

Full Length Research Paper

Bacterial content in the intestine of frozen common carp *Cyprinus carpio*

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The quantitative and qualitative analyses of bacterial flora associated with the intestine of common carp *Cyprinus carpio* in fresh and frozen storage conditions for 16 months was determined. Aerobic plate counts (APC) ranged from $1.8 \pm 2.7 \times 10^9$ to $1.1 \pm 2.9 \times 10^4$ colony forming unit (CFU) g^{-1} , $2.0 \pm 2.1 \times 10^{10}$ to $6.1 \pm 1.4 \times 10^4$ CFU g^{-1} , and $1.3 \pm 3.2 \times 10^{10}$ to $4.3 \pm 3.3 \times 10^4$ CFU g^{-1} each of 3 storage tank, respectively from the intestine of common carp in fresh and frozen storage. The APC decreased c. 3-log cycles after 1 month storage and then counts declined more slowly during frozen storage over 16 months. In total, 14 bacterial genera with 18 species were identified. Gram-negative rods bacteria (77%) dominated the population. *Aeromonas hydrophila*, *Aeromonas* sp., *Bacillus* sp., *Enterobacter* sp., *Micrococcus* sp., *Photobacterium damsela*, *Serratia liquefaciens*, *Shewanella putrefaciens*, *Staphylococcus* sp., *Streptococcus* sp. and *Vibrio* sp. survived after prolonged freezing. Two bacterial species viz. *Shewanella putrefaciens* and *Aeromonas hydrophila* were dominant with a prevalence of > 10% both in fresh and frozen fish. *S. putrefaciens* was the dominant bacteria (19% of the total isolates) throughout the period of study.

Key words: Bacterial enumeration, species composition, intestine, frozen common carp.

INTRODUCTION

Dense microbial populations occur within the intestinal content, with numbers of bacteria much higher than those in the surrounding water, indicating that the intestines provide favorable ecological niches for these organisms (Austin and Austin, 1999). For high quality fresh fish, the number of bacteria present on the surface varies from 3 to 4 \log_{10} CFU g^{-1} . Counts are normally one or two orders of magnitude higher in the gills and intestinal counts can reach 9 \log_{10} CFU g^{-1} (Sikorski, 1990). Microbiological quality of fresh and frozen fish can be assessed using several criteria (Kramer and Liston, 1987). Some seafood processors specify a 2-class plan for the acceptance of frozen fish, where APC should not be higher than 5 \log_{10} CFU g^{-1} (Elliot, 1987).

For fish, ICMSF (1986) proposed microbiological lower

(m) and maximum (M) limits of aerobic plate counts at 30°C as 5×10^5 (5.69 log) and 10^7 (7 log) CFU g^{-1} or cm^{-2} respectively. *Aeromonas hydrophila*, *Shewanella putrefaciens*, *Vibrio* spp., *Corynebacterium urealyticum*, *Staphylococcus* sp. and *Streptococcus* sp. were the major bacteria in the intestine of common carp (Al-Harbi and Uddin, 2008). Cahill (1990) reported the presence of *A. hydrophila*, *Flavobacterium* sp., *Pseudomonas* sp. and *Vibrio* sp. in common carp. Contamination of edible portions of fish may originate from *gastrointestinal* sources. The shelf-life of raw fish depends on the storage conditions, the intrinsic factors, and the qualitative and quantitative composition of the initial microflora, related to the environment where the fish live and are caught, the season, the fishing method and the early conditions of handling (Ward, 1988). Freezing is one method of preserving fish for long periods and frozen fish have become an important commodity both for domestic and export markets in a number of countries. The duration of bacterial viability in frozen fish tissues or the length of

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time tissues can remain frozen and still yield viable bacteria; upon partial thawing is an area warranting further study. In local markets, storage of frozen common carp without evisceration is most common. The quality of fish can be measured by bacterial numbers in fish organs, as these affect the storage life and fish product quality. Despite the number of surveys on the microbiology of fish, there have been few studies on frozen fish.

