Original Research Article

Commensal and pathogenic bacteria associated with farmed Rainbow Trout (*Oncorhynchus mykiss Walbum*) and aquaculture environment

Abstract

The bacterial microflora associated with farmed rainbow trout (O*ncorhynchus mykiss Walbaum*) and its aquaculture environment was characterized. The counts of commensal, indicator and pathogenic bacteria were determined and bacterial colonies were characterized. The mean mesophilic counts were 4.5 log CFU g^{-1} and 7.6 log CFU g^{-1} in skin with muscle and intestine respectively. Significantly high densities of faecal coliform, *E. coli* and Enterococci was detected in pond sediment, fish intestine and feed when compared to pond water and trout tissues. A total of 110 randomly selected isolates from TSA were identified and characterized. Motile and non-motile aerobic Gram-negative bacteria together with Enterobacteriaceae accounted for 70-75% of the mesophiles. The skin microflora of trout was dominated by Gram-negative aerobic rods belonging to Enterobacteriaceae, Aeromonadaceae, *Pseudomonas/ Shewanella, Moraxella, Acinetobacter* and *Flavobacterium*. Psychrotrophic bacterial isolates of the genera *Pseudomonas, Morganella, Hafnia* were identified in trout. Pathogenic bacteria such as *Clostridium botulinum, Yersinia enterocolitica* could not be detected. However, *Aeromonas hydrophila, A.veronii*biovar*sobria, A. veronii*biovar*veronii* were prevalent in farmed trout. Presence of bacterialpathogens in the trout farm envisages a strict hygienic handling and processing of fish from such culture systems for ensuring public health safety.

Key words: rainbow trout, aquaculture environment, bacterial flora, indicator bacteria, pathogenic bacteria

Introduction

UNDER PEER REVIEW

Fish are generally regarded as safe, nutritious and beneficial but aquaculture products have sometimes been associated with certain food safety issues (WHO 2007). Several studies have demonstrated many bacterial species encountered in different fish which are potentially pathogenic under certain conditions as reported for *Streptococcus sp*. (AL-Harbi, 1994). Disease cause economic losses not only from mortality but also for treatment expenses, postponement or loss of the opportunity to sell the fish and contraction of zoonotic diseases by the handler and final consumer of the affected fish.

It is generally accepted that the environment can influence the microflora associated with the skin, gills and intestines of finfish (Horsley, 1973). In the aquatic environment, hosts and microorganisms share a similar ecosystem where bacteria can either colonise the host (intestinal tract, gills, or skin) or not (Harris 1993). Therefore, fish grown in different aquaculture systems may harbor different species and number of bacteria. The culture practices such as pond fertilization, supplementary feeding with slaughter house waste or agricultural byproducts imposes a high probability of contamination on the aquacultured fish (Toor et al. 1991; Surendran et al. 1995). Freshly harvested aquaculture products, particularly those from tropical regions may harbor pathogenic bacteria, which forms a part of natural micro-flora of fish ponds (Clucas and Ward, 1996). The presence of human pathogenic bacteria in aquaculture products is dependent on a number of factors including method of production, rearing practices, environmental conditions and the methods used to harvest, process and distribute the products (ICMSF, 1998; Reilly and Ka¨ferstein, 1999). In recent times increased attention is given to the possibility of cultured fish as vector of human pathogenic bacteria (Apun*et al*., 1999; Islam *et al*., 2000).Farmed fish not only transmit disease to man but are themselves subject to many diseases and capable of transmitting many of the established food borne microbial infections and intoxications (FAO/WHO 1974).

India is the second largest producer of freshwater fish in the world (FAO 2005) and important farmed species include Indian major carps, exotic carps, Tilapia, murrels and catfish (magur, Singhi, Pangasius). One of the more recent species is rainbow trout (*Oncorhynchusmykiss* Walbaum) an exotic coldwater fish. Trout farming is gaining popularity in India. In India this species mainly supports recreational fishery although recently it has found its way to supermarkets in metro cities as an exotic food item which has demand among

UNDER PEER REVIEW

the urban population. Presently trout is considered to be a highly priced fish in India. However, there is dearth of information on the bacterial load of farmed trout in India. The microbial profile and incidence of pathogen on rainbow trout (O*ncorhynchus mykiss Walbaum*) from temperate countries has been investigated by a number of workers and has been the subject of a number of reviews (Austin and Al- Zahrani, 1988; Cahill, 1990; Ringø, et al. 1995 Gonzalez-Rodrguez et al. 2002; Mc Adams et al. 2005; Nam and Joh 2007; Kayis et al. 2009; Salgado – Miranda et al. 2010). However, the microbial profile of aquacultured rainbow trout from tropical countries has received little attention. Ignorance of the microbial profile of aquaculture products can also affect human health as evidenced by the recent transmission of Streptococcal infections due to S. *iniae*and *L. garvieae*from tilapia to humans, which resulted in several meningitis cases in Canadian fish processors (CDC 1996). Hence it is essential to investigate the bacterial flora associated with trout grow-out culture in order to develop safe farm management practices for the production of safe fish. The major aim of the present study was to identify and characterize the bacterial flora associated with farmed rainbow trout and its farm environment and to assess the incidence of bacterial pathogens in farmed trout.

