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Effect of adding isolates of probiotic bacteria *Lactobacillus acidophilus* 4453 and *Streptococcus thermophiles* 5935 on Performance of common carp fingerlings *Cyprinus carpio* linnaeus (1758)

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Abstract

This study was conducted to evaluate the efficiency of isolates of probiotic bacteria *Lactobacillus acidophilus* 4453 and *Streptococcus thermophiles* 5935 on performance of common carp fingerlings which had initial body weight attained (44±0.69) g. A total of 72 fish were used and randomly divided into 4 treatments with 3 replicates (6 fish per replicate). An experimental diet was prepared with a crude protein attained (29.14%) and gross energy of 417.95 kcal / g. The probiotics were prepared in the form of bacterial suspensions at a dilution of (10^{-7} cfu/ml) and were added to the suspension of 20% Arabic gum and phosphate buffer salt as an enveloping and adhesive material of bacterial cells to increase their stability and survival on feed pellets. The liquid probiotics were added to specified amounts of the experimental diets according to the following treatments (T0 control treatment, T1 *Lactobacillus acidophilus* 4453, T2 *Streptococcus thermophiles* 5935, and T3 *Lactobacillus acidophilus* 4453 + *Streptococcus thermophiles* 5935). Fish performance was assessed after 83 days of the experiment according to weight gain, daily growth rate, relative growth rate, specific growth rate, metabolic growth rate, feed conversion rate, feed conversion efficiency, and protein efficiency ratio. The results of the statistical analysis showed that all the probiotic treatments were significantly exceeding as compared with T0 in the following order T1 and T3 with no significant difference followed by T2 of the weight gain, daily growth rate, relative growth rate, specific growth rate, and metabolic growth rate, whereas T1, T2, and T3 gave the best feed conversion rate, feed conversion efficiency and protein efficiency ratio. Therefore, we conclude that the single and multi-bacterial isolate probiotics of *Lactobacillus acidophilus* 4453 and *Streptococcus thermophiles* 5935, within the conditions of this study, were highly influential in achieving the best utilization of the food intake and the best performance of common carp fingerlings.

Keywords : common carp , fingerlings , bacterial isolates , probiotic , performance, *Lactobacillus acidophilus* 4453 , *Streptococcus thermophiles* 5935.

Introduction

Lactic acid bacteria are a microorganism used as a probiotic in aquaculture and also colonize naturally in the gut of healthy fish (Iman *et al*.,2014). Most species are harmless, have beneficial effects on fish health, and have anti-pathogen activity (Gildberg *et al*.,1998). Most strains of lactic acid bacteria belong to the genus Lactobacillus and they are characterized by their ability to decompose organic matter, reduce ammonia, and inhibit the growth of pathogens by competing for binding sites within the gut (Nikoskelainen *et al*.,2001) and producing organic acids such as formic acid, acetic acid, lactic acid, hydrogen peroxide, and several other compounds, and stimulating the immune system of fish (Lara-Flores *et al*.,2010). The term "Probiotic" comes from the Greek suffixes pro and bios, which means supportive or life-enhancing (Schrezenmeir and DeVrese,2001). Probiotics are defined as beneficial microorganisms that beneficially influence host physiology by improving gut microbial and nutritional balance and systemic immunity (Geng *et a*l.,2012). It is also defined as whole, live, or dead microorganisms, parts, or extracts of it, when taken in an appropriate dose, enhances growth performance, feed conversion rate, and increases disease resistance (Hoseinifar *et al*.,2016). In the field of aquaculture, probiotics have been used to combat diseases and improve growth and in some cases, as an alternative to antibiotics as they are characterized by their ability to produce antibiotic compounds that inhibit the growth of pathogens, competition for binding sites and food, changes in the enzymatic activity of pathogens, as well as their role in immune stimulation, furthermore, it has a role in improving digestion and benefiting from food (Khalafalla,2013).The current study aims to evaluate the effect of using isolates of probiotics bacteria *Lactobacillus acidophilus* 4453 and *Streptococcus thermophiles* 5935 on performance of common carp fingerlings.

