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EFFECTIVENESS OF ENDOPHYTIC BACTERIA FOR SOME MEDICINAL PLANTS AS PROBIOTICS FOR NILE TILAPIA (*Oreochromis niloticus*)

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ABSTRACT

The main objective of this research is to study the possibility of using endophytic bacteria isolated from some medicinal plants (*onion*, *Allium cepa*, *brassicales*, *Salvadora persica*, *fenugreek*, and *Trigonella foenum-graecum*) as probiotics for Nile tilapia (*Oreochromis niloticus*). Fish growth was evaluated using fish growth performance indices and proximate fish composition. Fish health was assessed by quantifying some biochemical compounds in fish serum, and at the end of the experiment, a challenge test was performed with *Vibrio parahaemolyticus*. Endophytic bacteria increased all growth performance indicators compared to the control group. Endophytic bacteria of fenugreek recorded higher growth performance than other plants. Moreover, in all treatments except for onion, bacteria mixed with feed significantly supported fish growth performance compared to bacteria added to rearing water. Endophytic bacteria of onion and fenugreek recorded higher concentrations of protein in muscles than the control group by 56 and 49%, respectively. Furthermore, 88, 75 and 63% of the treatments recorded a decrease in albumin, ALT and AST concentrations compared to the control group. Although the concentration of urea in the blood was higher than the control group by about 7.4 to 44.3%, challenge test showed that all treatments had a 20% mortality rate compared to the control group (10%). As a result, the endophytic bacteria of onion, brassicales and fenugreek are recommended as probiotics for Nile tilapia. Further study is needed to elucidate the optimal bacterial concentration necessary for tilapia growth.

KEYWORDS: Endophytic bacteria; Probiotic bacteria; Medicinal plants; Nile tilapia

INTRODUCTION

Bacterial endophytes are present in all tissues of plants including flowers, seeds, leaves, stems, and roots (Elmagzob *et al*., 2019). Bacterial endophytes possess pathogenic, saprophytic, and mutualistic relationships. In the mutualistic (symbioses) relationship between the bacterial endophytes and the host plant, endophytes use the internal tissues of the plant to protect themselves from changing external environments, while bacterial endophytes promote plant growth by synthesizing plant hormones, fixing nitrogen, enriching phosphorus, and improving stress resistance (Rahman *et al*., 2021). Despite their importance to each other, the relationship of bacterial endophytes to their host plants remains poorly understood (Wu *et al*., 2021). Bacterial endophytes had been isolated from medicinal plants such as *Teucrium polium* (Hassan, 2017) and *Cinnamomum camphora* (Elmagzob *et al*., 2019). Medicinal plants were used in traditional medicine, with fewer side effects during treatment. Furthermore, medicinal plants are used in aquaculture as a natural source of food and immunostimulants for fish without causing any environmental problems (Tadese *et al*., 2022). Moreover, these plants can be used in Nile tilapia aquaculture to produce more natural products against heavy metal toxicity, such as *Trigonella foenum-graecum* (Abbas *et al*., 2019) and *Moringa oleifera* (Mekky *et al*., 2020).

The Nile tilapia, *Oreochromis niloticus*, is the most important species in Egypt because it is favored by the Egyptians, and of high commercial value. So, tilapia aquaculture is among the sectors of food pro-

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duction; hence many strategies have been developed to improve aquaculture, and one of these is the use of probiotic bacteria.

Probiotics are defined as microorganisms that have beneficial effects on the host. These bacteria in aquaculture can increase feed utilization efficiency, enhance nutritional value, improve surrounding water's quality, and stimulate the host's immune system to increase disease resistance (Verschuere *et al*., 2000). Of those, lactic acid bacteria can stimulate the immune system in fish (Gatesoupe, 2008), also, *Bacillus subtilis* (Zokaeifar *et al*., 2014) and *Pediococcus acidilactici* (Eissa *et al*., 2022) can improve water quality, fish growth performance, and immunity of Nile tilapia. In addition, *Lactobacillus casei* can reduce the adverse effects of nanomaterials (Hedayati *et al*., 2021).