Materials and Methods

Collection of samples

Rainbow trout (*O. mykiss*)of average weight 250g and average length 278mm were collected from aquaculture farm located in Idukki district, Kerala (India) using a drag net and aseptically transported to the laboratory within 6 h of harvesting, in insulated polystyrene boxes containing ice for analysis.Fish were fed on animal protein sources like livestock processing waste. Feed collected from trout farm was also analyzed.

Microbiological analysis

Bacteriological analysis was performed on muscle with skin, intestine and feed samples. Twenty-five-gram muscle with skin were aseptically weighed, added to sterile physiological saline (NaCl, 0.85% w/v) solution (225ml), and homogenized in a stomacher (Lab blender 400, Seward Medical, London) for 60 sec at room temperature. Fish intestine portion was excised, weighed and placed in sterile bags containing enough saline solution to make 1:10 dilutions. Ten-gram feed wasaseptically weighed,

UNDER PEER REVIEW

homogenized in mortar and pestle using sterile physiological saline (NaCl, 0.85% w/v) solution (90ml). Decimal dilutions in saline solution were prepared and plated onto agar or poured into tubes for the MPN method.

Commensal bacterial flora

For mesophilic and psychrotrophic bacteria, 0.5 ml of appropriate serial dilutions was spread plated onto the surface of preset tryptic soy agar plates (TSA, Oxoid, U.K (Spanggaardet al. 2000**;** Huber et al. 2004) and incubated at 37°C and 7°C for 2 and 10 days respectively (Austin and Al -Zahrani 1988; AOAC 2000). *Pseudomonas* were counted on Cetrimide-Fusidin-Cephaloridine (CFC) agar (Oxoid code CM 559, supplemented with SR 103; Oxoid U.K.) after 3 days of incubation at 20°C (Molin and Ternstrom1982; Mead 1985). *Brochothrixthermosphacta* was determined on Streptomycin sulfate-Thallous acetate – Actidione Agar (STAA Hi Media, India) after incubation at 20°C for 4days (Gardner 1966).

Enterobacteriaceae and H_2S-producing bacteria (including *Shewanella putrefaciens*) were counted on violet red bile glucose agar (VRBGA, Oxoid code CM 485) and Iron Agar (IA, Oxoid code CM 867), respectively by pour plate method and plates were incubated respectively at 30°C for 24 h. (Mossel 1987) and 20°C for 5 days (Gennari and Campanini 1991).

Indicator bacteria

Enterococci and *Staphylococcus aureus* counts were determined respectively on KF Streptococci Agar (Oxoid code CM 701) after incubation at 37°C for 2 days and on Baird Parker Agar (Oxoid code CM 275) incubated at 37°C for 2 days and typical colonies were confirmed (FDA 2001). Coliforms, faecal coliforms and *Escherichia coli* counts were determined by a 3-replicate tube MPN (Most Probable Number) procedure (APHA 1998). Positive EC tubes were confirmed by streaking onto Eosine Methylene Blue Agar (EMB Agar, Difco, Detroit, MI) and incubated at 37ºC for 24 h. Characteristic *E. coli* colonies were isolated and confirmed by biochemical tests as described by APHA (1998).

Pathogenic bacteria

Clostridium perfringens numbers were determined by the three tube MPN method using Lactose Sulphite Broth (West, 1989) and confirmed by streaking on to Tryptose Sulfite Cycloserine (TSC) agar and 3-4 characteristic colonies were confirmed by biochemical reactions as described by FDA (2001). For *Clostridium botulinum* detection, samples (approximately 5.0g) were inoculated into 25ml. Cooked Meat Media (CMM) and sterile paraffin oil was poured. Inoculated tubes were incubated at 30°C for 3-6 days. Skin/Shell with muscle portion of fish and intestinal portions of fish were analyzed separately. After incubation for 6 days at 30°C, cultures were centrifuged at 10000 x g. and 4°C for 20 min. and each supernatant was adjusted to pH 6.2 with 1N HCl and frozen at –20°C until tested for the presence of *C. botulinum* toxin. The methodology outlined in the U.S. Food and Drug Administration Bacteriological Analytical Manual (FDA, 1998) was employed for trypsinization, toxicity test by mouse bioassay and toxin neutralization tests using type specific monovalent antitoxins A-E obtained from National Institute for Biological Standards and Control (NIBSC) Herts, UK.

