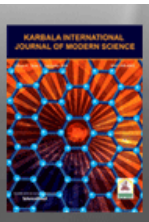

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Seasonal variation of microbiota in Shank Acanthopagrus latus living in both brackish and fresh water

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Seasonal variation of microbiota in Shank Acanthopagrus latus living in both brackish and fresh water

Abstract

The present study was conducted to monitor the microbiota in the posterior intestine of Shank fish living in the river (fresh water) and Al-Razzaza lake (salt or brackish water) in Kerbala, Iraq. Cultivable bacteria were calculated and identified from specimens obtained from the posterior intestine of mucosa (PM) and digesta (PD) during summer and winter seasons. The total culturable bacteria (TCB) of bacteria isolated from both intestinal regions from fresh water fish during summer time were higher than counterparts in winter time. In contrast, the TVC of bacteria isolated from the PM from salt water fish during winter time were higher than those reported in summer time for the same region.

Up to 13 species of bacteria were identified from the posterior intestine. *Aeromonas hydrophila* was the predominant microbe isolated in high percentages from all samples (e.g PD, PM, summer, winter, river and lake). *Grimontia hollisae, Pasteurella multocida, Enterobacter cloacae* and *Pseudomonas fluorescens* were isolated from the PM of fresh water fish during summer time. *Brucella* spp. were found only in PD of fish from salt water during summer time.

The mean log of lactic acid bacteria (LAB) in the PD varied between 5.4 ± 0.5 and 4.5 ± 0.1 CFU g^{-1} during summer and winter in the river location. The mean log of LAB populations in the PM ranged from 4.3 ± 0.9 to 5.9 ± 0.1 CFU g^{-1} during the seasons of summer and winter in the river location. The LAB population in the both regions of intestine in Shank living in the lake during both seasons were completely absent.

In conclusion, seasonal variation in river and lacustrine temperature and salinity influenced the TCB and LAB population and distribution of bacterial species during the study.

Keywords

Shank • Al-Razzaza lake • Posterior intestine • Lactic acid bacteria • Total viable count

1. Introduction

The gastrointestinal (GI) microbiota is a complex and dynamic microbial community that colonizes the gastrointestinal tract of animals. This complex microbial community is beginning to be identified in many fish species using molecular and biotechnological approaches.

The microbial community of fish can be divided into two major groups: autochthonous or indigenous bacteria which are firmly associated to the mucosal surface of the GI tract, and allochthonous or non-resident meaning microbes are rejected after some time because they are unable to adhere to the mucosal surface of the GI tract [1,2]. This microbial community comprises of bacteria that are aerobic, optional anaerobic and compulsory anaerobic, yeasts, viruses, and Archaea [3−5].

Microbiota of the GI tract of fish perform several important functions, e.g. aiding digestive function by promoting nutrient supply with enzymes, amino acids and vitamins [6], preventing colonization of pathogens by producing antibacterial materials, maintaining mucosal immunity [4] and is implicated in the improving of immune function [7].

In fish, microbiota of GI tract is sensitive to a series of exogenous and endogenous determinants including the GI structure, water environment factors (e.g. water chemistry, temperature, salinity) [8], the rearing environment and condition stress [9]. Furthermore, seasonal fluctuations in the rearing water are reported to affect the structure of GI microbiota [10,11].

Bacteria are deemed an important part of the fish of which the majority come from the water environment, soil or sediment, and ingested food [12]. Different growth phases, water habitat, and host trophic level are thought to influence the composition and diversity of GI microbiota of a fish species [13].

It is possible that differences in surrounding environments lead to variation in fish resident microbiota [1]. Previous studies have confirmed that diversity and structure of fish GI microbiota are strongly correlated with salinity [14].

Sea bream (*Acanthopagrus latus*), known locally in Iraq as Shank, has been widely distributed both in salty and fresh water by accident through an attempt to cultivate desirable fish species from Shatt Al-Arab in the south of Iraq to the Al-Razzaza lake in Kerbala city [15].

Shank is a marine fish belonging to the family Sparidae which is considered the most commercially important marine fish in Iraq due to easy adaptation for living in water bodies.

Due to colonization by a wide range of microbes, the distal part of the gut in fish is a favorable ecological niche for microorganisms [16].

