the volume to 250 ml. Thoroughly mix the solution. Cover the top part of the flask. Place the flask in an autoclave at a partial pressure of 15 and a temperature of 121°C for 20 minutes. After removing the flask from the autoclave, mix it well and pour the solution into Petri dishes, allowing it to solidify undisturbed. The Petri plates will exhibit a creamy to yellow-coloured media.

### Culturing of Bacteria

In a separate petri dish, samples were taken from the fish's gut. A solid gel-like consistency of culture media was attained by growing 20ml of culture media in Petri dishes, then leaving it for some time. Dissection of the fish gut was done using a sterilized apparatus, and the sample taken was transferred to a 9% saline solution in a flask. Shake well with an electric shaker. Serial dilutions were made, and ten microliters of suspension were placed on culture media (solidified) and inoculated in an incubator at 37°C. After incubation, the bacterial colonies were seen after about a one- or two-day period studied visually.

For the identification/characterization of the staining property of gut microbes, gram staining was performed. An average of three was considered an accurate count in standard lab analysis. So, three Petri dishes were prepared with agar media spreading uniformly in Petri plates using a sterilized rod. This formula can measure the total bacterial count: Total viable count = Average number of colonies × Dilution factor

### Microbial Count

The petri dish was coated with nutrient agar, and sterile rods or loops were used to disperse the liquid culture material evenly. We used a method that involved preparing three separate Petri dishes and averaging the results to get a reliable bacterial count. Every petri dish was inverted within the incubator to avoid moisture buildup. The

total number of live bacteria in each sample was then determined.

### **Gram Staining**

Dye is applied to microorganisms in a procedure called staining. Dye compounds were formerly utilized as stains. *Salmonella spp* Gram-negative rods were detected in the nutrition ager. A purple stain was used for Gram staining, revealing rod-shaped, gram-negative bacteria by making them appear pink or red.

### **Slide Preparation**

After sterilizing the culture plates, a loop full of culture was transferred to the slide. The colonies were selected using a platinum loop sterilized in a flame. The platinum loop was chilled when collecting the culture to prevent it from overheating and gently dispersing it. After the samples were smeared, they were dried in the air and fixed by heat by passing the slides over a flame; however, the slides were not heated for too long, as this could kill the bacteria. Gram staining was performed on the heat-fixed bacterial smear to identify cell shape and whether the bacteria were gram-native or gram-positive. After examining under the light microscope, we could see *Escherichia. Coli* and Enterobacteriaceae, a large family of gram-negative bacteria.

### **Gram Staining Principle**

After sterilizing the culture plates, a loop full of culture was transferred to the slide. The colonies were selected using a platinum loop sterilized in a flame. The platinum loop was chilled when collecting the culture to prevent it from overheating and gently dispersing it. After the samples were smeared, they were dried in the air and fixed by heat by passing the slides over a flame; however, the slides were not heated for too long, as this could kill the bacteria. Gram staining was performed on the heat-fixed bacterial smear to identify cell shape and whether the bacteria were gram-native or gram-positive.

### **Gram Staining Protocol**

For 60 seconds, crystal violet was used to rinse the glass slide retaining the heat-fixed smear thoroughly. The slides were cleaned to get rid of any excess discoloration. Drops of iodine were left on the slides for a full minute. The slides were cleaned again to remove any residual color from the marks. The slides were discoloured by briefly exposing them to acid alcohol. Due to the potential for misleading outcomes from overly aggressive decolourization, the slides were cleaned as soon as possible. Safranin counter stain was used to rinse the slid glass for 60 seconds. Bacterial morphology and staining features were studied by observing air-dried slides under a light microscope.

### **Blood Collection**

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 stored in an antiseptic centrifuge tube at the end of the experiment using a clean syringe.

### **Hematological Parameters**

Total red blood cell (RBC) and white blood cell (WBCs) counts were measured with a hematocytometer. The sample taken was divided into two parts; the first was with 10% EDTA (ethylenediaminetetraacetate) to determine various haematological indices like Hb (haemoglobin), Htc (hematocrit), RBCs, and WBCs. 100ml of the blood sample was incubated at 37°C in micro flat-bottomed plates for about 60 minutes to make the cell adhesion easier. After that, the saline buffer was used for washing. Washing was done three times to get an accurate count.

The smears were stained to determine each cell count (differential staining of leukocytes). The other section of the blood sample was permitted to clot at 4°C overnight. The clogged sample was centrifuged at 4000 rpm for 15 minutes, then stored until use.

### Physiochemical Parameters

Consistent monitoring of the water parameters was ensured by maintaining regulatory control. Electronic devices such as a pH meter and HANNA HI-8424 measured pH levels, temperature, and dissolved oxygen (DO). Standard measurement techniques were employed to assess the total alkalinity, water hardness, and concentrations of CO<sub>2</sub> and HCO<sub>3</sub>. The objective was to systematically track and analyze the various water quality indicators using appropriate instrumentation and recognized methods.