Aeromonas spp. were counted on starch ampicillin (SA) agar (Hi Media, India) containing 10ug/ml of ampicillin incubated at 28°C for 48 h (Palumbo *et al.* 1985) and 25 characteristic colonies were confirmed by biochemical tests as described by Kirov (1997) and API 20 NE system (Bio Merieux, France)

For detection of *Y. enterocolitica,* 10g muscle tissue was aseptically weighed into 90 ml Peptone sorbitol bile broth (PSBB; pH 7.6), homogenized for 30 s in a stomacher (Lab blender 400, Seward Medical) and incubated at 4^{0}C for 21 days (Singh *et al.* 2003). After 7-, 14- and 21-days incubation, alkaline post enrichment treatment was performed as described by FDA (2001), followed by spread plating on CIN agar (Oxoid CM 653 and Sr 109). Plates were incubated overnight at 30^{0}C. Small (1-2 mm diameter) red "bull's eye" colonies having deep red center with sharp border surrounded by clear colorless zone with entire edge were isolated into LAIA slant (FDA 2001). Twenty-five presumptive isolates were confirmed using biochemical tests prescribed by FDA (2001) and API 20E system (Bio Merieux, France).

Characterization of commensal and pathogenic bacterial flora

UNDER PEER REVIEW

The dominant aerobic microflora at the final sampling points was determined by isolating and identifying 20% of the colonies from TSA (30°C and 7°C) plates. 66-80 colonies were randomly selected from TSA plates sampled from rainbow trout skin with muscle, and intestine samples and 20 colonies were randomly selected from TSA plates sampled from feed samples. All colonies from a sector of the plate or all colonies from a whole plate were isolated, purified and characterized morphologically and biochemically. 20 typical colonies each were isolated from IA and VRBGA sampled from rainbow trout skin with muscle and intestine samples and subsequently identified biochemically. A total of 220 bacterial cultures were isolated from trout muscle with skin, intestine and feed samples. The strains were tested for Gram reaction, catalase and oxidase reactions, motility, oxidative/fermentative metabolism and presence of spores. They were then grouped according to the taxonomic schemes of Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984; Sneath et al., 1986), further tested for the most relevant characteristics of each group and identified using schemes proposed by several authors for identification (Allen *et al.,* 1983; Krieg and Holt, 1984; Sneath *et al*., 1986; Austin, 1988; Andrew and Mitchell, 1997; Kirov, 2001). The API 20E and 20 NE system (Bio Merieux, France) was employed for the confirmation of the isolates.

Twenty colonies randomly selected from TSA Plates incubated at 7°C were processed for 16S rRNA gene sequence analysis for identification. Crude DNA template was prepared using sterile Milli Q water in a single cycle of boiling for 10 min and freezing at -20ºC and stored until further use. Amplification of 16s rDNA gene (Partial sequence) was performed with the combination of 27F (GAGTTTGATCCTGGCTCAG) and 1544 R (AGAAAGGAGGTGATCCAGCC) as per Shigematsu et al. (2009). Polymerase chain reaction was performed in 50µl reaction mix with 1x concentration of Onetaq Quick load master mix with GC buffer (NEB, Cat.No.M0487), 0.5µM concentration of each primer (Sigma), 5µl of crude DNA prepared by cell lysate method and the Cycling conditions were standardized for one taq master mix as 94°C for 2min initial denaturation, followed by 30 to 35 cycles of denaturation (94°C for 30sec), annealing (50°C for 1 min) and extension (68°C for 1.5 min) and a final extension of

UNDER PEER REVIEW

68°C for 7 min. Amplicon of size 1500bp were purified using Gen Elute gel extraction kit (Sigma, NA1111) and sent for sequencing for the same primers. Blast analysis was performed in public domain NCBI to determine the homology and isolates were identified.

Statistical analysis

Bacterial numbers in cfu g^{-1} or MPN g^{-1} sample were transformed into log_{10} values. Statistical analysis of the bacterial parameters was performed using the statistical tool package of Microsoft Excel 97 software. Student's t-test analysis was used to evaluate the significance of differences between means of microbial counts performed at 30 and 7°C. A level of $P<0.05$ was considered statistically significant.