Material and methods

Fish

A total of 72 common carp fingerlings were brought from a local hatchery in Babylon governorate, with an average weight attained (44 ± 0.69) g . to the experimental site, fish were placed in a 3% salt bath for 5 minutes, then they were placed in floating cages until they were distributed to the experimental cages. After that, it was adopted for 14 days, as the fish were starved for three days, after that food was given to them at a rate of 1% of the biomass weight in each cage (two meals per day).

Environmental measurements

The following physicochemical parameters of water were measured during the duration of the experiment such as Water temperature, Dissolved oxygen, pH, Salinity, and total dissolved substances. Periodic measurements were made to assess the quality of the water and to indicate its suitability for fish rearing.

Field experiment

The experiment was carried out at the first agricultural research and experiment station in the Um Al-Akf region in Al-Muthanna Governorate for the period from 1/9/2021 to 3/6/2022 in an earthen pond with a length of 45 m, a width of 35 m, and a depth 1.5 m, and according to the coordinates. N.45.189309, E,31.321394, the pond was supplied with water by an electric pump with a capacity of 40 HP installed on the side of the Al-atshan river and pumped the water through a pipe with a diameter of 8 inches and with a tight nozzle covered with a plastic buckle to prevent the entry of aquatic organisms and foreign bodies coming from the river into the pond, the water partially and continuously changed from the pond, An iron bridge covered by wood with dimensions 24 m long and 60 cm wide, in the shape of the letter (T) was installed on the pond to facilitate the process of feeding, weighing fish and taking samples of water for the environmental measurements. A total of 12 Circular plastic cages with a diameter of 50 cm and a depth of 65 cm were equipped. As for the floating structure, plastic tubes with a diameter of 10 cm in a rectangular shape were used to make a plastic frame with dimensions 2.5 m x 1.250 m. A rectangular wooden board with dimensions 2.5 m x 1.250 m was attached to the plastic frame. Holes were made on that wooden board with a diameter of 53 cm to insert the cages from it and settle them in the water. The covers of the cages were made of an iron frame with a diameter of 50 cm and covered with a plastic net with a diameter of 0.5 cm was fixed on it to protect them from birds and at the same time allow food to be served through them.

Manufacturing of the experimental diet

The materials of the experimental diet as shown in table (1) were supplied from the local markets, as its were well crushed after knowing their chemical composition, as mentioned in table (2), and then a mixture with equal content of protein and energy was prepared, and then mixed well to ensure its homogeneity. After that, the mixture was pressed into pellets in Al-Diwaniyah Roughages Factory located in the south of the Diwaniyah Governorate into pellets with a diameter of 3 mm and then left to air-dry outside the factory. After drying, it was packed in plastic bags of 50 kg until use. A sample was taken from it for analysis to know its chemical composition, as shown in table. (1).

Table (1) Ingredients of experimental diet

No	Ingredients	percentage in the diet (%)
1	Soy bean meal	40
2	AL-Wafi protein concentration	20
3	Wheat bran	15
4	Corn	15
5	Barley	5
6	Wheat flour	3
7	Premix	1
8	Oil	1

Chemical composition of diet	
Component	**Result (%)**
Moisture	5.03
Dry matter	94.97
Crude protein	29.14
Crude fat	1.74
Crude fiber	4.48
Nitrogen free extract (NEF)	51.1
Ash	8.51
Gross energy (Kcal/g) *	417.95
Digestible energy (Kcal/g)**	313.46
Metabolizable energy (Kcal/g)***	341.26
Protein : Calorie ratio ****	92.96

* **Gross energy (Kcal/g)** it was calculated according to (NRC,1993) by using factors 5.65, 9.45 and 4.22 Kcal/g of protein, lipid and carbohydrate, respectively.

