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Bacterial flora of polycultured common carp (*Cyprinus carpio*) and African catfish (*Clarias gariepinus*)

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Abstract

Quantitative and qualitative analyses of bacterial flora associated with pond water, gills, and intestine of polycultured healthy common carp (*Cyprinus carpio*) and African catfish (*Clarias gariepinus*) were carried out and identified to species level where possible. Total viable bacterial counts in the pond water ranged from $9.2 \pm 5.5 \times 10^3$ to $6.6 \pm 5.1 \times 10^4$ colony-forming units (cfu)/mL; in the gill filaments of carp and catfish, $3.3 \pm 3.8 \times 10^6$ to $7.9 \pm 5.6 \times 10^6$ and $1.1 \pm 4.6 \times 10^5$ to $2.3 \pm 5.2 \times 10^6$ cfu/g, respectively; and in the intestine of carp and catfish, $1.4 \pm 2.9 \times 10^{10}$ to $1.7 \pm 6.0 \times 10^{11}$ and $2.7 \pm 3.4 \times 10^{10}$ to $1.0 \pm 4.5 \times 10^{11}$ cfu/g, respectively. Gram-negative rod-shaped bacteria dominated the populations: 90% in carp, 89% in catfish, 80% in water, and 86% in the total populations. Altogether, 14 bacterial species of 10 genera were identified in total populations. Pond water bacteria had a reflection on the bacterial composition of the gills and intestine of carp and catfish. *Aeromonas hydrophila*, *Shewanella putrefaciens*, *Vibrio cholerae*, *Staphylococcus* sp., and *Vibrio vulnificus* appeared as the common bacteria in the populations, where the first three were highly significantly abundant ($P < 0.0001$). Moreover, *A. hydrophila* was the most significantly dominant bacteria (32%; $P < 0.005$) among the total populations. *Pantoea* sp. and *Pasteurella pneumotropica* were present only in carp and catfish, respectively, but *Corynebacterium urealyticum* and *Micrococcus* sp. were present only in pond water.

Keywords: Bacterial flora, Polyculture, *C. gariepinus*, *C. carpio*, Earthen ponds

Background

Positive trend in tilapia aquaculture in Saudi Arabia brought the idea of introducing other culturable fish species to provide varieties to consumers. An attempt has been undertaken to introduce common carp (*Cyprinus carpio*) and African catfish (*Clarias gariepinus*) in 1981 and 1987, respectively. Culture of these species increased particularly in concrete tanks, and it can be produced in many confined water bodies throughout the country.

There is growing awareness on the influence of bacterial composition of fish, especially in the intestine, on the health and growth of the host. Extreme examples of the influence of the gut flora include the negative effects of the pathogenic organisms and, in contrast, the total reliance that ruminants have on their gut flora for the assimilation of organic carbon from the environment (Kennedy et al. 1991). The influence of the gut flora on the host is clearly of great interest in aquaculture, particularly where poor

productivity and/or stock losses are widespread (Sharmila et al. 1996; Moriarty 1997; Skjermo and Vadstein 1999; Lavens and Sorgeloos 2000).

The bacterial composition may change with age, individuals, nutritional status, environmental conditions, and the complexity of the fish digestive system (Cahill 1990; Ringø et al. 1995; Al-Harbi and Uddin 2004; Ringø et al. 2006). The intestinal flora may be of significance in fish spoilage (Kaneko 1971) and faecal contaminant spread (Al-Harbi 2003).

The intestinal flora of endothermic animals serves both a digestive function and as a protection barrier against disease (Sissons 1989). Assuming that the non-pathogenic microflora has a disease-preventive effect, this protection is likely to be mediated by microorganisms that are present in high numbers. Therefore, studies on the composition and characteristics of the dominant microflora are a crucial part in probiotic fish research.

In contrast to the intestine, little is known about the development or activity of bacterial flora on gills. Some normal bacterial flora of water such as *Pseudomonas fluorescens*, *Aeromonas hydrophila*, *Edwardsiella tarda*, *Vibrio* sp., and Myxobacteria (Sugita et al. 1985) can be found on the body surface or in the intestines of fish and may produce disease epizootics under environmental stress. The literature related to the bacterial flora and pathogens of fish shows a degree of confusion with similar bacteria being isolated from both healthy and diseased individuals. A thorough study on bacterial load and types in the internal organs of apparently healthy fish is needed to provide a predictive capability for possible disease outbreaks and provide an opportunity to support management action for preventive measure. The present study reports on the composition of aerobic bacterial flora, both quantitatively and qualitatively associated with pond water, gills, and intestine of common carp and African catfish. Bacterial flora in fish and water environment can be different in various geographical areas. Thus, the studies described in this paper are needed.