Results and discussion

Bacterial load on farmed trout and farm environment

The bacterial population associated with trout skin with muscle, intestine and feed samples was shown in table 1. In trout skin with muscle and intestine, the highest counts were shown by mesophilic aerobic bacteria. Their population was 4.5 log_{10}cfu g^{-1} in trout muscle and 7.6 log_{10}cfu g^{-1} in intestine. The population of psychrotrophic aerobic bacteria, H_2S producing bacteria, Enterobacteriaceae, *Aeromonas, Pseudomonas,* LAB and *B. thermosphacta*counts ranged between 10^2 and 10^3cfu g^{-1}. The intestine samples of trout harboured high bacterial loads; the highest counts were noted for mesophilic aerobic bacteria which were in the range of 10^7- 10^8cfu g^{-1}. Trout were fed with livestock processing waste and analysis of feed showed high counts of mesophilic aerobic bacteria (10^8cfu g^{-1}), H_2S producing bacteria, Enterobacteriaceae (10^5cfu g^{-1}) and *B. thermosphacta* (10^4cfu g^{-1}). *Pseudomonas* count was 10^3cfu g^{-1}. H_2S producing bacterial flora contributed 10-13 % of the total flora of fresh trout. The mesophilic counts on skin with muscle of trout were close to or lower than the m value ($5x10^5$cfu/g) recommended by the International Commission of Microbiological Specification for Foods (ICMSF 1998) for whole fresh water fish and indicates good quality of trout. The results confirmed the earlier reports from Spain (Gonzalez-Rodriguez et al. 2001; González et al. 1999), Greece (Chytiri*et al.* 2004; Savvaidis et al. 2002), U.K (Ozogul andOzogul 2002), USA (Mc Adams etal

UNDER PEER REVIEW

2005) and Iran(Rezaei et al. 2008) on aquacultured fresh trout. It is widely accepted that the initial microbial load of fresh water fish varies depending on water conditions and temperature.

Bacterial levels in farm water, feeder canal water and sediment samples are given in Table 2. Aerobic mesophilic counts at 37°C and 7°C for water and sediment samples differ significantly ($P < 0.05$). Enterobacteriaceae population in water and sediment samples was in the range of 10^{2} - 10^{3}cfu g^{-1}. Mesophilic counts (mean log cfu g^{-1} at 30 ºC) were significantly higher in sediment samples than in water ($P<0.01$) as reported earlier for trout farms in Denmark (Schmidt et al.2000). Schmidt et al. (2000) observed total bacterial counts of 10^{3}-10^{4} culturable bacteria per ml of water, while sediment samples yielded counts of 10^{6} -10^{8} CFU g ^{1}. Similar results were obtained in this study.The bacterial numbers in the intestine samples were 7.657 $\log_{10}$cfug ^{1}, a value much higher than that of the skin with muscle of trout and the surrounding waters ($P<0.01$). Generally, this result is consistent with those in the previous studies reported by Spanggaard et al. (2000) and Kim et al. (2007) for trout intestine.

H_2S producing bacterial flora contributed 10-13 % of the total flora of fresh trout. Significantly higher counts of Enterobacteriaceae, *Aeromonas, Pseudomonas,* LAB and *Brochothrixthermosphacta* were obtained in the intestine samples compared to skin with muscle sample ($P<0.01$).The results of this study confirmed the findings of González et al. (1999) Savvaidis et al.(2002), Arashisar et al. (2004) and Chytiri et al. (2004) that Enterobacteriaceae, *Brochothrixthermosphacta*and lactic acid bacteria were also found to be members of the microflora of fresh rainbow trout.In this Study, significantly higher counts of Enterobacteriaceae, LAB and *Brochothrixthermosphacta* was obtained which indicates that they are able to persist and multiply in the environment provided by the intestinal tract as reported earlier (Sugita et al. 1988; Cahill 1990). Mexis et al. (2009) also reported that LAB and Enterobacteriaceae were part of the microflora of fresh rainbow trout. Naviner etal (2006) reported counts in the range of 10^{2} – 10^{5} for LAB and Enterobacteriaceae in trout intestine samples from French farms. LAB is not considered as belonging to aquatic environments, but certain species (i.e. *Carnobacterium*, *Vagococcus*, *Lactobacillus*, *Enterococcus*, *Lactococcus*) have been found in freshwater fish and their surrounding environment (González et al., 1999, 2000; Ringø et al., 2000). Several species of

