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EFFECTS OF DIETARY SALT AND HERBAL SUPPLEMENTATION ON HYDROLYTIC BACTERIAL COMMUNITIES IN THE FILTRATION SYSTEM OF BONYLIP BARB (*Osteochilus vittatus*)

Pengaruh Suplementasi Garam dan Ramuan Herbal terhadap Komunitas Bakteri Hidrolitik dalam Sistem Filtrasi Ikan Nilem (*Osteochilus vittatus*)

Siti Khoirun Nisa[1], Syafrie Alfauzie[2], Mevira Sekar Chaerunissa[2], Hamdan Syakuri[1*], Taufik Budhi Pramono[1], Dewi Nugrayani[2], Emyliana Listiowati[2]

[1]Magister Program in Aquatic Resources, Fisheries Department, Universitas Jenderal Soedirman, [2]Aquaculture Study Program, Fisheries Department, Universitas Jenderal Soedirman

Dr. Soeparno Street, Karangwangkal, North Purwokerto, Banyumas, Central Java 53122

[*]Correspondent author: hamdan.syakuri@unsoed.ac.id

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ABSTRACT

Dietary supplementation with salt and herbal additives may influence not only the gut microbiota of cultured fish but also the bacterial communities in the surrounding water, including filtration systems. This study aimed to characterize the morphotypes and hydrolytic activity of bacteria isolated from the filtration system of Bonylip barb (*Osteochilus vittatus*) fed diets supplemented with salt and herbal concoctions. A completely randomized design (CRD) was employed with four treatments and three replicates: P1) Control; P2) 3% salt supplementation; P3) 10 mL/kg feed herbal supplementation; and P4) combined 3% salt and 10 mL/kg feed herbal supplementation. After 60 days of culture, bacterial isolates from the filtration system were assessed for colony morphotypes and hydrolytic activity, including proteolytic, amylolytic, cellulolytic, and lipolytic indices. Results indicated that all supplemented treatments (P2–P4) yielded a higher diversity of bacterial morphotypes compared to the control. Notably, proteolytic activity was significantly elevated in the salt-supplemented group (P2) relative to both the control and herbal-only treatments. However, salt and herbal supplementation did not significantly affect amylolytic, cellulolytic, or lipolytic activity indices. These findings suggest that dietary salt influences the functional composition of hydrolytic bacteria in aquaculture filtration systems.

Keywords: salt, herbs, Bonylip barb, hydrolytic bacteria, filtration system.

ABSTRAK

Suplementasi pakan dengan tambahan garam dan ramuan herbal dapat memodulasi tidak hanya mikrobiota usus ikan budidaya, tetapi juga komunitas bakteri di perairan sekitarnya, termasuk dalam sistem filtrasi. Penelitian ini bertujuan untuk mengkarakterisasi morfotipe dan aktivitas hidrolitik bakteri yang diisolasi dari sistem filtrasi pemeliharaan ikan nilem (*Osteochilus vittatus*) yang diberi pakan dengan suplementasi garam dan ramuan herbal. Rancangan acak lengkap (CRD) digunakan dengan empat perlakuan dan tiga ulangan: P1) Kontrol; P2) suplementasi garam 3%; P3) suplementasi herbal 10 mL/kg pakan; dan P4) kombinasi suplementasi garam 3% dan herbal 10 mL/kg pakan. Setelah 60 hari pemeliharaan, isolat bakteri dari sistem filtrasi dievaluasi berdasarkan morfotipe koloni dan aktivitas hidrolitik, meliputi indeks proteolitik, amilolitik, selulolitik, dan lipolitik. Hasil menunjukkan bahwa semua perlakuan suplementasi (P2–P4) menghasilkan keragaman morfotipe bakteri yang lebih tinggi dibandingkan kontrol. Aktivitas proteolitik secara signifikan meningkat pada kelompok yang mendapatkan suplementasi garam (P2) dibandingkan kontrol dan perlakuan herbal saja. Namun, suplementasi garam dan herbal tidak berpengaruh signifikan terhadap indeks aktivitas amilolitik, selulolitik, maupun lipolitik. Temuan ini menunjukkan bahwa garam dalam pakan memengaruhi komposisi fungsional bakteri hidrolitik di sistem filtrasi budidaya ikan.