### Temperature

Silver carp can only be found in the temperate waters of Asia. The best water temperature for Silver carp is between 6 and 28°C, which promotes rapid growth in the fish. Higher water temperatures have a detrimental effect on fish development and growth because they contain less dissolved oxygen.

### pH

The pH of the water was maintained within the safe range of 6.8-8.0 to ensure the proper keeping and management of freshwater fish. An acceptable range of 6.5-9.0 was also considered. It was observed that a significant drop or rise in pH, such as below 4.0 or above 11.0, caused fish to experience stress and ultimately led to their demise. Therefore, consistent efforts were made to prevent drastic fluctuations in pH levels, prioritizing the well-being and survival of the fish.

### Total Hardness

Hardness, which is a measure of alkalinity in water primarily attributed to the presence of calcium and magnesium, was observed. Calcium and magnesium are essential for triggering metabolic reactions, including bone formation and weight regulation. Maintaining appropriate water hardness levels was crucial to provide the necessary minerals for these physiological processes in the fish.

## RESULTS

### Haemoglobin

The minimum and maximum values of Hb were 5.33 and 6.21 (g/dL) after 15 and 60 days, respectively. Statistical analysis revealed significant differences in Hb counts among the treatments and over time (days) at a significance level of  $P \leq 0.01$ .

**Table 4.1 Haemoglobin for different treatments**

Hb (g/dL)			
DAYS	T0	T1	T2
15	5.19	5.09	5.33
30	5.06	5.19	5.78
45	5.03	5.21	5.56
60	5.01	5.28	6.21

### Hematocrit (HCT)

The minimum and maximum values of HCT were 37.41 and 48.00 % after 15 and 60 days, respectively. Statistical analysis revealed significant differences in HCT counts among the different treatments and over time (days) at a significance level of  $P \leq 0.05$ .

**Table 4.2 Hematocrit (HCT)**

HCT %			
DAYS	T0	T1	T2
15	29.79	32.56	37.41
30	21.32	24.89	44.28
45	22.31	27.70	48.00
60	25.65	39.26	41.44

### White blood cells (WBCs)

White blood cells' minimum and maximum values were 123 and  $148 \times (10^3 \mu\text{l})$  after 15 and 60 days, respectively. Statistical analysis revealed significant differences in white blood cell counts among the treatments and over time (days) at a significance level of  $P \leq 0.01$ .

**Table No.3 WBCs for different treatments**

WBCs $\times (10^3 \mu\text{l})$			
DAYS	T0	T1	T2
15	119	130	140
30	116	125	123
45	120	128	148
60	117	133	147

### Lymphocyte

The initial value of lymphocytes was recorded as 60 %. In the T0 group, the minimum and maximum values of lymphocytes after 15 and 60 days were 61.7 and 64.3 × (10<sup>6</sup> μl), respectively. In the T1 group, these values were 62.0 and 68.0 × (10<sup>6</sup> μl) after 15 and 60 days, respectively. In the T2 group, the minimum and maximum lymphocyte values were 67.1 and 73.1 × (10<sup>6</sup> μl) after 15 and 60 days, respectively.

Statistical analysis revealed significant differences in lymphocyte counts among the treatments and over time (days) at a significance level of P≤0.01.

**Table 3.4 Lymphocytes under different treatments**

Lymphocyte %			
DAYS	T0	T1	T2
15	61.7	62.0	67.1
30	62.5	65.1	68.7
45	63.1	67.9	71.5
60	64.3	68.0	73.1

### Physiochemical Parameters

Temperature plays a vital role in the growth and performance of fish, making it a crucial aspect to consider. The effects of water temperature on fish populations can be both direct and indirect. Each organism has a specific temperature range optimal for its functioning and stability. Cold-blooded fish, in particular, are influenced by ambient temperature, which affects their growth, reproduction, feed consumption, and various physiological functions. In the T0 group, the minimum and maximum values of temperature after 15 and 60 days were 15 and 20°C, respectively. In the T1 group, these values were 16 and 22°C after 15 and 60 days, respectively. In the T2 group, granulocyte's minimum and maximum values were 15 and 3 after 15 and 60 days, respectively.

**Table 3.5 Mean (±SED) temperature values for control and experimental groups for 8 weeks.**

TEMPERATURE			
Weeks	T0	T1	T2
Week 1	15	17	16
Week 2	18	19	17
Week 3	17	16	15
Week 4	20	22	21
Week 5	19	18	17
Week 6	24	21	22
Week 7	21	23	24
Week 8	20	22	21
Mean ± SED	19.56 ± 2.70	20.22 ± 2.82	19.56 ± 3.32

### pH

The pH value indicates the concentration of hydrogen ions present. Living organisms have specific ranges within which they can tolerate temperatures and pressures. Although not entirely pure, water contains additional components contributing to its acidity, alkalinity, or neutrality, which is beneficial for fish farming. Due to the high presence of carbonates and bicarbonates, the water generally exhibited alkaline properties. In all treatments, the pH levels ranged from 7.8 to 7.1, serving as the upper and lower limits.