UNDER PEER REVIEW

Streptococcus and *Lactococcus* bacteria including *S. iniae, S. agalactiae, S. dysagalactiae, S. parauberis, S. feacalis, L. garvieae*and *L. lactis* have been so far discriminated as the cause of Streptoococcosis/lactococcosis outbreaks in trout aquaculture (Austin and Austin, 2007; Haghighi Karsidani et al. 2010; Bekker et al. 2011)**.**

Characterization of microflora

Commensal bacterial flora

A total of 110 bacterial isolates selected from TSA plates were identified from rainbow trout skin with muscle and intestine. Of the 56 isolates from trout skin with muscle, 21.4% Enterobacteriaceae(*Enterobacter, Citrobacter, Klebsiella, Hafnia*), 14.3 % Aeromonadaceae, 39.4 %Gram-negative aerobic coccobacilli and rods (*Pseudomonas/ Shewanella, Moraxella, Acinetobacter, Flavobacterium*), 21.4% Gram positive cocci - *Micrococcaceae*(*Micrococcus* and *Staphylococcus*) and *Streptococcaceae* and 3.5% Gram positive spore forming rods (*Bacillus*). Identification of the 54 intestinal isolates showed that Enterobacteriaceae (30%) and Aeromonadaceae (18.5%) dominated the microflora followed by Gram-negative aerobic coccobacilli and rods (*Pseudomonas/ Shewanella, Moraxella, Acinetobacter, Flavobacterium*) constituting 33% of the flora. Gram positive bacteria were identified as Streptococcaceae (7.4%) and Bacillaceae (11.1%).

16s *rDNA* partial sequencing analysis of predominant psychrotrophic bacterial isolates found in Rainbow trout revealed that isolates belonged to *Pseudomonas fragi*, *Morganella morganii*, *Hafnia alvei* and *Aeromonas* spp.

The isolates from peptone iron agar plates (20 isolates) were identified as Aeromonadaceae (*A. hydrophila, A. veronii*biovar*sobria*)Enterobacteriaceae(*Enterobacter intermedius*, *Citrobacter*) and *Shewanella*. The VRBGA isolates (20 isolates) were identified as *Enterobacter agglomerans, E. intermedius,E. aerogenes, Providencia rettgeri,Klebsiella* spp. *Citrobacter* and *Hafnia*. Of the 20 isolates from feed collected from trout farm, the bacteria identified were Enterobacteriaceae(30%) Aeromonadaceae (25%), Gram-negative aerobic coccobacilli and rods 25%(*Pseudomonas/ Shewanella,*

UNDER PEER REVIEW

Flavobacterium), Gram positive cocci (Streptococcaceae) 10% and Gram-positive spore forming rods (*Bacillus*) 10%.

González et al. (1999) reported predominance of *Acinetobacter, Pseudomonas, Staphylococcus*, *Enterococcus* and *Bacillus* in rainbow trout from Spain. Kayis et al. (2009) isolated *Pseudomonas/ Shewanella, Flavobacterium, Aeromonas, Enterobacter, Citrobacter, Hafnia* and *Yersinia* from rainbow trout from farms in Turkey. Bacterial groups *Aeromonas* and *Shewanella* were reported to be the specific spoilage organism (SSO) in trout from temperature and tropical waters (Chythiri et al. 2004; Ninan et al. 2011). Naviner etal (2006) reported abundance of *A. hydrophila*and *A. sobria*in the intestinal microbiota of trout in France. The results of the present study agree well with that of Spanggaard et al. (2000) who reported predominance of Enterobacteriaceae (*Citrobacter, Hafnia, Proteus, Serratia*) and Aeromonadaceae in the intestinal microflora of rainbow trout from Denmark farms. They also reported predominance of other Gram-negative bacteria of the genera Acinetobacter, *Flavobacterium*, *Shewanella*, *Pseudomonas* in some farms. Gram positive bacteria were identified as *Arthrobacter, Carnobacterium, Streptococcus* and *Staphylococcus.* In contrast, Huber et al. (2000) reported *Acinetobacter*, *Aeromonas*, *Flavobacterium*, *Shewanella*, *Pseudomonas* and *Carnobacterium* as the common bacterial genera in the microbiota of rainbow trout intestine from Denmark farms. It has been shown that the microbiotas of the fish intestine is highly dependent on the bacterial colonization during early development, environmental conditions and dietary changes (Ringø et al., 1995; 2006; Olafsen, 2001).