Kata Kunci: garam, herbal, ikan Nilem, bakteri hidrolitik, sistem filtrasi.

INTRODUCTION

Bonylip barb (*Osteochilus vittatus*) is a key aquaculture commodity in Indonesia with promising market potential and high domestic demand for consumption in various product forms (Aryani *et al*., 2017; Saepuloh *et al*., 2021). Meeting this demand requires increasing culture productivity. One strategy involves dietary supplementation with salt and herbal additives to enhance growth and fish health (Gasco *et al*., 2018; Pratama & Mukti, 2021).

Salt supplementation in Bonylip barb feed (2 % NaCl) shifted digestive tract bacterial composition toward Gram-positive dominance (69.99 %) versus Gram-negative (21.69 %) (Listiowati *et al*., 2023). Moreover, salt addition can improve production efficiency in Bonylip barb culture (Syakuri *et al*., 2024). Herb supplementation accelerates growth, enhances health, improves feed utilization, and increases disease resistance of fish (Andriani *et al*., 2022), and in Gibel goldfish (*Carassius auratus* Gibelio) it strengthens microbial balance by suppressing pathogens and promoting beneficial bacteria (Wu *et al*., 2018).

Intensive Bonylip barb farming often suffers from deteriorating water quality due to accumulation of uneaten feed and fecal waste, necessitating effective water management (Setyaningrum *et al*., 2019). Filtration systems play a crucial role by reducing physical and chemical residues of organic matter and hosting diverse microbial guilds, including nitrifying, heterotrophic, and hydrolytic bacteria (Sugita *et al*., 2005; Yosmaniar *et al*., 2018). Hydrolytic bacteria produce enzymes—protease, amylase, lipase, and cellulase—that decompose protein, carbohydrates, fat, and cellulose, preventing toxic buildup in culture water (Ojovan *et al*., 2021).

In catfish (*Clarias gariepinus*) ponds, hydrolytic bacteria have been shown to reduce levels of protein, fat, carbohydrates, biochemical oxygen demand (BOD), and chemical oxygen demand (COD) (Prayogo *et al*., 2018). Hydrolytic bacteria play a crucial role in maintaining water quality, yet the impact of dietary salt and herbal supplementation on their diversity and activity within filtration systems remains unexplored. Therefore, this study aimed to determine the morphotype diversity and hydrolytic activity index of bacteria in the filtration system of Bonylip barb (*Osteochilus vittatus*) fed diets supplemented with salt and herbal concoctions.

RESEARCH METHODS

Experimentation and Fish Rearing

The experiment was conducted as a completely randomized design (CRD) with four dietary treatments and three replicates each. Treatment P1 served as the control with commercial feed only, P2 received commercial feed supplemented with 3% salt, P3 was fed commercial feed with 10 mL kg feed^{-1} herbal concoction, and P4 combined both 3% salt and 10 mL kg feed^{-1} herbal supplementation in the commercial feed. The concentration of salt was determined based on our previous result (Syakuri *et al*., 2024) and the concentration of herbal suplementation was determined based on suggestion from a shrimp farmer in Purworejo who provided the herbal concoction. This work was conducted in July-September 2023 in a private fish farm in Bojongsari, Kembaran, Banyumas, Central Java. The tools for the main work included autoclave, incubator, micropipet, vortex, 30 L plastic tubs, aeration systems, petridish, reaction tubes, and hot plate. The materials for the main work included trypticase soy agar (TSA), trypticase soy broth (TSB), NaCl 0.9% solution, KOH 3%, carboxymethyl cellulose (CMC), gum Arabic, olive oil, starch, $CaCl_2$, neutral red, Congo red, skim milk, and aquadest.