**Table No. 6 Mean (±SED) pH values for control and experimental groups for 8 weeks.**

pH			
Weeks	T0	T1	T2
Week 1	7.7	7.5	7.6
Week 2	7.3	7.8	7.5
Week 3	7.8	7.2	7.4
Week 4	7.6	7.6	7.1
Week 5	7.1	7.7	7.4
Week 6	7.5	7.1	7.3
Week 7	7.2	7.4	7.5
Week 8	7.8	7.7	7.6
Mean ± SED	7.49 ± 0.26	7.48 ± 0.24	7.39 ± 0.19

### Total hardness

The concentration of divalent metal ions is typically reported as milligrams per litre of calcium carbonate equivalent, indicating the amount of water containing these ions. Water hardness significantly influences the breeding success of fish. Across all measurements, the overall hardness ranged from 221 mg/L to 264 mg/L.

**Table No. 7 Mean ( $\pm$ SED) total hardness values for control and experimental groups for 8 weeks.**

TOTAL HARDNESS	
Weeks	T0
Week 1	225
Week 2	252
Week 3	261
Week 4	240
Week 5	257
Week 6	243
Week 7	221
Week 8	239
Mean $\pm$ SED	243.22 $\pm$ 13.68

### DISCUSSION

This study was conducted at the Microbiology and Immunology Laboratory, Department of Zoology, Wildlife, and Fisheries, University of Agriculture in Faisalabad. Its primary objective was to examine the effect of sericite on the gut microbes and haematological parameters of Silver carp.

Previous research found that feeding Nile tilapia a diet rich in natural minerals like Macsumuk increased their resilience to illness (Shahkar *et al.*, 2015). Natural zeolite has also been shown to significantly reduce microbiological parameters in the water (such as Salmonella) in experimental recirculating aquaculture systems compared to microbiological parameters in conventional recirculating aquaculture systems (Sirakov *et al.*, 2015). The present research aligns with the findings of (Hassaan *et al.*, 2020), who found that the total count of gut microbiota in

the gastrointestinal of experimental fish showed upright decreases in reaction to nutritive sericite levels, respectively.

Haematological indices are valuable biological markers that indicate how the body responds to changes in nutrition (Hassaan *et al.*, 2018). Fish anaemia is typically diagnosed when the levels of red blood cells (RBC) and haemoglobin fall below the normal range (Maita, 2007). In the present study, there were statistically significant differences in the total erythrocyte count, red blood cell count, or haemoglobin concentration between the control group and the group of fish fed with a diet supplemented with sericite. Present research aligns with the findings of (Jawahar *et al.*, 2016), who found that the zeolite supplementation was administered to striped snakehead (*Channa striata*), significantly increasing Hb, Htc, and RBC levels. This current research aligns with the findings of (Hassaan *et al.*, 2020), who found that all the diets with sericite supplements showed higher white blood cell counts (WBCs) and their differentials compared to the control diet. These numbers can indicate fish well-being since an increase in non-specific or innate immunity could explain this trend. The striped snakehead-fed zeolite-enriched diets also considerably increased their white blood cell count (Jawahar *et al.*, 2016).

Kim *et al.* (2022) discovered that sericite treatment inhibited cell division reduced reactive oxygen species and decreased tumour volume in TNBC cells and mice. These findings suggest sericite's potential as a therapeutic agent and for developing anticancer drugs for TNBC. (Choi *et al.*, 2016) investigated using methyl-esterified sericite (ME-sericite) for harvesting microalgae. Activation of sericite with methyl-hydrochloric acid increased surface area, facilitating efficient absorption of microalgae. ME-sericite successfully harvested 78-99% of microalgae across various pH ranges.

Nazari *et al.* (2018) found that clay treatment improved happiness levels and reduced depression and anxiety in individuals with physical impairments compared to the control group. Clay treatment offers potential benefits for enhancing well-being and mental health in individuals with disabilities.

Currently, a limited number of studies assess the impact of clay minerals on the overall composition of fish. Therefore, the available findings have few extensive comparisons to draw upon. Nevertheless, these findings strongly indicate the positive influence of incorporating specific clay minerals such as sericite into fish diets. This addition significantly enhances gut microbiota, improving the fish quality in terms of their entire body, and contributes to their overall health and fitness.

#### CONCLUSION

In conclusion, the study on the effect of sericite on gut microbes and hematological parameters of Silver carp reveals promising findings, suggesting that sericite supplementation can positively influence the gut microbiome and hematological health of the fish. By modulating gut microbiota and improving hematological parameters, sericite may be a valuable feed additive in aquaculture, promoting fish health and well-being. Further research is necessary to fully understand the mechanisms and optimal dosage of sericite for Silver carp, but the potential benefits for sustainable aquaculture practices are significant.

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