Although the presence of native GI tract microbiota in different species of fish has been recognized, to the authors' knowledge, there is no information available on the association of gut microbiota in Shank living in the environment of Iraq.

Therefore, this study was conducted to describe the influence of variations of environmental seasonal on the bacterial composition in the GI of wild Shank (*A. latus*) by the application of conventional microbiological methods.

2. Materials and methods

The water temperature and salinity of river and lake were measured during the study. During the sampling period from winter 2014 to summer 2014, temperature degrees of water were 16 °C and 31 °C in winter and summer, respectively. The Total Dissolved Solids (T.D.S.) of lake were 12,904 mg/L and 37,194 mg/L in winter and summer, respectively. On the other hand, the T.D.S of Hindya river were 600 mg/L and 760 mg/L in winter and summer, respectively.

2.1. Fish sampling and dissection

Shank fish were collected from a local river called Hindya river and Al-Razzaza lake in Kerbala, Iraq during summer and winter. Fish from each sampling were transferred alive to the laboratories of College of Sciences, Kerbala University by using polythene bags filled with oxygenated water. Once in the laboratory, fish were euthanized by an overdose (4 drops in 100 ml of water) of clove oil (GOPALDAS VISRAM & CO. LTD, INDIA) followed by hit on the brain. Six fish from each location at each season time were dissected as described elsewhere in the study of Al-Hisnawi et al. [17]. In order to reduce variation three segments of each section were pooled into one sample and analyzed for bacterial community [18,19]. After that, mucosa and digesta materials from each sample were immediately processed for conventional microbiological analysis.

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2.2. Microbiology

2.2.1. Isolation of intestinal bacteria

For bacteriological studies, intestinal homogenates were serially diluted in PBS up to 10^7 and aliquots of 100 µL of the appropriate dilutions for each homogenized gut sample was plated in triplicate on to tryptone soy agar (TSA; Oxoid) and de Man, Rogosa and Sharpe agar (MRS; Oxoid) to determine total culturable bacteria (TCB) and lactic acid bacteria (LAB), respectively. The incubation temperature was 16 ± 1 °C and 36 ± 1 °C in winter and summer time, respectively for 24–48 h. The log of total cultivable bacteria present in the samples was calculated by counting colony-forming units per gram from plates.

2.2.2. Identification of bacterial isolates by conventional isolation techniques

A total of 160 and 80 colonies of TCB and LAB having apparently different color and shape were randomly selected and purified by streaking and re-streaking on fresh media until purity was achieved.

Bacterial isolates were first placed into genera or species on the basis of colony shape, cell shape, Gram stain, movement, endospore formation, ability to produce catalase, oxidase, and glucose fermentation [20,21]. Furthermore, analytical profile index API 20NE bioMérieux France tests (which is a classification of bacteria based on biochemical tests, allowing fast identification) were conducted according to the manufacturer's instructions.

2.2.3. Identification of LAB isolates by conventional isolation techniques

LAB isolates were examined for physiological properties including gas production from glucose, their capability for growing in MRS broth at 10 °C for 10 days, at 45 °C for 48 h, at pH 9.6 for 48 h. Furthermore, they were tested with biochemical tests mentioned above. In addition, API 20NE/E tests of LAB were carried out in accordance with the recommendations from the producer (BioMerieux, USA), [22].

3. Statistical analysis

All results are presented as mean ± SD of the replicates. An independent two samples t-test was applied to search for the significant differences for TCB and LAB in each gut region between two seasons in each type of water. The statistically significant differences in microbial community (TCB or LAB) in gut regions between both seasons and type of water were tested by use of two-way ANOVA followed by Duncan's multiple range tests. All statistics were conducted using MiniTab statistical software version 17 (IBM, Pennsylvania, USA). The accepted levels of significance in all cases were $P < 0.05$.