The fish samples were maintained indoors in twelve 30 L plastic tubs, each filled with 20 L of well water (≈23 cm depth) and fitted with an aerator and internal filter. Bonylip barb fry (3–8 cm), sourced from a local cultivator in Purbalingga and confirmed healthy, were acclimated for 15 minutes in an aerated drum before being stocked at a uniform density of 10 fish per tub. For the first 10 days, all tubs received only commercial feed to complete acclimation; thereafter, fish were fed their respective test diets at 3% of biomass. Every 10 days, 50% of the water volume in each tub was replaced.

Preparation of Test Feed

This study employed a commercial pellet (0.7–1 mm) containing 39 % protein, 5 % fat, 6 % crude fiber, 12 % ash, and 10 % moisture, which was supplemented per treatment with either a coarse salt solution, an herbal concoction, or their combination. The herbal blend—sourced from a shrimp farmer in Purworejo Regency—comprised garlic (*Allium sativum*), turmeric (*Curcuma longa*), ginger (*Curcuma xanthorrhiza*), red ginger (*Zingiber officinale*), honey, brown sugar, and anchovy extract in fixed proportions. The specific composition of the herbal concoctions has not been made publicly available, as it constitutes proprietary knowledge retained by the farmer. For each treatment, 1 kg of feed was uniformly sprayed with 250 mL of the respective solution, while the control diet was sprayed with 250 mL of distilled water. After spraying, all diets were air-dried at room temperature before use.

Water Sampling

Aseptically, water was sampled from inside each treatment's filter and immediately transferred into sterile 5 mL microtubes. The tubes were kept chilled and transported to the laboratory.

Hydrolytic Test Media

Proteolytic medium was prepared by dissolving 40 g of trypticase soy agar (TSA) and 20 g of skim milk powder (2% w/v) in 1 L of distilled water in an Erlenmeyer flask. Amylolytic medium comprised 40 g TSA and 10 g soluble starch (1% w/v) dissolved in 1 L of distilled water. For lipolytic medium, 1 L of distilled water was heated to 100 °C and stirred for 15 min with 1% olive oil, 1% gum arabic, and 0.5% $CaCl_2$, then 40 g TSA was added once the emulsion was homogeneous. Cellulolytic medium was made by combining 40 g TSA and 1% carboxymethylcellulose (CMC) in 1 L of distilled water and stirring at 100 °C for 15 min. Each medium was microwaved, then sterilized in an autoclave at 121 °C for 15 min. Composition details are summarized in Table 1.

Table 1. Composition of Hydrolytic Media (Atlas 1995)

Media	Composition
Proteolytic	40 g of TSA + 20 g of skim milk powder + 1 L aquades
Amylolytic	40 g of TSA + 10 g pati (tepung tapioka) + 1 L aquades
Lipolytic	40 g of TSA + 10 mL of olive oil + 10 g gum arabic + 0,5% $CaCl_2$ + 0,06 g of neutral red + 1 L aquades
Cellulolytic	40 g of TSA + 10 g CMC + 1 L aquades

Bacterial Isolation and Morphological Observation

Bacterial isolation was performed aseptically using serial dilution and pour plate techniques. Homogenized water samples (0.5 mL) were transferred into 4.5 mL of sterile physiological solution and vortexed to achieve a 10^{-1} dilution, then serially diluted in the same manner up to 10^{-5}. From the 10^{-3}–10^{-5} dilutions, 0.5 mL aliquots were inoculated into molten TSA medium (Sanders, 2012), gently swirled to mix, and allowed to solidify. Plates were incubated inverted at 28 °C for 24 h, and resulting colonies were characterized by color, size, shape, elevation, and margin (Tóth *et al*., 2013).

Observation of Gram Properties Using 3% KOH

The KOH Gram test was performed by transferring an 18–24 hour-old bacterial colony (one loopful) into a drop of 3 % KOH on a sterile glass slide. The colony was emulsified thoroughly in the KOH suspension and stirred continuously for 60 seconds. The inoculating loop was then withdrawn slowly to assess string formation. The appearance of a viscous, mucoid "string" indicates a positive KOH reaction (Gram-negative bacteria), whereas no string formation indicates a negative reaction (Gram-positive bacteria) (Buck, 1982).