Methods

Experimental pond conditions

The present study was performed in three artificial earthen ponds at the Fish Culture Station located in the Al-Qassim region of Saudi Arabia. These ponds were constructed in 1993 and are completely dependent upon the supply of groundwater as a water source throughout the year. Limited vegetation exists in the shallow shore areas, and there is mud of 14 to 23 cm on the bottom. Water was added in the ponds to compensate for the loss from evaporation and seepage. Originally, common carp, *C. carpio*, and African catfish, *C. gariepinus*, were received from the Al-Qassim Fish Farm and held in these ponds. Fertilizers together with artificial feed were used. The area of each pond was 1,200 m^2 with an average depth of 1.6 m.

Physico-chemical parameters

Surface water temperatures, dissolved oxygen (DO), pH, and total dissolved solids (TDS) of the ponds were measured using a Universal Pocket Meter Multiline P4 (WTW, Weilheim, Germany). Salinity was determined with a refractometer (A366ATC, Atago, Co., Ltd, Itabashi-ku, Japan). NO_2-N and hardness were recorded using a HACH DR/2000 analysis unit (HACH Co., Ames, IA, USA). All determinations were done weekly between 0800 and 0900 hours.

Bacteriological sampling and analyses

The samplings were done three times at 2-week intervals for bacterial investigations of polycultured pond water, gills, and intestine of common carp and African catfish in each of the three ponds, and means ± standard deviations were estimated.

Pond water

Pond water samples were collected in sterile glass bottles (250 mL) 15 to 20 cm below the water surface from three different locations in each pond in every sampling. Upon arrival at the laboratory, bacteriological analysis was performed with the three water samples of a pond separately and averaged. Serial dilutions of up to 10^{-4} were made using sterile 0.85% (*w*/*v*) NaCl. Aliquots of 0.1 mL of the serial dilutions were seeded onto tryptone soya agar (TSA; Difco, Detroit, MI, USA) plates in duplicate by spread plate technique.

Gills and intestine of common carp and African catfish

For every sampling, 9×2 fish (three in each group) were chosen randomly for each pond for bacterial counts in the gills and intestine. None of the fish sampled had gross lesions, and all were assumed to be clinically normal. The fish were killed by physical destruction of the brain, and the number of incidental organisms was reduced by washing the fish skin with 70% ethanol before taking the gills and opening the ventral surface with sterile scissors to expose the body cavity. Desired amount of gills and intestine were taken aseptically and homogenized in a mortar separately. Then, *ca.* 2 g of each homogenate was suspended in 25 mL of sterile saline. Desired 10-fold dilutions, namely 10^{-6} for gills and 10^{-9} for intestine, were made and treated as the pond water sample.

Total aerobic heterotrophic plate count

Total aerobic heterotrophic bacterial counts of pond water, gills, and intestine of carp were determined by the incubation of all the inoculated plates at 25°C, and colonies were counted using Leica Quebec Darkfield Colony Counter (Leica, Inc., Buffalo, NY, USA) at 24 and 48 h after inoculation. The plates having ≥30 to 300 colonies were used to calculate bacterial population numbers, expressed as colony-forming units per unit of sample.

Isolation of bacteria

Bacterial isolates were recovered from cultured water, gills, and intestine of carp and catfish from the three ponds at each sampling. To determine the percent composition of bacteria types in the samples, we divided the bacterial colonies into different groups according to colony characteristics, namely shape, size, elevation, structure, surface, age, color, and opacity, and counted the number of colonies of each recognizable type. With some exception, three to five representatives of each colony type were then streaked repeatedly on TSA plates until pure cultures were obtained. For all populations, an average 3% of primary isolates failed to grow despite repeated attempts on subsequent subculturing. Purified cultures were inoculated onto TSA slants and kept at 4°C for stock; these were resubcultured on slants every 6 weeks.

Identification of bacteria

To identify purified isolates to genus or species level, basic tests, namely Gram's stain, motility, morphology, oxidase test, catalase, glucose oxidation-fermentation, amylase, gelatinase, lipase, indole, H_2S production, and nitrate reduction, were performed following the criteria described in the *Bergey's Manual of Systematic Bacteriology* (Holt et al. 1994). The presumptive vibrio species were confirmed by their growth in different concentrations of NaCl and thiosulphate-citrate-bile sucrose agar (Oxoid Limited, Thermo Fisher Scientific, Basingstoke, UK) and by their sensitivity (0 out of 129 survived) to a vibriostatic agent (Oxoid Limited, Thermo Fisher Scientific). Further identification was performed using the commercial API 20E, API 20 STREP, (bioMerieux sa, Marcy l'Etoile, France) and BIOLOG (BIOLOG, Inc., Hayward, CA, USA).