Indicator bacteria

Among the indicator organisms, significantly higher levels of faecal coliform, *E. coli* and faecal streptococci were detected in pond sediment, fish intestine and feed (Table 3). Faecal Streptococcal (2.3 $\log_{10}$ cfu g^{-1}) and *S. aureus* (1.3 $\log_{10}$ cfu g^{-1}) populations were low in fresh trout and were within the acceptable limit. *C. perfringens* was detected in sediment and intestine samples in low numbers. High levels of faecal coliforms and faecal streptococci were previously reported for fish farms in India (Surendran *et al.,* 1995; Lalitha and Surendran 2004; 2006). *E. coli* count in trout muscle was below the m limit (9.5 g^{-1}) recommended by the ICMSF (1998) for good quality fish.

UNDER PEER REVIEW

Pathogenic bacteria

Motile aeromonads are a part of the microflora of fresh trout and their population was 2.692 $\log_{10}$cfu g^{-1}. and 5.6 $\log_{10}$cfu g^{-1} respectively in skin with muscle and intestine samples. Among pathogenic bacteria, *A. hydrophila, A. veronii*biovar*sobria*and *A. veronii*biovar*veronii* were isolated from farmed trout. *Y. enterocolitica* and *C. botulinum* were not detected in farmed trout samples. Naviner et al. (2006) reported Aeromonas counts of 10^2-10^6cfu g^{-1} intestinesample from farmed trout in France and *A. hydrophila*and *A.sobria*were identified in the samples. Nam and Joh (2007) observed *Aeromonas* counts of 10^2 - 10^3cfu/ml of water from trout farms in Korea and found dominance of *A. sobria* in water and trout intestine. In the same study, they isolated *A. hydrophila* from farm water. *A sobria* was identified as the causative agent of Epizootic Ulcerative Syndrome (EUS) in fish farms in South East Asian countries such as Bangladesh and India (Chacon etal 2003). *A. hydrophila*, *A. veronii* biovar *veronii* and *A. veronii* biovar *sobria* are the strains more often associated with gastroenteritis in humans.

Fish are susceptible to a wide variety of bacterial pathogens. *S.putrefaciens, A.hydrophila*and *A. sobria*, potential pathogens of fish, have been isolated from the skin and intestine of rainbow trout *Oncorhynchus mykiss* as reported earlier (Austin and Austin 1999 ; Huber et al., 2004; Koziñska and Pêkala, 2004; Pond et al., 2006). Pond et al. (2006) proposed that fish digestive tract is a reservoir for many pathogens. The prevalence of *A. hydrophila*and *A. sobria*, which are generally recognized as agents of gastroenteritis, wound infections, septicemia and endocarditis (Kirov 2001) in humans, are of concern in the culture environment because it may pose threat to public health. Motile aeromonassepticaemia / hemorrphagicsepticemia is probably the most common bacterial disease of freshwater fish that has been associated with several members of the genus *Aeromonas,* including *A. hydrophila, A. sobria, A. caviae, A. schuberti,* and *A.veronii*(Austin and Austin 1999).*A. hydrophila* was reported in diseased trout from Korea (Lee et al. 2000). Salgado – Mirranda etal (2010) isolated Aeromonads most frequently from gills and intestine of farmed trout in Mexico. Rehulka (2002) reported severe skin lesions in Rainbow Trout (*Oncorhynchus mykiss*) caused by *A. sobria*and *A. caviae.A. hydrophila*and *A. sobria*are psychrotrophic pathogens andcan produce different virulence factors, not only at optimal growth temperature, but also at

UNDER PEER REVIEW

refrigeration temperatures. This may be of importance for refrigeratedfood products that usually have an extended shelf-life at this temperature. Although adequate cooking will inactivate these bacteria, handling and cross-contamination could be a health hazard, especially for susceptible populations like children, the elderly, immunocompromised and unhealthy adults.

In this study, *C. botulinum* and *Y. enterocolitica* could not be detected in farmed trout skin with muscle and intestine samples. In trout samples, majority of the *Yersinia* strains isolated belonged to *Y. intermedia.* Kapperud and Jonsson(1976) detected *Y. enterocolitica* in brown trout from Norway. Mc Adams et al (2005) did not isolate *Y. enterocolitica* from aquacultural rainbow trout from farms in Virginia. Lalitha and Gopakumar (2000) reported distribution of *C. botulinum* in sediments and fish from farms located in and around Cochin (Kerala, India). Mc Adams etal (2005) identified C.*botulinum* in trout farms in Virginia. In a study on *C. botulinum*from Finnish trout farms, Heilm et al. (1998) found*C. botulinum*type E in 68% of the farm sediment samples, in 15% ofthe fish intestinal samples, and in 5% of the fish skin samples.An outbreak of botulism by *C. botulinum*type E was reported in rainbow trout, *Salmo gairdneri* Richardson, farmed in Denmark (Huss et al. 1974) and in Britain (Cann and Taylor 1982). Botulism outbreak was not reported in fish farms in India.