Hydrolytic Bacterial Activity Test

Hyrolytic activities were assessed by spot-inoculating each bacterial isolate (one loopful) onto respective media. After incubation, the clear zone and colony diameter was measured. For cellulolytic index, plates were flooded with 0.1 % Congo red solution and left for 20 minutes, then rinsed with 0.2 M NaCl for 15 minutes to remove unbound dye (Murtiyaningsih & Hazmi, 2017). Hydrolysis of CMC was indicated by a clear zone around the colony. The hydrolysis index (IH) was calculated using the formula: IH = (diameter of the clear zone) ÷ (diameter of the colony) as previously described (Hastuti *et al*., 2017).

Data Analysis

Data on bacterial morphotypes and hydrolysis index values (proteolytic, amylolytic, lipolytic, and cellulolytic) were summarized descriptively and presented in figures and tables. To compare treatment groups, statistical analyses were performed in SPSS. One-way ANOVA followed by Tukey's post-hoc test was used for datasets meeting normality and homogeneity assumptions; otherwise, the non-parametric Kruskal–Wallis test was applied. All tests were conducted at a significance level of $\alpha = 0.05$.

RESULTS

Number of Hydrolytic Bacterial Morphotypes

Observation of bacterial colonies from the filtration media of Bonylip barb (*Osteochilus vittatus*) yielded several distinct morphotypes, classified by Gram reaction and colony characteristics (color, shape, margin, elevation, and size). From filtration systems of rearing fish, 112 hydrolytic bacterial isolates were recovered, and functional screening showed these isolates possessed proteolytic, amylolytic, lipolytic, and cellulolytic activities. As illustrated in Figure 1, both salt and herbal treatments increased morphotype diversity compared to the control: the herbal concoction alone produced 15 morphotypes, the 3 % NaCl treatment yielded 14, the combined 3 % NaCl + 10 mL herbal treatment gave 12, and the control exhibited only 9 (Figure 1).

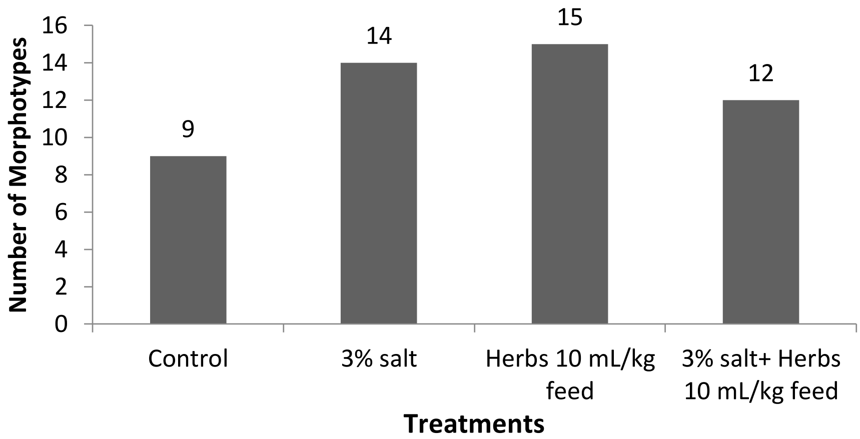

Figure 1. Number of Hydrolytic Bacterial Morphotypes isolated from the Filtration System of Bonylip Barb (*Osteochilus vittatus*)

Hydrolytic Bacteria Index Value

Of the 112 bacterial isolates tested for protein hydrolysis activity, 80 exhibited proteolytic capabilities. The hydrolysis index values of these proteolytic bacteria ranged from 0.1 to 1.7, indicating low to moderate enzymatic activity. Mean hydrolysis indices varied across dietary treatments, ranging from 0.3 ± 0.2 to 0.8 ± 0.3 (Figure 2). One-way ANOVA revealed a statistically significant difference in proteolytic activity among treatments ($P < 0.05$), suggesting that feed supplementation influenced bacterial enzyme expression in the filtration system. Post-hoc Tukey's HSD analysis confirmed that the 3 % salt treatment yielded a significantly higher proteolytic index than both the control and herbal-only treatments ($p < 0.05$), indicating that salt supplementation more strongly promoted proteolytic bacterial activity.