Results

Physico-chemical parameters

The values for physico-chemical parameters of the water samples taken from the cultured ponds are shown in Table 1. No significant variation was observed in the parameters between the ponds. Water temperature during the sampling period ranged from 24.8 ± 0.7°C to 25.7 ± 1.0°C. Dissolved oxygen varied from 7.2 ± 0.4 to 7.9 ± 0.5 mg/L. The pond water pH was slightly alkaline (7.3 ± 0.2 to 8.2 ± 0.3). The salinity was 5.0 ± 0.16 to 5.2 ± 0.15 ppt. Total dissolved solids in water were in the range of $3{,}324 \pm 76$ to $3{,}847 \pm 93$ mg/L. The range of NO_2-N was 0.22 ± 0.02 to 0.26 ± 0.04 whereas the hardness was $1{,}193 \pm 64$ to $1{,}331 \pm 55$ mg/L

Quantitative bacterial flora

The results of the quantitative aerobic heterotrophic bacterial flora in pond water, gill filaments, and intestine of carp and catfish are presented in Figure 1. During the study period, mean bacterial counts in pond water ranged from $9.2 \pm 5.5 \times 10^3$ to $6.6 \pm 5.1 \times 10^4$ cfu/mL; in the gill filaments of carp and catfish, $3.3 \pm 3.8 \times 10^6$ to $7.9 \pm 5.6 \times 10^6$ and $1.1 \pm 4.6 \times 10^5$ to $2.3 \pm 5.2 \times 10^6$ cfu/g, respectively; and in the intestine of carp and catfish, $1.4 \pm 2.9 \times 10^{10}$ to $1.7 \pm 6.0 \times 10^{11}$ and $2.7 \pm 3.4 \times 10^{10}$ to $1.0 \pm 4.5 \times 10^{11}$ cfu/g, respectively. Each count was the mean of viable bacterial colonies grown on duplicate agar plates made per individual sample. Individual pond results are shown in Figure 1.

Qualitative bacterial flora

The bacterial composition from pond water, gills, and intestine of common carp and African catfish were identified to species level where possible. Their percentage

Table 1 The values for physico-chemical parameters of cultured pond waters from each of the three ponds

Pond	Temperature (°C)	DO (mg/L)	pH	Salinity (ppt)	TDS (mg/L)	NO_2-N (mg/L)	Hardness (mg/L)
1	24.8 ± 0.7	7.2 ± 0.4	7.3 ± 0.2	5.2 ± 0.15	3,324 ± 76	0.26 ± 0.04	1,331 ± 55
2	25.7 ± 1.0	7.9 ± 0.5	8.2 ± 0.3	5.1 ± 0.08	3,847 ± 93	0.22 ± 0.02	1,193 ± 64
3	25.5 ± 0.8	7.7 ± 0.4	7.8 ± 0.2	5.0 ± 0.16	3,452 ± 81	0.23 ± 0.03	1,229 ± 77

Values given are mean ± SD. DO, dissolved oxygen; TDS, total dissolved solids.

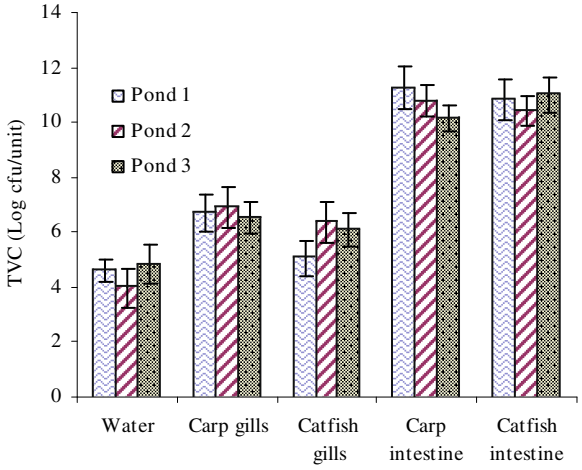

Figure 1 Log values of average bacterial counts in pond water (cfu/mL), gills (cfu/g), and intestine (cfu/g).

distribution (Table 2) is given as the mean bacterial flora of the three ponds because all the ponds and data obtained from each were very similar. Gram-negative rod-shaped bacteria dominated in every population: 90% in carp, 89% in catfish, 80% in water, and 86% in the total populations. Ten bacterial genera and fourteen species were identified from pond water, gills, and intestine of carp and catfish. Members representing five species (*A. hydrophila*, *Shewanella putrefaciens*, *Vibrio cholerae*, *Staphylococcus* sp., and *Vibrio vulnificus*) were common to all the populations with *A. hydrophila* being the most dominant, covering around one-third (32%) of the total bacterial populations.