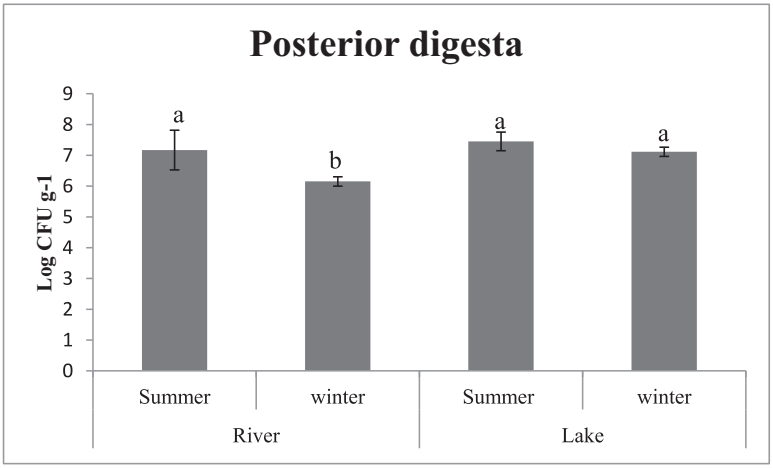
4. Results

4.1. Isolation of intestinal bacteria

The microbial structure of digesta and mucosa of the posterior intestine in Shank was investigated during season time (summer and winter) for fish living at both river and lake locations. The results of the TCB in the PD of Shank at both river and lake are presented in Fig. 1. The TCB counts in the PD during the study period ranged from log 7.2 ± 0.6 and 6.2 ± 0.2 CFU g^{-1} in the summer and winter to log 7.5 ± 0.3 and 7.1 ± 0.1 CFU g^{-1} in the summer and winter at both fresh and salt water, respectively. Significant differences in the TCB levels in the PD were found between the summer and winter times for fish living in the river ($P < 0.05$). In contrast, the TCB levels were not significantly different between summer and winter in fish living in the lake. Furthermore, the bacterial counts were significantly higher in the lake ($P < 0.05$) compared to those reported in winter time from fish in the river. Significant differences were also observed between the TCB levels in respect of season time ($P < 0.05$) and type of water (river and lake) ($P < 0.05$) but there were no significant differences for an interaction effect ($P < 0.05$) (Fig. 1).

The results of TCB in the PM of Shank at both fresh and salt water are presented in Fig. 2. The mean log of TCB counts in the PM during the study period ranged between 6.1 ± 0.4 and 4.5 ± 0.1 CFU g^{-1} in the summer and winter to 3.6 ± 0.2 and 4.7 ± 0.2 CFU g^{-1} in the summer and winter at river and lake, respectively. The highest counts of TCB were found in the PM of Shank living in the river during summer time which were significantly higher compared to other season at the river and the lake locations. TCB levels were significantly lower in Shank during summer time living in the lake compared to other season at the river ($P < 0.05$). TCB levels were not affected by season time ($P < 0.05$), but were affected by type of water (river and lake) ($P < 0.05$), in addition an interaction effect ($P < 0.05$) was shown (Fig. 2).

The results of the LAB in the PD of Shank at both the river and the lake are presented in Fig. 3. The mean log of LAB counts in the PD during the study period ranged between 5.4 ± 0.5 and 4.5 ± 0.1 CFU g^{-1}

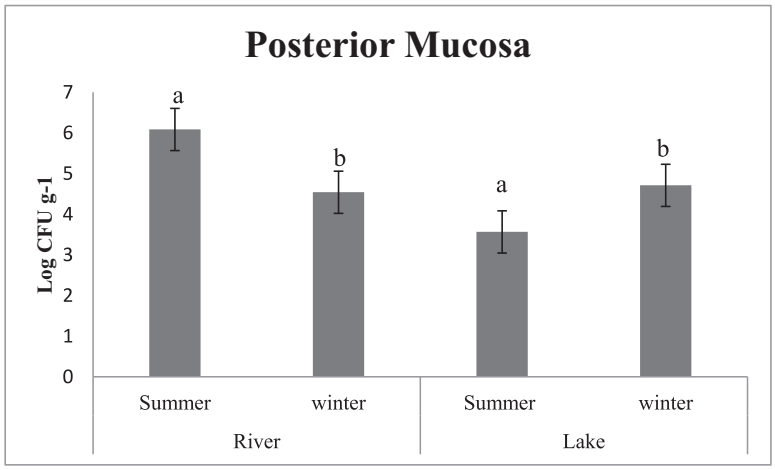


Two-way ANOVA						
	P value		Seasons		Type of water	
Seasons	Type of water	Interaction	Summer	winter	River	Lake
0.017	0.025	0.169	a	b	b	a