This study aims to evaluate the possibility of using endophytic bacteria isolated from some medicinal plants (onion, *Allium cepa*; brassicales, *Salvadora persica*; and fenugreek, *Trigonella foenum-graecum*) as probiotics for fish Nile Tilapia (*Oreochromis niloticus*).

MATERIALS AND METHODS

Isolation of endophytic bacteria from some medicinal plants

Endophytic bacteria were isolated from some medicinal plants such as; onion (*Allium cepa*), brassicales (*Salvadora persica*) and fenugreek (*Trigonella foenum-graecum*). All plant samples (root, stem, and shoot) were washed thoroughly under running tap water, surface-sterilized by dipping sequentially in 95% ethanol for 10 seconds, then 0.5% sodium hypochlorite for 2 minutes, and 70 % ethanol for 2 minutes then rinsed several times with sterile distilled water (Youssef *et al*., 2004). Sterility tests of samples were performed to ensure the removal of surface microorganisms. Surface-sterilized samples were cut off about 2-3 mm, then placed on a Luria-Bertani (LB) broth medium (Atlas, 1946) and incubated for 24-48 h at 32 °C. The bacteria were isolated by streaking on LB agar medium, and their ability to produce indole-3-acetic acid (IAA) was examined according to Acuña *et al.* (2011).

Experimental design

The aquaculture experiment was carried out at the Aswan Research Station, National Institute of Oceanography and Fisheries, Aswan, Egypt. It was designed with three replications. A number of 540 live and apparently healthy Nile Tilapia fish (*Oreochromis niloticus*) was obtained from the General Authority for Fish Resources Development, Aswan (mean fish weight 17.5 ± 5.6 g). Fish samples were acclimated in glass aquaria for 15 days and fed on a basal diet (20% fish meal powder, 20% soybean, 10% corn, 45% bran, 2% vitamins and elements and 3% oils). The experiment was conducted for 40 days using glass aquaria (80 x 60 x 50 cm) filled with fresh water (directly from Nasser Lake) and supplied with continuous air. During the study period, water temperature was kept at 25± 3°C and pH at 7.2 ± 0.1. During the experiment, 10% of the water tanks were replaced daily, and feces were siphoned every two days. Fish were fed twice daily at a rate of 3% body weight.

Fish diets (treatments)

Fish were distributed into nine groups (treatments) as presented in Table (1). The bacterial biomass of selected endophytic bacteria for each plant was produced using the batch culture technique and Luria-Bertani broth medium. For treatments that received bacteria with the commercial fish feeds, the bacterial biomass was mixed well with the commercial fish feeds where 1 ml of culture was added per 1 g of the commercial fish feeds, then dried at room temperature (30–35 °C) for 48 hours. Bacterial load was estimated at 10^7 cells/g diet. For treatments that received bacteria in the rearing water, the bacterial biomass was added to the rearing water, adding 1 mL of culture per 1 liter of rearing water. The bacterial load was estimated as10^7 cells/ml. For treatments that received a mixture of endophytic bacteria isolated from onion, brassicales, and fenugreek either with diets or in rearing water, equal amounts of each bacterial cultured in batches isolated from onion, brassicales, and fenugreek are mixed prior to use in diets or in rearing water. Experimental feed was prepared weekly and stored in a refrigerator at 4 °C.

Fish analysis

Fish growth performance

Fish growth parameters (fish length and weight) were recorded for each treatment at the end of the experiment. The total length was measured (in cm) from the head to the end of the tail. Also, weight was measured in grams on a digital scale. Growth indices of weight gain (WG), specific growth rate (SGR), feed conversion efficiencies (FCE), feed conversion ratio (FCR), and the condition factor (CF) were calculated according to the following formula (Priestley *et al*., 2006) :

Weight gain (WG) = final weight – initial weight

Specific growth rate (SGR) = 100* (ln final weight – ln initial weight)/days of feeding

Feed conversion efficiencies (FCE) = 100* (weight gain/ feed intake)

Feed conversion ratio (FCR) = feed intake / weight gain

Condition factor (CF) = (total weight/total length^3)*100

Proximate composition

Fish carcass samples were analyzed for ash content, total protein using an automatic Kjeltech analyzer, and total fat using the Soxhlet-Randall method with immersion diethyl ether extraction method according to AOAC (2019).