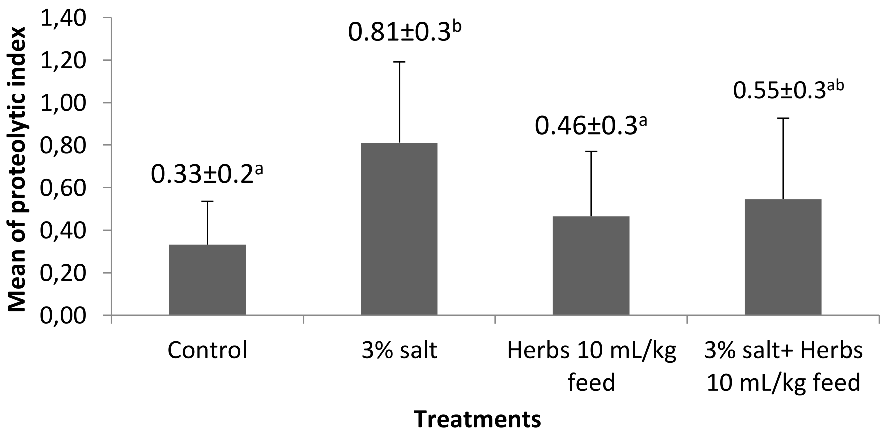

Figure 2. Mean Hydrolysis Index of Proteolytic Bacteria isolated from the Filtration System During Bonylip Barb (*Osteochilus vittatus*) Culture with Salt and Herbal Feed Supplementation. Means followed by different superscript letters are significantly different at $p < 0.05$.

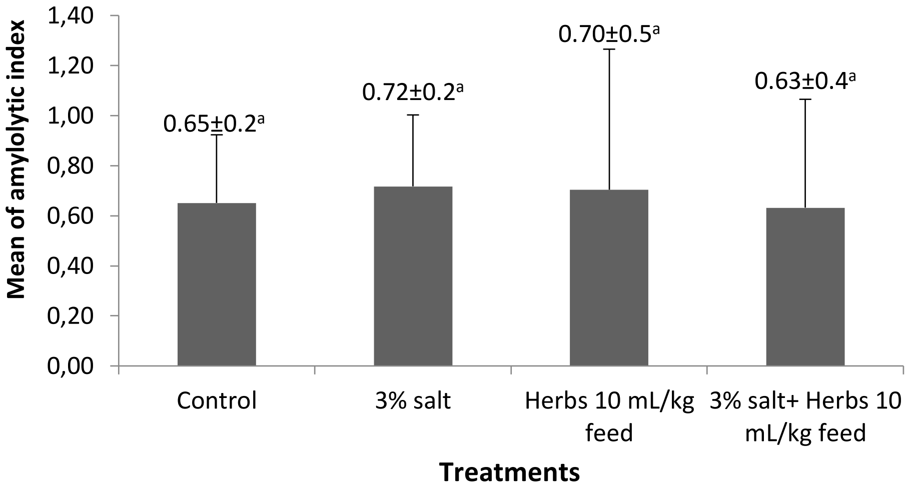

Figure 3. Mean Hydrolysis Index of Amylolytic Bacteria isolated from the Filtration System During Bonylip Barb (*Osteochilus vittatus*) Culture with Salt and Herbal Feed Supplementation. No statistically significant differences were observed among treatments ($p > 0.05$)

Starch-degrading activity was detected in the majority of bacterial isolates, with 95 out of 112 exhibiting amylolytic capabilities. The corresponding hydrolysis index values spanned from 0.1 to 1.8, reflecting low to moderate enzymatic efficiency. Mean index values across feed supplementation treatments ranged from 0.6 ± 0.2 to 0.7 ± 0.5 (Figure 3). According to the Kruskal–Wallis test, no statistically significant variation was observed among treatment groups ($P > 0.05$), indicating that dietary supplementation did not substantially affect amylolytic bacterial activity in the filtration system.