Fig. 1. Number of the TCB isolated from the PD of Shank fish living in the river (fresh water) and the lake (salt water) during the summer and winter time. Results are presented as mean log values ± SD in each time (n = 3). Bars which do not share letters within each type of water are indicated for significant differences ($P < 0.05$). The table displays the two-way ANOVA analysis of season, type of water and interactions.

during the summer and the winter respectively, in the river location. The LAB population in the PD from Shank fish living in the lake were completely absent.

The results of the LAB in the PM of Shank at both the river and the lake are presented in Fig. 4. The mean log of LAB counts in the PM during the study period ranged between 4.3 ± 0.9 and 5.9 ± 0.1 CFU g^{-1} during the summer and the winter respectively, in the river location. Similar to the PD, the LAB population in the PM from Shank were completely absent in the



Two-way ANOVA						
	P value		Seasons		Type of water	
Seasons	Type of water	Interaction	Summer	winter	Lake	River
0.320	0.000	0.000	a	a	a	b

Fig. 2. Number of the TCB isolated from the PM of Shank fish living in the river (fresh water) and the lake (salt water) during the summer and the winter time. Results are displayed as mean log values ± SD in each time (n = 3). Bars which do not share letters within each type of water are indicated for significant differences ($P < 0.05$). The table displays the two-way ANOVA analysis of season, type of water and interactions.

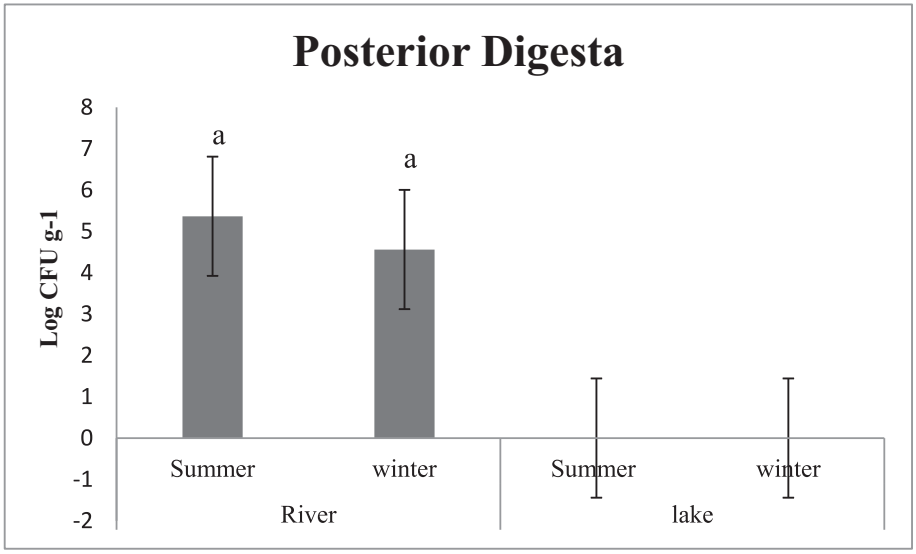


Fig. 3. Number of the LAB isolated from the posterior digesta of Shank fish living in the river (fresh water) and the lake (salt water) during the summer and the winter time. Results are displayed as mean log values ± SD in each time (n = 3). Bars which do not share letters within each type of water are indicated for significant differences ($P < 0.05$).

salt water. For fish living in the river, no significant variations in LAB levels in the PD were identified between summer and winter time ($P > 0.05$).