Fish serum biochemistry

At the laboratory of the Magdi Yacoub Heart Center, Aswan, Egypt, a colorimetric assay was used for the quantitative determination of albumin (g/dl), alanine aminotransferase (U/L), aspartate aminotransferase (U/L), creatinine (mg/dl), urea (mg/dl), total bilirubin (mg/dl), direct bilirubin (mg/dl), C-reactive protein (mg/dl) in fish serum on a Roche/ Hitachi cobas c system (Cobas integra 500565).

Challenge test with bacteria

Challenge testing was performed with *Vibrio parahaemolyticus* (Joshi *et al*., 2014). At the end of the experiment, thirty Nile tilapia fish per treatment were injected intra-peritoneally with 0.2 ml of a suspension containing 5×10^7 CFU/ml live *V. parahaemolyticus*. The challenged fish were observed for fifteen days to record mortality rates.

Statistical analysis

Data were statistically analyzed using analysis of variance (ANOVA) using the STATISTICA computer program.

Table 1. Experimental layout

Treatments	Code	Experimental diets (Treatment description)
1	C	Fish feeding with commercial fish feeds without endophytic bacteria
2	O	Fish received endophytic bacteria isolated from onion mixed with the commercial fish feeds
3	OL	Fish received endophytic bacteria isolated from the onion added to the rearing water
4	B	Fish received endophytic bacteria isolated from brassicales mixed with the commercial fish feeds
5	BL	Fish received endophytic bacteria isolated from brassicales added to the rearing water
6	F	Fish received endophytic bacteria isolated from fenugreek mixed with the commercial fish feeds
7	FL	Fish received endophytic bacteria isolated from fenugreek added to the rearing water
8	M	Fish received a mixture of endophytic bacteria isolated from onion, brassicales and fenugreek together with the commercial fish feeds
9	ML	Fish received a mixture of endophytic bacteria isolated from onion, brassicales and fenugreek added to the rearing water

RESULTS AND DISCUSSION

Fish growth performance

Many researchers have used natural products, medicinal plants and probiotic bacteria as feed additives to enhance fish growth and fish health (Ali *et al*., 2011; Kazuñ *et al*., 2018 and Abbas *et al*., 2019). This study used endophytic bacteria isolated from some medicinal plants as probiotic bacteria for Nile tilapia.

The effects of the endophytic bacteria on *O. niloticus* weight gain (WG), specific growth rate (SGR), feed conversion efficiencies (FCE), feed conversion ratio (FCR), and condition factor are illustrated in Figure (1).

All the experimental treatments recorded higher weight gains than the control (11.7 g). Significantly, the highest values were recorded in ML, F, and FL treatments (21.6, 20.4, and 19.9 g, respectively), with increases of 85, 74, and 70%, respectively, over the control group. Also, more than 75% of the experimental treatments had a higher specific growth rate than the control. Significantly, the highest SGR values were recorded for the M, F, FL and B treatments (2.4, 2.3, 2.3 and 2.1%/day, respectively). Moreover, the control had the lowest feed conversion efficiency (56%), while M treatment had the highest value (103%), followed by F and FL treatments (97 and 95% respectively). On the contrary, M, FL and F treatments recorded the lowest feed conversion ratios (1.06, 1.06 and 1.14). Fortunately, the changes in the condition factor values were minimal, ranging from 1.4 to 1.6 g/cm^3 as shown in Figure (1). In all treatments except O and OL, the addition of bacteria mixed with the feed recorded significant increases in weight gain, specific growth rate and feed conversion efficiencies compared to bacteria added to the rearing water (Fig. 1). Regardless of the techniques used (mixing with feed or rearing water), results indicated that endophytic bacteria increased all growth performance indicators, in the following order F > M $\geq$ B > O.