Limited information on microbiological conditions of fish during frozen storage in Saudi Arabia led to this research. Scientific knowledge on quality changes in fish during freezing and subsequent frozen storage will provide a basis for supplying good quality fish. The objective of this research study was to evaluate quantitative and qualitative bacteriological results from the analysis of the intestine of both fresh and frozen common carp.

MATERIALS AND METHODS

Fish

Four hundred and fifty of common carp *Cyprinus carpio* weighing between 700 to 1100 g were collected from three 10 m² (3.8 x 2.64 x 1.2 m) outdoor aerated concrete tanks (150 fish from each tank). The tanks water level was 1 m, and about 20% of the tanks water was exchanged daily with fresh water (ground water) to maintain good water quality. The fish for all the three tanks were fed daily with a tilapia pelleted feed (34% crude protein) at the rate of 3% of body weight. The water temperature was 30°C.

Bacteriological sampling and analyses

Three samplings, one in each tank were done simultaneously to harvest the fish by hand net for microbial investigations of common carp intestine for fresh and frozen conditions. Immediately after catch, 16 sample bags of each of the 3 tanks, a total of 48 bags and each containing 9 representatives (size, condition, sex etc.) fish were preserved in a freezer at $-20 \pm 1^\circ\text{C}$ for frozen sampling from May, 2005 to August, 2006. Samples were tested monthly for quantitative bacterial analyses and every four months for qualitative analyses. One bag of frozen fish was randomly taken from each unit and microbial quality determined.

Common carp intestine

Microbial examination of fresh fish was done initially at the beginning of the storage period (fresh) and at 4, 8, 12, and 16 months following storage at $-20 \pm 1^\circ\text{C}$. From each of the 3 tanks, 15 representative fish (three replicates, three x five sample times in each tank) were used. They were killed by physical destruction of the brain. At each sample time, 9 fish collected from each of 3 tanks (three fish in each replication) were used for bacteriological tests. Frozen fish were thawed at room temperature (c. 1 to 2 h) until it was soft enough to take out the intestine. The number of incidental organisms was reduced by washing the fish skin with 70% ethanol before opening the ventral surface with sterile scissors to expose the body cavity. Around 5 g of intestine from sampled fish was taken aseptically, mixed, and homogenized in a mortar. Intestines were taken from three (interior, mid and posterior) parts of the

whole intestines to standardize the sampling. Around 2 g of homogenate was suspended to a bottle containing 25 ml of sterile (121°C , 15 min) 0.85% (w/v) NaCl prepared in de-ionized water. One milliliter (1 ml) of the suspension was serially diluted to 10^{-9} . Volumes (0.1 ml) of the dilutions were spread onto tryptone soya agar plates (TSA, Oxoid, UK) in duplicate.

Total viable count (TVC)

Total aerobic heterotrophic bacterial counts of common carp intestine were determined by incubation of all the inoculated plates at 25°C for 48 h and colony forming units (CFU) were counted with a Quebec Darkfield Colony Counter (Leica, Inc., Buffalo, New York) equipped with a guide plate ruled in square centimeters. The plates having ≥ 30 to 300 colonies were used to calculate bacterial population numbers, expressed as CFU.

Isolation of bacteria

Bacterial isolates were recovered from the intestine of fresh and frozen common carp. To determine the percent composition of bacteria types in the samples, we divided the bacterial colonies into different groups according to colony characteristics (shape, size, elevation, structure, surface, age, color, and opacity), and counted the number of colonies of each recognizable type. With some exceptions, 3 to 5 representatives of each colony type were then streaked on TSA plate repeatedly until pure cultures were obtained. Around 2% of primary isolates failed to grow despite repeated attempts on subsequent sub-culturing. Purified cultures were inoculated onto TSA slants and kept at 4°C for stock; these were sub-cultured on slants every 6 weeks.