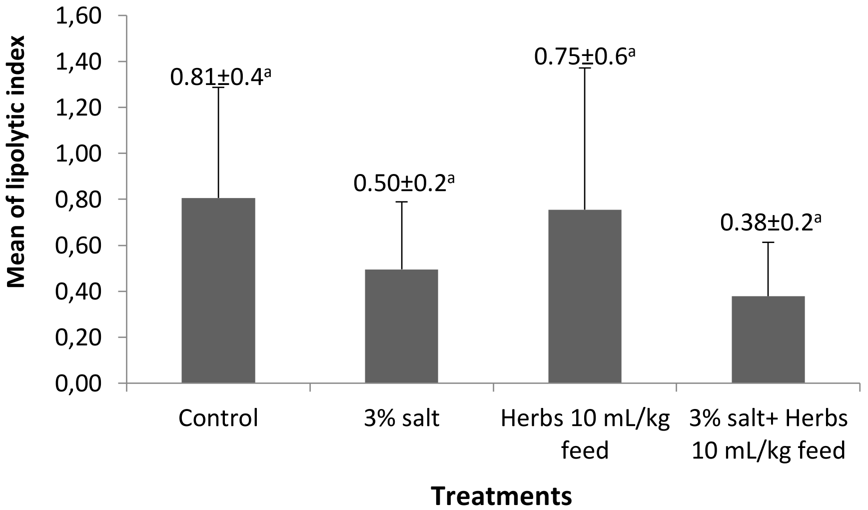

Figure 4. Mean Hydrolysis Index of Lipolytic Bacteria isolated from the Filtration System During Bonylip Barb (*Osteochilus vittatus*) Culture with Salt and Herbal Feed Supplementation. No statistically significant differences were observed among treatments ($p > 0.05$)

Fat-degrading activity was observed in 65 of the 112 bacterial isolates, with the highest number (23 isolates) recovered from the 3% salt supplementation treatment. Lipolytic hydrolysis index values ranged from 0.1 to 2.0, indicating low to moderate enzymatic activity.

Across the different feed treatments, mean lipolytic indices varied between 0.3 ± 0.2 and 0.8 ± 0.4. Statistical analysis using the Kruskal–Wallis test revealed no significant differences among treatment groups (P > 0.05), suggesting that dietary supplementation did not markedly influence lipolytic bacterial performance within the filtration system (Figure 4).

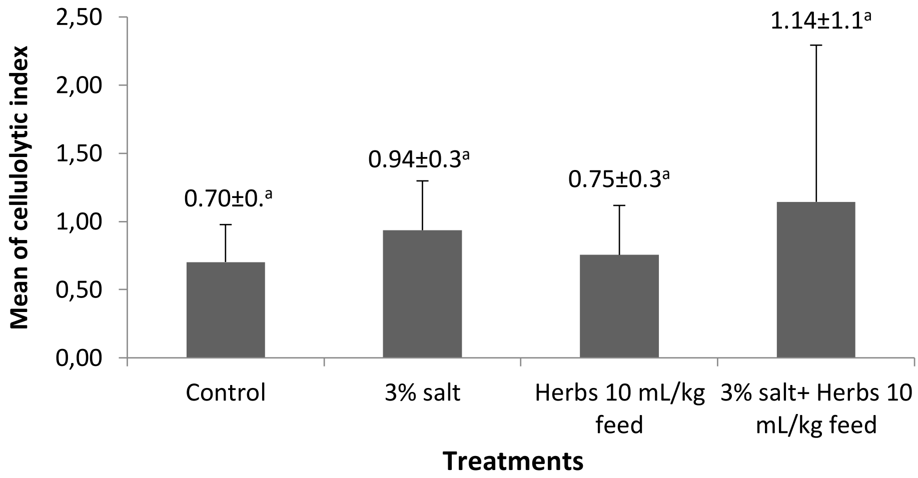

Figure 5. Mean Hydrolysis Index of Cellulolytic Bacteria Isolated from the Filtration System During Bonylip Barb (*Osteochilus vittatus*) Culture with Salt and Herbal Feed Supplementation. No statistically significant differences were observed among treatments (p > 0.05)