4.2. *Identification of bacterial isolates by conventional isolation techniques*

Representative pure isolates of TVC recovered from the posterior intestine of Shank during summer and winter living in the river and lake were identified to genus or species level and their proportion distribution is presented in Table 1. *Aeromonas hydrophila* was the predominant microbe isolated in high percentages from all samples (e.g. PD, PM, summer, winter, river and lake). *Hafnia alvei, Escherichia coli, Enterobacter amnigenus, Klebsiella terrigena* and *Bacillus* spp. were present in most of intestine regions and seasons in the river and the lake in different percentages. *Grimontia*

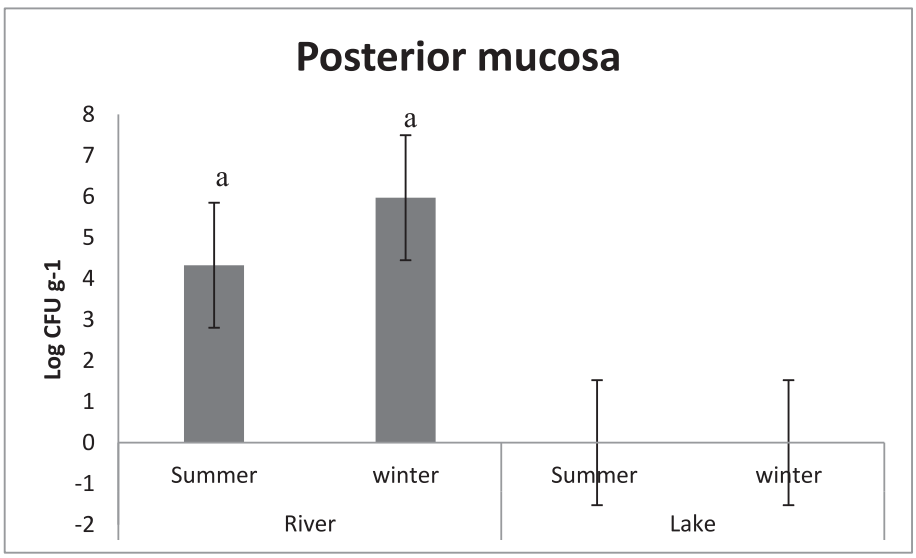


Fig. 4. Number of the LAB isolated from the PM of Shank fish living in the river (fresh water) and the lake (salt water) during the summer and the winter time. Results are displayed as mean log values ± SD in each time (n = 3). Bars which do not share letters within each type of water are indicated for significant differences ($P < 0.05$).

Table 1
Composition percent of the total culturable of bacteria in the distal digesta of Shank fish living in the river and the lake during both seasons of the year.

Bacterial isolates	River				Lake			
	Summer		Winter		Summer		Winter	
	PD%	PM%	PD%	PM%	PD%	PM%	PD%	PM%
Grimontia hollisae	10							
Pasteurella multocida	10							
Enterobacter cloacae	10′							
Aeromonas hydrophila	50	33.3	85	45.5	71.4	40	10	10
Enterococcus faecium	10							
Hafnia alvei	10		5	9.1			10	
Pseudomonas fluorescens		33.3	10					
Brucella spp.					14.3			
Vibrio fluvialis	10				14.3			
Escherichia coli				9.1			30	10
Enterobacter amnigenus				9.1			10	10
Klebsiella terrigena		33.3		9.1				10
Bacillus spp.				18.2		60	40	60

hollisae, Pasteurella multocida, Enterobacter cloacae and Enterococcus faecium were present only in the PD of Shank living in the fresh water of summer time present in 10% of samples. *Pseudomonas fluorescens* was isolated only during summer from PM of fish living in fresh water (Table 1). *Brucella* spp. was found only in PD of fish living in salt water during summer time.

4.3. Identification of LAB isolates by conventional isolation techniques

Representative pure isolates of LAB recovered from the posterior intestine of Shank during summer and winter living in the river and lake were identified to genus or species level and their proportional distribution are presented in Table 2. *Klebsiella pneumoniae* was the only bacterial species isolated from all intestine regions of fish reared in the river during both seasons. *E. cloacae* were predominant bacterial species in all intestinal samples except PM of fish living in the river during winter time. *E. faecium* and *E. coli* were isolated only from the PM of fish living in the river during summer and winter season. *Kluyvera* spp., *Enterobacter sakazakii* and *Klebsiella oxytoca* were isolated from fresh water fish during summer time. Finally, *Streptococcus* spp. were the most predominant microbe in the PM of fish during winter time.

5. Discussion

5.1. Isolation of intestinal bacteria

The obtained data showed that the bacterial numbers increased during summer season compared to the winter season for TCB and LAB at both river and lake (salt water). The high numbers of bacterial populations present in almost all samples at summer

Table 2
Composition percent of the LAB in the distal intestine of Shank fish living in the river and the lake during both seasons.