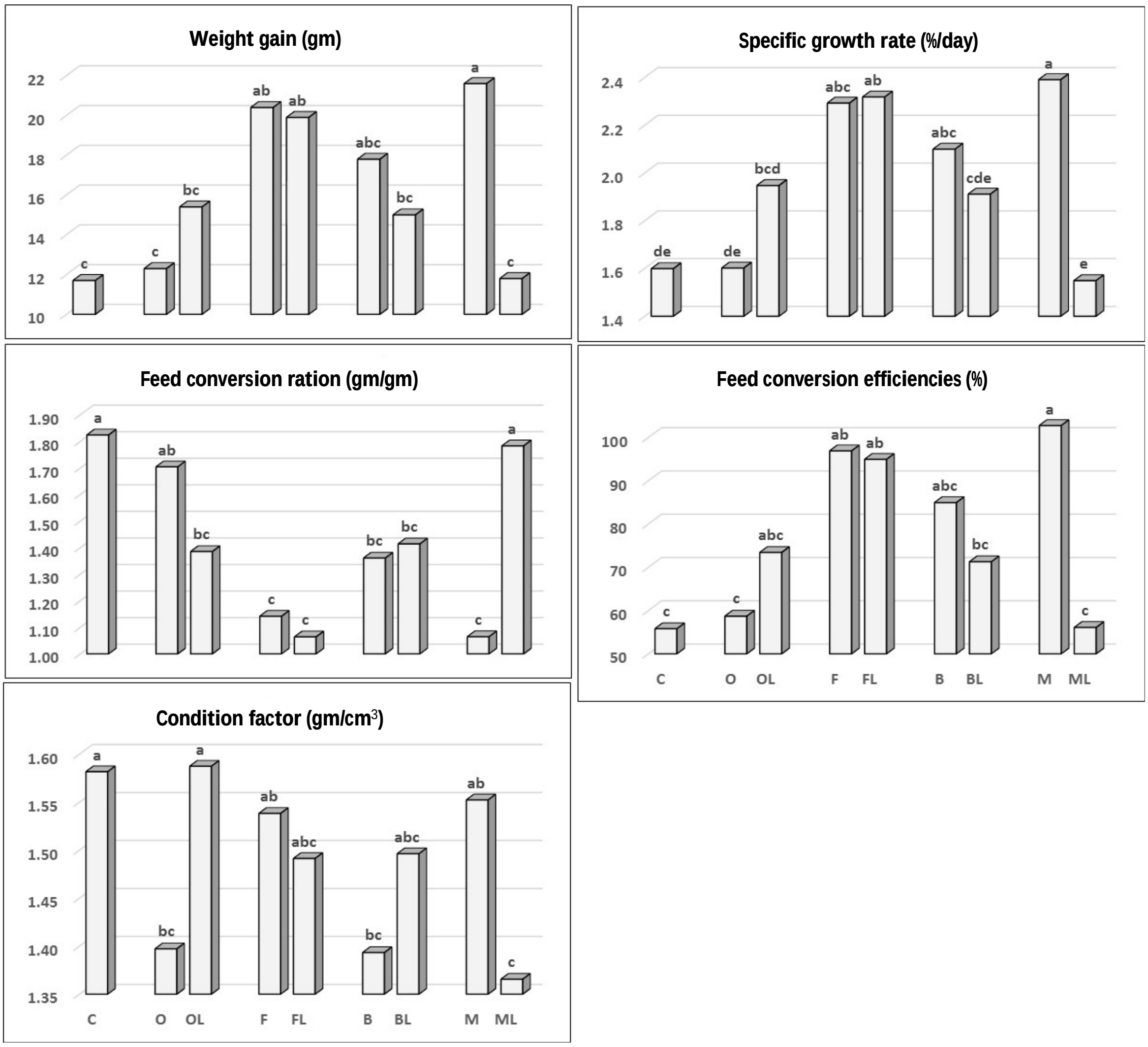

Figure 1. Growth performance (weight gain, specific growth rate, feed conversion ratio, feed conversion efficiency and condition factor) of *Oreochromis niloticus* fed with endophytic bacteria either with feed or with rearing water.

C, O, OL, F, FL, B, BL, M and ML are illustrated in Table (1).