Twelve bacterial species were found in pond water with the dominant bacteria (prevalence > 11%) *A. hydrophila*, *V. cholerae*, *S.* putrefaciens, and *Staphylococcus* sp., of which the first one was the most dominant (30%). Pond water bacteria had the

Table 2 Percentage (%) composition of bacteria

Bacteria	Pond water	Carp gills	Catfish gills	Carp intestine	Catfish intestine
A. hydrophila	29.93	29.79	25.71	36.81	41.84
Corynebacterium sp.	1.36	3.19	0	0	0
Corynebacterium urealyticum	2.04	0	0	0	0
Edwardsiella sp.	2.72	2.13	2.14	1.23	0
Micrococcus sp.	2.04	0	0	0	0
Pantoea sp.	0	2.13	0	1.84	0
Pasteurella pneumotropica	0	0	3.57	0	0
S. putrefaciens	12.25	20.21	21.43	19.63	17.73
Staphylococcus sp.	11.57	9.58	8.57	6.75	7.80
Streptococcus sp.	2.72	0	6.43	0	0
Vibrio alginolyticus	1.36	0	2.14	2.45	0
V. cholerae	14.29	19.15	19.29	19.63	16.31
Vibrio sp.	7.48	0	0	3.68	6.38
V. vulnificus	6.80	8.51	6.43	3.07	7.09
Unidentified G^- rods	5.44	5.32	4.29	4.91	2.84

Recovered from samples of pond water, gills, and intestine of *C. carpio* and *C. gariepinus* from the three studied ponds.

reflection on the bacterial composition of the gills and intestine of carp and catfish. Nine bacterial species were found in carp intestine. The dominant bacteria (prevalence ≥ 20%) *A. hydrophila*, *S. putrefaciens*, and *V. cholerae* were observed. In carp gills, eight bacterial species were observed, which followed the intestine in bacterial dominancy with additional dominant bacterium, *Staphylococcus* sp. (10%).

Ten bacterial species were identified in the gills of catfish with an indication of higher bacterial diversity in the gills than in the intestine (six). *A. hydrophila*, *S. putrefaciens*, and *V. cholerae* were the dominant bacteria (prevalence ≥ 16%) in both cases. *Pantoea* sp. was present only in carp. *P. pneumotropica* was present only in catfish gills. *Streptococcus* sp. was present in catfish gills but not in carp and vice versa for *Corynebacterium* sp. *C. urealyticum* and *Micrococcus* sp. were not detected in both fish but were present in pond water.

Discussion

Variations in bacterial load of pond water, gills, and intestine of common carp and catfish were observed. Bacterial load was high and it is not a disadvantage at all time. If the bacteria are not pathogenic, high bacterial abundance may indicate a potential of organic matter recycling, self-cleaning potential, and re-mineralization. The composition and quantity of the microorganisms also vary depending on water temperature (Rheinheimer 1985; Al-Harbi and Uddin 2004). A favorable season (24.8°C to 25.7°C) which is optimal for common carp and African catfish was selected to isolate the bacteria at maximum level. Chowdhury et al. (1994) observed a bacterial count of 1.3×10^4 to 5.6×10^5 cfu/mL in *C. batrachus* pond water. The quality of fish can be measured by monitoring the water bacteria of growing ponds, since these affect the storage life and the quality of fish products. Hagi et al. (2004) observed a bacterial load of 1.9×10^9 cfu/g in the intestine of common carp. Chowdhury et al. (1998) reported a bacterial load of up to 2.2×10^8 cfu/g in the slime of hybrid catfish (*C. batrachus* x *C. gariepinus*). Wu et al. (2010) found that the total viable counts were 3.4×10^8 cfu/g in the intestinal content and 2.1×10^7 cfu/g in the intestinal mucus of yellow catfish (*Pelteobagrus fulvidraco*). High metabolic activity of fish associated with increased feeding rates at higher temperatures might be a cause for high bacterial load in the gills and intestine of fish. The variations in bacterial counts between individual fish have been observed previously (Spanggaard et al. 2000) and were confirmed by our results. Our studies showed a maximum difference of 2 log units, which is much lower than that observed by Spanggaard et al. (2000), up to a difference of 5 log units in healthy rainbow trout.