** **Digestible energy (Kcal/g)** it was calculated by applying the coefficient of 0.75 to convert gross energy to digestible energy according to (Hepher *et al*.,1983).

*** **Metabolizable energy (Kcal/g)** it was calculated using a value of 4.5 Kcal/g proteins, 8.51 Kcal/g fat and 3.48 Kcal/g carbohydrates according to (Jauncey and Ross,1982).

**** **Protein : Calorie ratio** it was calculated by below equation

P:E = crude protein x 10000/digestible energy, according to (Hepher *et al*.,1983).

Preparation of probiotics

Bacterial isolates *Lactobacillus acidophilus* 4453 and *Streptococcus thermophiles* 5935 were obtained from Al-Ameen Center for Biotechnology in Al-Najaf Province and diagnosed according to biochemical tests. After that, an antagonistic test was conducted between the bacterial isolates to ensure their compatibility with each other, as the study requires a double combination of them in the preparation of a probiotic. This test was conducted by using the cross streaks method, as the bacterial isolates were grown in the form of two straight lines intersecting with a sign (X) on a Petri dish containing the nutrient agar medium, and then the dishes were incubated at a temperature 30 °C for a period 48 hours. The probiotics were prepared according to the method mentioned by Mohapatra *et al* (2014) with some modifications. the bacterial isolates were activated by using 30 test tubes of each bacterial isolate, each test tube containing 9 ml of nutrient broth, and then each tube inoculating with 1 ml of each isolate, then incubating at 30 °C. ° for 48 hours until the density of the medium changes from clear to turbid, then the tubes isolates were centrifuged at 6000 rpm for 20 minutes, The supernatant was discarded and the bacterial particles were washed by using phosphate buffer salt (PBS: pH=7.2), then a decimal dilution process was performed to reach the required dilution (10^{-6}), the number of colonies forming units (cfu) was measured of each probiotic by Pour plate method on nutrient agar medium. the bacterial colonies were counted manually within

the range (30-300) of bacterial colonies (Al-Dulaimi,1988) by using the APD Colony App Lite. then, the probiotics were formed with the bacterial combinations mentioned in table (3) In equal proportions (1) for the first and second probiotics and (1:1) for the third probiotics with final cfu ($1*10^{-7}$ cfu/ml) In the form of final treatments T0 without any addition, T1: *Lactobacillus acidophilus* 4453 ,T2: *Streptococcus thermophiles* 5935 and T3: *Lactobacillus acidophilus* 4453 + *Streptococcus thermophiles* 5935.

Table (3) Bacterial combination of probiotics

Probiotics	**Bacterial isolates**	
	***L. acidophilus* 4453**	***S. thermophiles* 5935**
Probiotic 1	✓	-
Probiotic 2	-	✓
Probiotic 3	✓	✓

After that, a suspension of 20% of Arabic gum-phosphate buffer salt was used as an enveloping and adhesive material for bacterial cells to increase their stability and survival on feed pellets (Fazilah *et al*.,2019), the suspension was prepared by dissolving 20 g of Arabic gum powder in 100 ml of a phosphate buffer salt solution and then 10 ml of it was added to a 60 ml test tube, the probiotics with the combinations indicated in the table (3) were added at a rate of 10 ml per 100 g to a specified amount of diet as a carrier for the preparation probiotics of (15) days, the probiotics were mixed with the experimental diet according to the method mentioned by Wanka *et al* (2018). the experimental diets were left to dry at a temperature of 25° C, probiotics were kept at a degree of (4)° C. The fish were fed on them daily at a feeding level of 5% of the live mass.

Growth parameters

Weight gain = Final Weight- Initial Weight

Daily growth rate = Final Weight- Initial Weight/ ΔT (Schmalhousen ,1926).

Relative Growth Rate = Final Weight*Initial Weight/ Initial Weight (Uten,1978).

Specific growth Rate = Ln Final Weight- Ln Initial Weight / ΔT (Brown,1957).