Identification of bacteria

To identify the selected bacterial isolates to genus or species level, the purified isolates were observed for cell shape, motility, flagellation, spores, encapsulation, and Gram staining. The isolates were then subjected to biochemical tests (oxidase, catalase, amylase, gelatinase, lipase, indole, H₂S production and nitrate reduction) following the criteria described in the Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). The presumptive vibrio species were confirmed by their growth in different concentrations of NaCl, thiosulphate-citrate-bile sucrose (TCBS) agar (Oxoid) and by their sensitivity to the vibriostatic agent (O/129) (Oxoid). In parallel, the commercial API 20E, API 20 STREP, (bioMerieux, France) and Biolog (biolog, Hayward, CA, USA) methods were also used.

RESULTS

Quantitative bacterial flora

Quantitative results of APC in common carp intestine at fresh and different storage conditions for 16 months are summarized in Table 1. APC decreased from $1.8 \pm 2.7 \times 10^9$ to $1.1 \pm 2.9 \times 10^4$ CFU g⁻¹, $2.0 \pm 2.1 \times 10^{10}$ to $6.1 \pm 1.4 \times 10^4$ CFU g⁻¹, and $1.3 \pm 3.2 \times 10^{10}$ to $4.3 \pm 3.3 \times 10^4$ CFU g⁻¹ in the intestine of common carp collected from tank 1, tank 2, and tank 3 respectively during the period of study. For all groups of fish, APC decreased c. 3-log

Table 1. Counts of bacteria in the intestine of *Cyprinus carpio* in fresh and frozen storage conditions for 16 months.

Tank number	Fresh intestine CFU g ⁻¹	Month frozen (CFU g ⁻¹)															
		1 st	2 nd	3 rd	4 th	5 th	6 th	7 th	8 th	9 th	10 th	11 th	12 th	13 th	14 th	15 th	16 th
1	1.8x10 ⁹ (2.7)	5.2x10 ⁶ (3.4)	1.3x10 ⁶ (2.8)	5.8x10 ⁵ (3.8)	8.3x10 ⁵ (1.4)	3.2x10 ⁵ (2.4)	1.9x10 ⁵ (3.6)	2.2x10 ⁵ (4.0)	3.4x10 ⁵ (3.1)	2.0x10 ⁵ (1.7)	5.5x10 ⁴ (3.1)	1.9x10 ⁵ (2.2)	8.0x10 ⁴ (2.9)	1.8x10 ⁴ (2.5)	3.2x10 ⁴ (3.7)	5.3x10 ⁴ (3.1)	1.1x10 ⁴ (2.9)
2	2.0x10 ¹⁰ (2.1)	1.0x10 ⁷ (2.1)	4.8x10 ⁶ (2.7)	2.1x10 ⁶ (2.6)	2.7x10 ⁶ (3.4)	1.0x10 ⁶ (2.2)	4.2x10 ⁶ (1.9)	1.4x10 ⁶ (2.5)	8.9x10 ⁵ (2.0)	1.5x10 ⁵ (1.0)	6.1x10 ⁴ (1.4)	1.1x10 ⁵ (2.3)	1.2x10 ⁵ (0.9)	7.9x10 ⁴ (1.7)	8.1x10 ⁴ (3.5)	6.4x10 ⁴ (2.8)	2.3x10 ⁵ (4.1)
3	1.3x10 ¹⁰ (3.2)	1.3x10 ⁷ (2.9)	6.2x10 ⁶ (3.3)	3.1x10 ⁶ (3.0)	1.3x10 ⁶ (2.0)	1.4x10 ⁶ (2.8)	7.5x10 ⁵ (2.7)	6.8x10 ⁵ (1.6)	1.1x10 ⁶ (1.8)	6.6x10 ⁵ (2.3)	3.7x10 ⁵ (3.5)	7.2x10 ⁵ (3.8)	4.3x10 ⁴ (3.3)	3.6x10 ⁵ (2.4)	8.9x10 ⁴ (4.2)	1.1x10 ⁵ (3.9)	6.7x10 ⁴ (2.5)

Parentheses indicate SD.

cycles after 1 month frozen storage; thereafter, counts slowly declined during frozen storage. Each count was the mean value of viable colonies that appeared in duplicate agar plates for each sample. Analysis of variance of the regressions showed no statistically significant difference between the tanks in the slopes with a common regression co-efficient of -0.129, but there was a significant difference in intercepts. The data for tanks 2 and 3 were not significantly different from each other with a mean value of 6.83, but that for tank 1 at 6.27 was significantly different from the other two. But the reasons were not clear.