The prominent feature of this study is that the bacterial flora of both fish is remarkably similar. Pond water bacteria had the reflection on the bacterial composition of the gills and intestine of carp and catfish. Gram-negative bacteria dominated in every population: 90% in carp, 89% in catfish, and 80% in water. Here, 10% more Gram-negative bacteria were found in both fishes than that of singly cultured fish (Al-Harbi and Uddin 2008, 2010). *A. hydrophila*, *S. putrefaciens*, *V. cholerae*, and *Staphylococcus* sp. were the most predominant isolates recovered from this study as shown in Table 2. Also, in monoculture, all these four bacteria dominated in the same pattern (Al-Harbi and Uddin 2008, 2010). Chowdhury et al. (1994) identified *Micrococcus* and coryneforms as

dominant bacteria from catfish pond water. Bacteria in the carp intestine did not show a big species diversification than those in the gills. Here, the dominant bacteria *A. hydrophila*, *S. putrefaciens*, and *V. cholerae* were observed. Our results are in partial agreement with a previous report (Sugita et al. 1990). In addition, it has been reported a wide variety of bacterial species in the intestine of common carp (Mahmoud et al. 2004; Namba et al. 2007).

Catfish gills showed a higher bacterial diversity than the intestine. *A. hydrophila*, *S. putrefaciens*, and *V. cholerae* dominated in catfish. Our results are in partial agreement with a previous report (Chowdhury et al. 1998) where *Aeromonas* and *Micrococcus* were the dominant bacteria in hybrid catfish. Wu et al. (2010) reported a wide variety of bacterial species with the predominant genera being *Plesiomonas*, *Yersinia*, *Enterobacter*, *Shewanella*, and *Aeromonas* in the intestine of yellow catfish (*P. fulvidraco*).

In an overall point of view, our studies indicate that nonspecific bacteria with the same group of host, typical in the aquatic system, are represented in the intestine and gill flora of fish cultured in mono- or polysystem. However, *Micrococcus* sp. and *C. urealyticum* were not detected in polycultured carp and catfish though were isolated in monocultured ones (Al-Harbi and Uddin 2008, 2010). The influence of physiological stress and environmental factors on the gut floral composition is an important consideration, particularly in aquaculture. Ingestion of free-living bacterial community under otherwise natural, stable conditions may possibly lead to the establishment of non-indigenous gut floral species (Lynch and Hobbie 1988). Differences in sampling, microbiological techniques, fish habitat, age, and conditions of fish are some of the probable reasons for all these variations in results. Gill disease may be initiated by opportunistic bacteria present on the surface of the gill. This study showed that the potential disease-causing bacteria *A. hydrophila* (Austin and Austin 1993; Sarker et al. 1999) was present in carp, catfish, and culture system as the most dominant figure. Noguchi et al. (1987) reported that *V. alginolyticus* produced tetrodotoxin in the intestine of fish.

Recovered *A. hydrophila*, *Vibrio* spp., *S. putrefaciens*, *Staphylococcus* sp., *Streptococcus* sp., and *Edwardsiella* sp. bacteria are facultative pathogens or agents of food poisoning, and spoilage is of importance. Although the presence of these bacteria is not often associated with fish diseases or enteric diseases in man, the health implications of the introduction of these organisms into natural water via the fish faeces in the aquaculture wastewaters should not be ignored. Spoilage bacteria *S. putrefaciens* and Vibrionaceae are able to utilize trimethylamine oxide, resulting in off odors and flavors (Gennary et al. 1999). In order to prolong the shelf life of fish, it is essential to control these spoilage bacteria. *S. putrefaciens* count is probably not only a sufficient parameter for shelf life prediction, but is also useful in quality determination of fish. The results indicate that the microflora of the aqueous environment could influence the bacterial flora of apparently healthy carp and catfish and that the commensal bacterial flora which included facultative pathogens could give rise to fish epizootics at any stress condition. These findings may contribute to aquaculture management practices and can also help in controlling the storage life and food safety. Further studies need to be conducted to observe the bacterial nature in diseased and healthy carp and catfish as well as how the bacterial pathogens contribute to the pathology of fish and aquaculture environment.

Conclusions

On the basis of the results obtained in this work, it can be concluded that 14 bacterial species of 10 genera were identified from pond water, gills, and intestine of carp and catfish. Members representing five species (*A. hydrophila*, *S. putrefaciens*, *V. cholerae*, *Staphylococcus* sp., and *V. vulnificus*) were common to all the populations with *A. hydrophila* being the most dominant, covering around one-third (32%) of the total bacterial populations.

Competing interests
Both authors declare that they have no competing interests.

Authors' contributions
Both authors planned the research, interpreted the results, and drafted the manuscript. AH supervised the whole work and edited and revised the manuscript. Both authors read and approved the final manuscript.

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