Bacterial isolates	River				Lake			
	Summer		Winter		Summer		Water	
	PD%	PM%	PD%	PM%	PD%	PM%	PD%	PM%
Enterobacter cloacae	28.6	41.7	66.7					
Klebsiella pneumoniae	28.6	16.7	33.3	11.1				
Kluyvera spp.	28.6							
Enterobacter sakazakii	14.3							
Enterococcus faecium		25		33.3				
Escherichia coli		18.3		22.2				
Klebsiella oxytoca		8.3						
Streptococcus spp.				33.3				

season might be associated with the increase of water temperature. Supporting the present data, Al-Harbi & Uddi [23] demonstrated that high levels of bacterial populations were associated with increased water temperature which was deemed to be optimum for many mesophilic bacteria. Furthermore, in accordance with the current results, Neuman et al. [24] showed that numbers of bacterial population generally increases with water temperature.

Numbers of bacterial population varied seasonally between the intestinal samples of fish living in fresh and salt water. In agreement, the intestinal microbiota in farmed Atlantic salmon varied seasonally [8]. Seasonal variation was also reported in the intestinal microbiota of hybrid tilapia in different seasons (autumn, summer and winter) [10]. A change in the rearing environment including water temperature can affect the composition of gastrointestinal tract microbiota [9,25].

LAB are a major component of commensal intestinal microbiota of healthy fish, though their composition depends on the rearing environment among seasons [11]. In the present study, LAB were completely absent in the lake during summer and winter. The intestinal structure of LAB within Atlantic salmon showed variation with seasons [26]. However, these studies examined the intestinal microbiota under water temperature only. Other studies have examined the impact of salinity on the composition of intestinal microbiota. For example, Sullam et al. [14] showed that the salinity had deep impact on the distribution of intestinal microbiota of fish and animals. In addition, Zhang et al. [27] reported the influence of different concentration of salinity on intestinal microbiota of Nile tilapia and Pacific white shrimp. The results showed that the composition of intestinal microbiota decreased when the concentration of salinity increased. In agreement with the present study, high populations of TVC in pond water, intestine of Nile tilapia and common carp and low populations of LAB were reported [28]. The reasons behind disappearance of LAB in the present study are not clear but might be attributed to temperature and salinity of the water which are considered the two important factors that influence the microbial community of GI in fish including LAB. However, further studies are needed to investigate this hypothesis.

It is worth noting that quality of fish is the main criterion for aquaculture industry from both producers and consumers. However, freshness is influenced by seasonal variation which in turn affects fish chemical composition [29]. As a result of the fact that surrounding temperature possibly affects the amount of deposited lipids, and content of fatty acids, these changes could affect the quality of fish [30]. In study of Ali et al. [31] variations of seasonal influence were found on the moisture, ash, fat and fatty acids, whereas the crude protein and minerals were not influenced by these conditions.

5.2. Identification of bacterial isolates by conventional isolation techniques

Up to 13 bacterial species were isolated and identified to species level from the intestinal samples of Shank and diversification was distinguished in both seasons. *A. hydrophila* were isolated in high percentages from posterior mucosa and digesta of Shank fish living in the river (fresh water) and lake (salt water) during winter and summer time. *A. hydrophila* as fish pathogens are Gram-negative, motile and produce a range of enzymes including arginine dihydrolase, catalase, β-galactosidase, 'tryptophanase', lysine decarboxylase, cytochrome oxidase, and phosphatase, and produce enterotoxin, which causes cell death and eventual tissue destruction [32].

Aeromonas spp. are the most common bacteria distributed in freshwater habitats and marine environments and *A. hydrophila* are the most common opportunistic pathogens of GI tract of fish which can cause several diseases when the conditions become unfavorable including big temperature changes, change in water quality, stress, higher densities and high levels of NO_3 and CO_2 [32]. These diseases including hemorrhagic septicemia and red sore disease [33]. Symptoms of these diseases are summarized as ulcers, abdominal inflation, accumulation of fluid, anemia, and hemorrhaging, which lead to huge mortality in fishes worldwide [34].