The same letters above the columns are not significantly different ($P > 0.05$)

In general, results showed that all fish treatments receiving endophyte bacteria enhanced the growth performance of fish compared to the control group. This is because bacteria isolates were selected according to their ability to produce indole-3-acetic acid (IAA). Tripathy *et al*., 2005 and Ali *et al*., 2011 reported that the presence of these bacteria (which produce IAA) caused an increase in nutrients (nitrates and phosphates) and increases in phytoplankton and zooplankton, and thus increases in fish growth.

Fish proximate composition

The final proximate compositions (total protein, total lipids and total ash) for the experimental fish are shown in Table (2). Total proteins ranged from 51 to 63% and the highest concentration was recorded in the control group (63%), while the lowest was recorded in the OL treatment (51%). With the exception of fenugreek (F and FL) treatments, the addition of bacteria with feed recorded significant increases in both protein and lipid contents compared to bacteria added to rearing water. In general, onion and fenugreek recorded higher lipid concentrations (means of 13 and 12%, respectively) than the other treatments (9 and 8% for brassicales and mixed treatments, respectively), which are comparable to the control group (8%). Ash content ranged from 14 to 23%. The highest concentration was recorded in the F treatment (23.4%), followed by OL and M treatments (22.8%), while the lowest concentration was recorded in the control group (13.9%).

The percentage of total protein in fish muscles ranged from 44 to 98%. In general, fish received endophytic bacteria from onion and fenugreek recorded higher protein concentrations in muscles (means of 88 and 78%, respectively) than the other treatments (means of 58 and 55% for fish that received endophytic bacteria from brassicales and mix treatments, respectively), compared to the control group (63%).

In general, the approximate composition of fish showed that the highest protein concentration was recorded in the control group, this agrees with Ali *et al*., 2011;2022. Significantly, the condition factor showed that about 63% of treatments were comparable to the control group, implying the physiological well-being of the fish (Ricker, 1975).

Table 2. Proximate composition of all bodies or muscles of Nile tilapia fish (*Oreochromis niloticus*) fed with endophytic bacteria either with diets or in rearing water.

	Total protein (%) in all bodies	Total lipids (%)	Ash (%)	Total protein (%) in muscles
C	63 a	8 cd	13.9 g	63 e
O	57 c	15 a	16.3 e	98 a
BL	51 e	10 bc	18.4 d	78 c
F	54 d	8 cd	23.4 a	93.8 b
FL	59 bc	15 a	14.4 f	63 e
B	60 b	12 b	18.4 d	44 g
OL	57 c	6 d	22.8 b	71 d
M	61 ab	8 cd	22.8 b	61 e
ML	52 de	7 d	20.2 c	48 f

O, BL, F, FL, B, OL, M and ML are illustrated in Table (1)

In the same column, the means followed by the different letters are significantly different ($P \leq 0.05$).

Biochemical profiles of fish blood

Concentrations of albumin (g/dl); alanine aminotransferase (U/L); aspartate aminotransferase (U/L); creatinine (mg/dl); urea (mg/dl); total bilirubin (mg/dl); direct bilirubin (mg/dl); C-reactive protein (mg/dl) in the experimental fish sera are shown in Table (3). Serum albumin concentration ranged from 0.61 to 1.02 g/dL. Significantly, all treatments except M treatment recorded a lower serum albumin concentration than the control group. In all treatments except M treatment, fish received endophytic bacteria with feed recorded a significant decrease in albumin concentration than those with the rearing water. About 75% of the treatments recorded lower ALT concentrations than the control group. In all treatments, the addition of bacteria to the rearing water recorded significant decreases in ALT concentrations compared to bacteria added to the feed. While in all treatments except O and OL, the addition of bacteria to rearing water recorded significant decreases in AST concentrations compared to bacteria added with feed. About 63% of treatments recorded lower AST values than the control group. The serum creatinine concentration ranged from 0.19 to 0.46 mg/dL. The values were in the following order F > O > OL ≥ B, while other treatments recorded values equal to the control group (0.19 mg/dL). Adding bacteria to rearing water significantly decreased the creatinine concentration compared to bacteria added to the feed. The serum urea concentration ranged from 3.0 to 4.3 mg/dL. Significantly, the control group recorded lower concentration (3.0 mg/dL) than the other treatments, which were higher than the control group by about 7.4 - 44.3%. In all treatments except B and BL, the addition of bacteria to the feed significantly decreased the urea concentration compared to bacteria added to the rearing water. Serum C-reactive protein in the control and all treatments were < 0.60 mg/dl. Serum total bilirubin was detected in about 55.5% of samples, as shown in Table (3). In all treatments except M and ML, the addition of bacteria to the aquarium significantly decreased the total bilirubin concentration compared to bacteria added with feed. Direct serum bilirubin was < 0.0 mg/dl in the control and all treatments.