Conclusion

The study provides the first insight on the commensal and pathogenic bacteria associated with trout from aquaculture farm located in Idukki district, Kerala (India). The study confirms that farmed rainbow trout carry significant numbers of faecal coliforms and enterococci in the intestine. Significant numbers of faecal coliforms and enterococci were also found in the farm sediment and feed. However, water does not contain high numbers of these organisms. The rearing practices such as feeding and pond fertilization could have influenced the microflora of trout. The study reveals the presence of pathogenic bacteria as part of the natural microflora of farmed trout. *A. hydrophila*and *A. sobria* that are potential pathogens of both rainbow trout and humans are prevalent in farmed trout.These pathogens are potential hazards to fish handlers and consumers. The results of the present study have practical significance in trout husbandry with regards to feed management and control of pathogens. Further study on the

UNDER PEER REVIEW

antibiotic resistanceprofile of these bacterial pathogens could establish the real threat posed by these organisms.

References

1. AL-Harbi, A.H. (1994)First isolation of Streptococcus sp. from hybrid tilapia (Oreochromis niloticus x O. aureus) in Saudi Arabia. *Aquaculture* 128, 195-201.
2. Allen D.A., Austin B. & Colwell R.R. (1983) Numerical taxonomy of bacterial isolates associated with a freshwater fishery. J General Microbiol 29, 2043-2062.
3. Andevari, G. T. and Rezaei, M. (2011), Effect of gelatin coating incorporated with cinnamon oil on the quality of fresh rainbow trout in cold storage. Int J Food Sci & Technol. 46, 2305–2311.
4. Andrew P.W. &MitchellT.J. (1997) The biology of Streptococci and Enterococci. Journal of Applied Bacteriology Symposium Supplement, Vol. 83. The Society for Applied Bacteriology, Blackwell Science, Osney Mead, Oxford, UK.
5. AOAC, 2000. Official methods of analysis (17th ed.), Association of Official Analytical Chemists, Washington, DC.
6. APHA (1998) Standard Methods for the examination of water and waste water, 20th edn. American Public Health Association,Washington, DC, USA.
7. Apun, K., A.M, Yusof., J. Kumbang, 1999. Distribution of bacteria in tropical freshwater fish and ponds. Int. J. Environ. Health Res., 9: 285-292.
8. Arashisar, S., Hisar, O., Kaya, M., & Yanik, T. (2004). Effects of modified atmosphere and vacuum packaging on microbiological and chemical properties of rainbow trout (Oncorhynchus mykiss) fillets. Int J Food Microbiol, 97, 209–214.
9. Austin B. (1988) Identi ¢ cation. In:Methods in Aquatic Bacteriology (ed. by B. Austin), pp. 95-112. JohnWiley& Sons, London, UK.

UNDER PEER REVIEW

10. Austin B. and Al- Zahrani A.M.J. 1988. The effect of antimicrobial compounds on the gastrointestinal microflora of rainbow trout, *Salmo gairdneri* Richardson. J. Fishery Biol. 33: 1-14.
11. Austin B., Austin D.A.(2007)Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish. Springer Praxis Publishing, UK, 552pp.
12. Bekker, A., Hugo, C., Albertyn, J., Boucher, C. E. and Bragg, R. R. (2011), Pathogenic Gram-positive cocci in South African rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis, 34: 483–487
13. Cahill, M.M., 1990. Bacterial flora of fishes. A review. Microbial Ecology, 19, 21-41.
14. Cann, D.C. and Taylor, L.Y. (1982) An outbreak of botulism in rainbow trout *Salmo gairdneri* Richardson farmed in Great Britain. *J. Fish Dis*. 5, 393-399.
15. Centers for Disease Control and Prevention (CDC) (1996). Invasive infection with *Streptococcus iniae* –Ontario 1995-1996. Morbidity and Mortality Weekly Report **45** (30): 650–53
16. Chacon, M.R., M.J. Figuras, G. Castro-Escarpulli, L. Soler, and J. Guarro. 2003. Distribution of virulence genes in clinical and environmental isolates of *Aeromonas* spp. Antonie van Leeuwenhoek 84, 269-278
17. Chytiri, S., Chouliara, I., Savvaidis, I. N., &Kontominas, M. G. (2004). Microbiological, chemical and sensory assessment of iced whole and filleted aquacultured rainbow trout. Food Microbiology, 21, 157–165.
18. Clucas, I.J. and A.R. Ward, 1996. Post-Harvest Fisheries Development. A guide to handling, preservation, processing and quality. National Resources Institute, Central Avenue, Chatham Maritime, UK., pp: 35-36.
19. Donald, B. and D.M. Gibson, D.M 1992. Preliminary observations on the flora of fresh vacuum packed salmon steaks. In "Quality Assurance in the Fish Industry" (H.H. Huss, Editor) Elsevier, London, p107-114.