Qualitative bacterial flora

Bacterial isolates recovered from the intestine of fresh and frozen common carp were identified to

genus or species level. Their percentage distribution (Table 2) is given as the mean bacterial flora of 3 tanks because in all the tanks and data obtained from each was very similar. Table 2 reports the isolation frequency of the recognized bacterial groups at fresh and different storage times. There were wide variations in the types of bacteria taken at different sampling times. Bacteria identified in this study were predominantly Gram-negative rods (77%). In total, 14 bacterial genera and 18 species were identified from the intestine of common carp. For fresh and frozen conditions, two bacterial species viz. *S. putrefaciens*, and *A. hydrophila* were the dominant bacteria with a prevalence of > 10% at all time. *S. putrefaciens* was the most dominant organisms (19% of the total isolates). Out of 18 species of bacteria 11 survived 16 months frozen storage. *Pseudomonas fluorescens*, *Vibrio alginolyticus*, and *Vibrio*

vulnificus were not recovered after 12 months frozen storage while after 16 months, *Cellulomonas* sp., *Corynebacterium urealyticum*, *Escherichia coli*, and *Pseudomonas* sp. were not found.

DISCUSSION

In this trial, bacterial load in common carp intestine varied in fresh and frozen conditions. The APC for all the groups of fish decreased c. 3-log cycles after 1 month frozen storage and c. 5-logs after 16 months. Al-Harbi and Uddin (2005) observed that the APC decreased c. 2-log cycles in frozen tilapia after 1 month storage and c. 3-logs after 1 year. According to Suvanich et al. (2000), APC decreased from c. 10⁷ to 10⁵ CFU g⁻¹ after 2 months frozen storage of channel catfish

Table 2. Composition and percentage distribution of bacteria in the intestine of *Cyprinus carpio* in fresh and frozen storage conditions at 4 month intervals for 16 months.

Bacteria	Fresh intestine		4 month frozen		8 month frozen		12 month frozen		16 month frozen	
	Number	%	Number	%	Number	%	Number	%	Number	%
<i>Aeromonas hydrophila</i>	38	18.18	17	11.26	14	10.45	17	10.18	13	10.66
<i>Aeromonas</i> sp.	12	5.74	9	5.96	8	5.97	9	5.39	7	5.74
<i>Bacillus</i> sp.	4	1.91	6	3.97	2	1.49	4	2.40	3	2.46
<i>Cellulomonas</i> sp.	0	0	2	1.33	4	2.99	5	2.99	0	0
<i>Corynebacterium urealyticum</i>	7	3.35	8	5.30	5	3.73	10	5.99	0	0
<i>Enterobacter</i> sp.	8	3.83	10	6.62	11	8.21	13	7.78	14	11.48
<i>Escherichia coli</i>	9	4.31	5	3.31	4	2.99	14	8.38	0	0
<i>Micrococcus</i> sp.	6	2.87	7	4.64	6	4.48	4	2.40	4	3.28
<i>Photobacterium damsela</i>	4	1.91	5	3.31	7	5.22	9	5.39	3	2.46
<i>Pseudomonas fluorescens</i>	3	1.44	4	2.65	2	1.49	0	0	0	0
<i>Pseudomonas</i> sp.	10	4.79	6	3.97	6	4.48	4	2.40	0	0
<i>Serratia liquefaciens</i>	4	1.91	7	4.64	3	2.24	2	1.20	8	6.56
<i>Shewanella putrefaciens</i>	39	18.66	20	13.25	24	17.91	41	24.55	23	18.85
<i>Staphylococcus</i> sp.	5	2.39	8	5.30	6	4.48	12	7.19	8	6.56
<i>Streptococcus</i> sp.	9	4.31	11	7.29	5	3.73	10	5.99	16	13.11
<i>Vibrio alginolyticus</i>	12	5.74	4	2.65	4	2.99	0	0	0	0
<i>Vibrio</i> sp.	17	8.13	9	5.96	9	6.72	7	4.19	12	9.84
<i>Vibrio vulnificus</i>	15	7.18	5	3.31	4	2.99	0	0	0	0
Unidentified Gram-negative rods	7	3.35	8	5.30	10	7.46	6	3.59	11	9.02

frame mince and then remained nearly unchanged.