Table 3. Quantitation of some serological parameters of Nile Tilapia fish (*Oreochromis niloticus*)

	Albumin (g/dl)	Alanine aminotransferase (U/L)	Aspartate aminotransferase (U/L)	Creatinine (mg/dl)	Urea (mg/dl)	C-Reactive protein (mg/dl)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)
C	1.02 a	111.2 c	516.8 d	0.19 c	2.98 g	< 0.60	0.00 e	< 0.0
O	0.62 f	66.2 f	479.7 f	0.45 a	3.94 c	< 0.60	0.02 d	< 0.0
OL	0.76 d	60.1 g	497.6 e	0.30 b	4.24 a	< 0.60	0.00 e	< 0.0
F	0.61 f	178.0 a	700.0 a	0.46 a	4.07 b	< 0.60	0.09 b	< 0.0
FL	0.83 b	61.3 g	356.6 g	0.19 c	4.30 a	< 0.60	0.03 d	< 0.0
B	0.65 e	102.5 d	599.7 b	0.30 b	3.59 d	< 0.60	0.06 c	< 0.0
BL	0.78 c	73.0 e	346.9 h	0.19 c	3.44 e	< 0.60	0.00 e	< 0.0
M	1.02 a	160.5 b	544.1 c	0.19 c	3.21 f	< 0.60	0.00 e	< 0.0
ML	0.76 d	43.5 h	236.8 i	0.19 c	4.03 bc	< 0.60	0.17 a	0.00

O, OL, F, FL, B, BL, M and ML are illustrated in Table (1)

In the same column, the means followed by the different letters are significantly different ($P \leq 0.05$)

The results indicated that the existence of some endophytic bacteria decreased albumin levels and thus improved liver function (Oluwalola *et al*., 2020). Although the results showed higher ALT and AST levels than those reported by Julinta et al., 2019 (23.5 IU/L and 24.7 IU/L, respectively), about 75 and 63% of total treatments had lower levels of ALT and AST, respectively, compared to control group. This indicates that endophytic bacteria can improve ALT and AST levels. Also, the creatinine levels in the tested fish were lower than that of normal *O. niloticus* serum creatinine (0.88 mg/dL), as Julinta *et al*. (2019) reported.

Results also indicated that about 50% of treatments reported similar creatinine levels to the control group (0.19 mg/dL). It is interesting that C-reactive protein (CRP) was <0.6 for all treatments, and was lower than the serum CRP level of normal *O. niloticus* (2.51 mg/L) as Julinta *et al*. (2019) reported, indicating that no inflammation occurred in all of the studied fish, where Li *et al*. (2022) reported that C-reactive protein is an acute-phase protein that can be used as an early diagnostic marker for inflammation. In addition, direct bilirubin was not detected, with minimal values for total bilirubin. In general, significant differences were observed in fish growth performance, protein in muscles, and biochemical profiles of blood. This may be due to different endophytic bacterial community, which produces different metabolites and/or different amount of metabolites (Ek-Ramos *et al*., 2019).