Metabolic growth Rate = Weight gain (g) /[**{(** Initial Weight/1000)$^{0.8}$ + Final Weight/1000)$^{0.8}$ }/2]/study duration (g/kg 0.8/d) (Dabrowski *et al*.,1986).

Feed Conversion Ratio = weight of food intake / weight gain of fish (Uten,1978).

Feed conversion efficiency = weight gain of fish / weight of food intake *100 (Uten,1978).

Protein Efficiency Ratio = Weight gain / protein intake (Gerking,1971).

Statistical analysis

The statistical program SPSS version (26) was used to analyze the data according to the Complete Randomized Design (CRD) ,significant differences between means were

tested by using (Duncan,1955) multiple range test, at a significance level of (0.05).

Results and discussion

Environmental measurements

The water temperature ranged between (16.5-30) °C, while the dissolved oxygen values ranged between (7.2-7.8) mg/L. The pH also recorded values within the range of (7-8.1) and the salinity values ranged between (4.981-6.730) g/L, and the total dissolved substance values ranged between (3015-4105) mg/L. The values of environmental factors recorded during the study period are considered appropriate for the growth of common carp, according to Froese and Pauly (2011) who mentioned that common carp can tolerate a wide range of temperatures ranging from (3-35) °C, and according to Al-Salman (2000) who indicated the minimum level of dissolved oxygen in the water of Cyprinidae is not less than 3 mg/L. according to FAO (2018), common carp is also characterized by its ability to live within a range of pH ranging from (6.5-9), Mangat and Hundal (2014) also indicated the possibility of rearing common carp fingerlings in coastal waters with salinity exceeding more than 6 ppt with a survival rate attained 100%. As for the values of the total dissolved substance recorded during the experiment period, it was considered within the normal limits of fish rearing as common carp can resist high concentrations of it up to 20000 mg/L (Al-Salman,2000).

Growth parameters

Table (4) shows significant differences (P≤0.05) between the experimental treatments**,** as all probiotic treatments, were exceeding than T0 of some parameters, T1 and T3 were exceeded on other treatments with no significant differences (P≤0.05) between them and recorded the highest means of weight gain attained (179.31±2.6)g (180.21±2.06)g, and for Daily growth rate attained (2.13±0.03)g/day (2.14±0.02) g/day , and for Relative growth rate attained (408.40±5.82)% (412.92±2.84)% , and for Specific growth rate attained (1.93±0.01) %/day (1.94±0.006) %/day , Metabolic growth rate attained (11.13±0.07) g/kg/day (11.18±0.04) g/kg/day respectively, as well as, T1,T2and T3 were exceeding than T0 on Feed conversation rate and recorded the highest means of it attained (1.82±0.04)g (1.90±0.02) (1.83±0.0)g, Feed conversation efficiency (54.83±1.42)% (52.54±0.56)% (54.42±0.97)%, and Protein efficiency ratio (1.88±0.04) (1.80±0.01) (1.86±0.03) respectively with no significant difference(P≤0.05) between them. The reason of exceeding of probiotic treatments may be due in weight gain, Daily growth rate, Relative growth rate and Specific growth rate to what mentioned by Ayyat *et al*. (2014) who found that the use of the bacterial strains *Lactobacillus acidophilus*, *Streptococcus thermophilus* and *Bifidobacterium bifidum* in the diet of Nile tilapia *Oreochromis niloticus*, as achieved the highest value in the weight gain and improved food consumption with recording the lowest value of the feed conversion factor. Or may be due to the role of Lactic acid bacteria including the isolates used in this study in aquaculture which play an important role as they promote growth, immune response, disease resistance, regulate microbial balance within the gut, and inhibit pathogens (Zuo *et al*., 2018; Hoseinifar *et al*., 2019). Or may be due their production of a group of vitamins that are

important in many biological processes that occur in all living organisms, which includes two groups. the first group of fat-soluble vitamins which includes A, D, E, and K, vitamins and the second group of water-soluble vitamins which includes C and complex B vitamins such as Thymine, Riboflavin, Niacin, Pantothenic acid, Pyridoxine, Biotin, Folic acid and Cobalamin (Leblanc *et al*.,2013) that improve fish performance during the duration of the experiment.