Cellulose-degrading activity was identified in 81 of the 112 bacterial isolates. The hydrolysis index values of these cellulolytic bacteria ranged from low to high, with control group measurements spanning 0.2 to 5.0. Across the different feed supplementation treatments, the mean cellulolytic indices of bacteria isolated from the filtration system ranged from 0.7 ± 0.2 to 1.1 ± 1.1 (Figure 5). One-way ANOVA analysis indicated no statistically significant differences in cellulolytic activity among the treatment groups (P > 0.05), suggesting that feed supplementation did not substantially influence cellulose degradation capacity in the bacterial community.

DISCUSSION

The filtration system used for Bonylip barb (*Osteochilus vittatus*) culture with salt and herbal feed supplementation exhibited greater hydrolytic bacterial morphotype diversity than the control (Figure 1), suggesting that these additives promote microbial heterogeneity. NaCl has been shown to influence bacterial composition, with higher concentrations enhancing microbial diversity (Pang *et al*., 2020), and dietary salt increasing gut microbiota richness in tilapia (Hallali *et al*., 2018). Herbal supplementation similarly boosts microbial diversity, as observed in rainbow trout fed with Espelette pepper powder (Mougin *et al*., 2023). This diversity in the digestive tract often mirrors that of the surrounding aquatic environment, with dominant genera such as Proteobacteria, Cyanobacteria, and Firmicutes found in both fish intestines and water samples (Zeng *et al*., 2020).

The filtration system for Bonylip barb culture supplemented with 3% salt showed a higher average proteolytic index than both the control and herbal treatments (Figure 2), likely due to salt-enhanced protease activity. Salt supplementation can stimulate the presence and enzymatic function of proteolytic bacteria, as evidenced by increased proteolysis in *Lactobacillus acidophilus* with rising salt levels (Gandhi & Shah, 2014). Protease activity typically peaks at low to moderate NaCl concentrations, with optimal bacterial enzyme performance observed around 20 g/L, beyond which activity declines due to microbial stress (Pang *et al*., 2020). Temperature, pH, and bacterial strain also modulate protease efficiency (Sun *et al*., 2021).

The study showed no significant differences in amylolytic, lipolytic, and cellulolytic bacterial indices across supplementation treatments, suggesting limited impact from salt or herbal additives. High NaCl concentrations are known to inhibit enzyme activity by disrupting enzyme structure and function, reducing amylase, lipase, and cellulase performance (Febriani *et al*., 2019; Pang *et al*., 2020) However, certain halophilic and halotolerant bacteria, such as *Bacillus* sp. and *Alkalilimnicola* sp., exhibit optimal hydrolytic enzyme production at specific salt levels (Bandal *et al*., 2021; Bora & Bora, 2012; Mesbah & Wiegel, 2017) Herbal supplementation, alone or combined with salt, also showed no significant effect, likely due to phytochemicals like curcumin that suppress bacterial hydrolytic activity by reducing enzyme-producing populations (Najafian, 2015).

CONCLUSION

A total of 112 hydrolytic bacterial isolates representing 34 distinct morphotypes were identified in this study. Filtration systems supporting Bonylip barb (Osteochilus vittatus) fed with salt, herbal supplements, or their combination exhibited greater bacterial morphotype diversity compared to the control. Salt supplementation notably enhanced the proteolytic index of bacteria, while amylolytic, cellulolytic, and lipolytic activity indices remained relatively consistent across all treatments.

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