Bacterial challenge

The mortality rate of *Oreochromis niloticus* injected with *Vibrio parahaemolyticus* was followed for more than fifteen days after forty days of feeding with endophytic bacteria either mixed with diets or added to rearing water. *Vibrio parahaemolyticus* injections showed that all treatments had a 20% mortality rate compared to the control group (10%). The addition of bacteria with feed has greater resistance than bacteria added to the rearing water, the mortality rate was observed during the first three days when bacteria added to the rearing water, as shown in Table (4). In general, fish that received endophytic bacteria from fenugreek have greater resistance than the other treatments, where deathal in the eleven days was comparable to the control in twelve days.

Significant differences were observed between the methods of adding endophytic bacteria. The addition of endophytic bacteria with feed recorded higher growth performance indices than the addition of endophytic bacteria in rearing water, a significant increase in the mixture treatment, an insignificant increase in fenugreek and brassicales, and insignificant decrease in onion as shown in Fig. (1). Also, the addition of endophytic bacteria with feed recorded a higher percentage of protein in fish muscles than the addition of endophytic bacteria in rearing water except in brassicales-endophytic bacteria (Table 2). In all treatments, the addition of endophytic bacteria in rearing water recorded lower values of creatinine and ALT, as well as in AST (except for onion-endophytic bacteria) than the addition of endophytic bacteria with the feed. While the mixture of endophytic bacteria with feed recorded lower albumin concentration (in all treatments except the mixed treatment); and urea concentration (in all treatments except for brassicales-endophytic bacteria) compared to the addition of endophytic bacteria in rearing water, as shown in Table (3). The difference between the two methods used (mixing with feed or rearing water) may be due to the difference in the number of endophytic bacteria that pass inside the fish's body. Opiyo *et al*. (2019) recorded that the probiotics have different effects depending on the level of application; therefore it was necessary to estimate the amount of microbes inside the fish's organs, or may be the mixing of bacteria with food raises the nutritional value of the diet, so it was necessary to conduct a careful analysis of the diet after mixing. Despite these differences, the results showed, in general, the possibility of using both methods. This has been authenticated in a challenge test with *Vibrio parahaemolyticus*. All treatments recorded high resistance (20% mortality rate after fifteen days of challenge testing), but the addition of bacteria with feed had greater resistance than bacteria added to rearing water as shown in Table (4). Fish that received endophytic bacteria from fenugreek had greater resistance than the other plants. As well as fenugreek has higher weight gain, specific growth rate and feed conversion efficiencies compared to the other plants. This may be because the endophytic bacteria are affected by the genotype, morphology and life history of medicinal plants (Mitter *et al*., 2013).

Table 4. Mortality rate of *Oreochromis niloticus* injected with *Vibrio parahaemolyticus* for more than fifteen days after forty days of feeding with endophytic bacteria

Days	C	O	OL	F	FL	B	BL	M	ML
1	0	0	0	0	**1**	**1**	**1**	**1**	**1**
2	0	0	**1**	0	**1**	0	**1**	0	**1**
3	0	0	**1**	0	0	0	0	0	0
4	0	0	0	0	0	0	0	**1**	0
5	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0
7	0	**1**	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	**1**	0	0	0
11	0	0	0	**1**	0	0	0	0	0
12	**1**	**1**	0	0	0	0	0	0	0
13	0	0	0	**1**	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0
Total death	**1**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**
Mortality rate (%)	**10**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**

C, O, OL, F, FL, B, BL, M and ML are illustrated in Table (1)

CONCLUSION

The addition of endophytic bacteria of onion, brassicales and fenugreek improved the growth performance of Nile tilapia, raises protein concentration in fish muscles, and improves the biochemical profiles of fish blood. This indicates the possibility of using these bacteria in the aquaculture of Nile tilapia. However, further study is needed to elucidate the optimal bacterial concentration necessary for tilapia growth.

Declarations

Ethics approval and consent to participate: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors. All the contributing authors consent to participate in this study.

Competing interests: The authors have no conflicts of interest to declare.

Authors Contributions: SMA, EMY and HAAE contributed to the study design and preparation of the materials. SMA performed the microbial and aquaculture experiments. EMY performed the proximate composition. HAAE performed fish serum biochemistry. SMA, EMY and HAAE have read and approved the final manuscript.

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Data availability: All data generated or analyzed during this study are included in this published article.

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