As for the metabolic growth rate, the reason for the exceeding of probiotic treatments to this parameter may be due to the nature of the action of probiotics on modifying the community of microbiota that colonized within the gut of fish in association with environmental factors (Jauncey and Ross,1982) and modifying that community by increasing the colonization of beneficial probiotic bacteria which creates a kind of synergistic relationship between its, that is reflected in the production of some microbial metabolic products such as short-chain fatty acids, indoles, propionates, acetates, and butyrate (Zhang and Davies,2016) that affect the metabolism and also affect the modification of the secretory activity of enterocyte and thus affect the production of intestinal peptides that modulate intestinal motility and its enzymatic secretions (Borre *et al*.,2014) .this relationship may also result in an effect on neurotransmitters released from enteric neurons in the digestive tract of animals, such as serotonin, dopamine, and norepinephrine (Asano *et al*.,2012 ; Yano *et al*.,2015) which in turn influence feeding behavior, gastrointestinal motility, function, and hormone secretion (Yang and Chiu,2017 ; Strandwitz,2018) and this indicates an increase in the surface of absorption within the gastrointestinal tract that increases the absorption of nutrients.As for Feed conversation rate, Feed conversation efficiency and Protein efficiency ratio, we noticed all probiotic treatments, were exceeding on T0 of mentioned parameters and reason may be due to the action of probiotics is ambiguous, as it is believed that they may increase the fish's feeling of hunger or that they stimulate the fish's appetite and improve the digestion of indigestible food compounds, thus achieving the benefit of nutrients and increasing their absorption capacity and increasing the production of vitamins and removing toxins from foodstuffs. It is also believed that the mechanism of its action It combines the aforementioned things (Irianto and Austin, 2002; Krishna *et al*.,2018).

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Table (4) Some growth parameters (mean ± standard error) of common carp fingerlings fed on probiotics during the duration of the experiment

Studied parameters	Experimental treatments			
	T0	T1	T2	T3
Initial weight (g)	43.64±0.24	43.90±0.14	44.52±0.37	43.64±0.22
Final weight (g)	180.80±0.60 c	223.22±2.70 a	213.36±1.67 b	223.85±2.27 a
Weight gain (g)	137.15±0.42 c	179.31±2.6 a	168.84±1.99 b	180.21±2.06 a
Daily growth rate (g/day)	1.63±0.005 c	2.13±0.03 a	2.01±0.02 b	2.14±0.02 a
Relative growth rate (%)	314.25±1.38 c	408.40±5.82 a	379.35±7.52 b	412.92±2.84 a
Specific growth rate (%/day)	1.69±0.003 c	1.93±0.01 a	1.86±0.01 b	1.94±0.006 a
Metabolic growth rate (g/kg/day)	9.71±0.01 c	11.13±0.07 a	10.76±0.09 b	11.18±0.04 a
Food intake (g)	296.36±4.47 b	327.67±12.58 a	321.51±7.23 ab	331.27±4.49 a
Feed conversation rate (g)	2.16±0.02 b	1.82±0.04 a	1.90±0.02 a	1.83±0.03 a
Feed conversation efficiency (%)	46.29±0.59 b	54.83±1.42 a	52.54±0.56 a	54.42±0.97 a
Protein efficiency ratio	1.58±0.02 b	1.88±0.04 a	1.80±0.01 a	1.86±0.03 a

Conclusion

The two isolates of probiotic bacteria *Lactobacillus acidophilus* 4453 and *Streptococcus thermophiles* 5935 and the dual combination of its and within the conditions of this study were highly effective in achieving the best performance of common carp fingerlings

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