The initial killing rate of microorganism during freezing is rapid then followed by a gradual reduction (Frazier and Westhoff 1990). This research appears closer to our work due to similar observation in the decrease of viable microorganisms with the frozen storage period of common carp. Throughout the period studied in this trial, Gram-negative rods in particular two bacterial species viz. *S. putrefaciens*, and *A. hydrophila* dominated the bacterial flora with a prevalence of > 10% as shown in Table 2. In

another study, *S. putrefaciens*, *Coryne. urealyticum*, *A. hydrophila*, and *Flavobacterium* sp. were the dominant bacteria with a prevalence of c. 10% in tilapia frozen for 1 year (Al-Harbi and Uddin, 2005). *Streptococcus agalactiae* was recovered from frozen tilapia after 6 months storage (Evans et al., 2004). Recovery and revival of different types of bacteria from frozen common carp after prolonged freezing in the present study supports the idea that frozen fish is a viable alternative to fresh fish for microbiological sampling in instances when fresh, healthy or moribund fish are unavailable or impractical

(Evans et al., 2004). Brady and Vinitnantharat (1990) found viable pathogenic Gram negative bacteria could be recovered from channel catfish *Ictalurus punctatus* frozen at -20°C for over 20 days. The high prevalence of *S. putrefaciens*, *Aeromonas* spp., *Vibrio* spp., *Enterobacter* sp., *Streptococcus* sp., and *Staphylococcus* sp. in this study suggests that these bacteria may be common in the intestine of carp. *S. putrefaciens* is considered as one of the main spoilage bacteria in fish (Gennary et al., 1999). Jørgensen and Huss (1989) characterized *S. putrefaciens* cultures in order to examine the spoilage activity and found a

good correlation between bacterial counts, detection time in conductance measurements and production of trimethylamine and off-odour. López-Caballero et al. (2001) also reported a correlation between the highest microbial counts (>9 log cfu/ml) of *S. putrefaciens* and TMA concentrations (45 mg N-TMA/100 ml), and strong putrid off-odours. *S. putrefaciens* and *Pseudomonas* spp. are the specific spoilage bacteria of iced fresh fish regardless of the origin of the fish (Gram and Huss, 1996). *P. fluorescens* is an important odour producer and the most frequently isolated proteolytic species (Edwards et al., 1987). Although, *S. putrefaciens* counts alone are not a sufficient predictor of shelf-life, they can be used as a quality determinant of fish. Although the numbers of food-borne outbreaks caused by *Aeromonas* spp. are low (Altwegg et al., 1991; Krovacek et al., 1992), the presence of *Aeromonas* spp. in the food chain should not be ignored. *A. hydrophila* have been recovered in other studies on frozen fish (Figueras et al., 2000, Castro-Escarpulli et al., 2003) and the significance of the bacteria isolated varies. The bacteria isolated included *S. putrefaciens*, *A. hydrophila*, *Enterobacter* sp., *Streptococcus* sp., *Staphylococcus* sp., *E. coli*, *V. vulnificus*, and *P. fluorescens* which are facultative pathogens or agents of food poisoning and spoilage. Shannon (1986) reported the presence of pathogenic *Bacillus cereus*, *E. coli*, *Staphylococcus aureus* and *V. cholerae* in catfish. The vibrios can cause different types of disease and are transmitted through foods and through cross-contamination during handling and processing of foods. Al-Harbi (2003) reported significant coliform bacteria including *E. coli* in fish ponds. Note that in the present study, *E. coli* could not be recovered after 16 months frozen storage. This research helps understand the quality of fish during frozen storage. During processing and handling of fish products, the presence of pathogenic bacteria is of concern to fish processors.

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