Different kinds of infections are caused by these bacteria in humans including gastroenteritis, hemolytic uremic syndrome, peritonitis, skin infections, bacteremia, meningitis, and necrotizing fasciitis [35]. *A. hydrophila* were isolated from different species of fresh water fish [10,23,17].

H. alvei, Vibrio fluvialis, E. coli, E. amnigenus, K. terrigena and *Bacillus* spp. were present in most seasons from fish living in salt and fresh water. All these bacteria were found in large numbers indicating that they may be prevalent bacterial species of the GI tract of Shank.

G. hollisae, P. multocida, E. cloacae and *E. faecium* were isolated from the posterior digesta of fish living in the river during summer only. This could be because of

suitable ambient temperature during summer season. Some of these bacteria including *A. hydrophila*, *E. coli*, *Vibrio* spp. and *P. fluorescens* are facultative pathogens.

Brucella spp. were present at very low frequency in posterior digesta of fish living in salt water during summer season. Supporting the present data, Ramos-Ramírez et al. [36] isolated *Brucella* spp. from water and skin of tilapia fish. The detection of Brucella in Shank fish in Iraq in the current study is associated with a public health risk for human beings [36].

Additionally, *V. fluvialis*, *E. coli* and *E. amnigenus* were present in winter season, while *K. terrigena* and *Bacillus* spp. were present in summer season in posterior digesta and mucosa of fish living in salt and fresh water.

Vibrio spp. have been reported to be a fish pathogen both in salt and fresh water which can cause different types of disease [32]. In agreement with the present study, previous studies have demonstrated the isolation of *Vibrio* spp. from salt and fresh water fish [34,8,7,37,38].

Furthermore, some of *Vibrio* spp. are deemed as important bacterial genera in aquaculture as probiotic (health-promoting).

Coliform bacteria and *Bacillus* spp. were also identified from different fish species [17,18,39]. Most bacterial genera in the current study were more frequently identified than in the previous studies which especially indicated that these bacteria are well adapted to their surrounding aquatic environment, both salt or fresh water [1].

5.3. Identification of LAB isolates by conventional isolation techniques

LAB belong to phylum Firmicutes which are Gram-positive, non-endosporing, non-motile with rod-shaped or coccid morphology, and have no ability to produce catalase- and/or oxidase enzymes [40].

Characteristics including production of inhibitory substances such as bacteriocins, hydrogen peroxide and short chain fatty acids (SCFAs) etc., and inhibition of reproduction of and reducing attachment and colonization of pathogens in the GI tract of animals are deemed as favorable traits of LAB [40,41].

In fish intestines, lactic acid bacteria (LAB) have also been found [39,18,11]. Several endogenous LAB strains were examined for their potential probiotic role [7]. In the present study, LAB were found in the posterior digesta and mucosa of fish living only in fresh water at both seasons. Ringø & Gatesoupe [42] demonstrated that environmental factors, stress, nutrition and salinity could affect the distribution of the LAB population. Furthermore, the same authors have reported that some other factors were related to the process of LAB isolation including the nutrient medium, the incubation temperature and incubation time. Therefore, it could be the high temperature beside high salinity hinder the growth of LAB in the summer. Further research using independent culture techniques are required to determine the impact of temperature changes and salinity on the gut microbiota of Shank fish.

Lactic acid bacteria (LAB) such as *E. faecium* are widely applied worldwide as probiotics because of their production of bacteriocins [43] and enzymes that enable fish to digest food, decreasing the acidity of the GI tract and thus enhancing the absorption of minerals [44].

The important capabilities of *E. faecium* which allow them to dominate the GT tract microbiota are their ability to tolerate high acidity and high concentration of bile salt in the environment and inhibit pathogenic bacteria either by high competitiveness for gaining nutrients or by production of antimicrobial substances [45].

6. Conclusions

The dominance of Proteobacteria and Firmicutes members was significantly noticed in the lake during summer and winter in the current study which reflects their tolerance to high concentrations of salt. The results obtained from the present study showed that the microbiota of apparently healthy Shank could be influenced by the surrounding environmental conditions. These results may contribute to understand the deep impact of environmental conditions on microbiota of fish in Iraq which could be considered as a data base for further studies.

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