UNDER PEER REVIEW

20. FAO/WHO(1974)Food and Agriculture Organization of the United Nations and World Health Organization Technical Report series (No. 550) Fish and Shellfish Hygiene report of WHO expert committee convened in cooperation with FAO
21. FAO. 2005. National Aquaculture Sector Overview – India. FAO 2010-2016. Fisheries and Aquaculture Department. In: *FAO Fisheries and Aquaculture Department* [online]. Rome. Updated. [Cited 13 January 2016]. http://www.fao.org/fishery/countrysector/naso_india/en
22. FDA., *Bacteriological Analytical manua*l 8thedn. (Revised), US food and Drug Administration. Rockville, Md., USA, 2001.
23. Flick, G.J.Jr.2008. Microbiological safety of farmed fish. Global Aquaculture Advocate p 33-34.
24. Gardner, G.A. (1966) A selective medium for the enumeration of Microbacteriumthermosphactum in meat and meat products. Journal of Applied Bacteriology 29, 455-460.
25. Gennari, M., Campanini, R.(1991) Isolamento e caratterizzazione di *Shewanella putrefaciens* da pesce fresco e alterato, carnifresche e alterate, prodottilattiero- caseari, acqua e suolo. *Industrial Alimenteria*, **30**, 965- 976, 988.
26. Gennari, M., Tomaselli, S., Cortrona, V., 1999. The microflora of fresh and spoiled sardines (*Sardina pilchardus*) caught in Adriatic (Mediterranean) sea and stored in ice. Food Microbiol. 16, 15-28.
27. González C.J., Lòpez T.M., García M.L., Prieto M. & Otero A. 1999. Bacterial microflora of wild Brown Trout (*Salmo trutta*), Wild Pike (*Esox lucius*), and Aquacultured Rainbow Trout (*Oncorhynchus mykiss*). J.Food Protect. 62, 1270- 1277.
28. Gonzalez CJ, Encinas JP, Garcia-Lopez ML & Otero A (2000) Characterization and identification of lactic acid bacteria from freshwater fishes. Food Microbiol 17: 383–391.
29. Gonzalez-Rodrguez M.N.; Sanz J.J.; Santos J.A.; Otero A.; Garca-Lopez M.L.2002. Foodborne pathogenic bacteria in prepackaged fresh retail portions of farmed rainbow trout and salmon stored at 3 ^{o}C. Int J Food Microbiol 76, 135-141.

UNDER PEER REVIEW

30. Gonzalez-Rodriguez M N, Sanz J J, Santos JA, Otero A, Garcia-Lopez M L (2001). acteriological quality of aquacultured freshwater fish portions in prepackaged trays stored at 3°C. J. Food Prot. 64: 1399-1404.

31. Gram, L.. et al. (1996). Microbial spoilage of fish and fish products. Int J Food Microbiol, 33:121-137.

32. Haghighi Karsidani S1, Soltani M1*, Nikbakhat-Brojeni G2, Ghasemi M3, Skall HF 2010. Molecular epidemiology of zoonotic streptococcosis/lactococcos in rainbow trout (Oncorhynchus mykiss) aquaculture in Iran. Iranian J. of microbial. 2, 198-209.

33. Harris, J.M. 1993. The presence, nature, and role of gut microflora in aquatic invertebrates: A synthesis, Microb. Ecol. *25,* 195–231.

34. Hielm, S., Hyytiä, E., Andersin, A.-B., Korkeala, H. 1998. A high prevalence of *Clostridium botulinum* type E in Finnish freshwater and Baltic sea sediment samples. J. Appl. Microbiol. 84: 133-137.

35. Horsely R.W. (1973) The bacterial flora of the Atlantic Salmon (Salmo salar, L) in relation to its environment. J Appl Bacteriol 36, 377-386.

36. Huber, I., Spanggaard, B., Appel, K.F., Rossen, L., Nielsen, T. and Gram, L..2004. Phylogenetic analysis and in situ identification of the intestinal microbial community of rainbow trout (Oncorhynchus mykiss, Walbaum). J Appl Microbiol 96, 117–132.

37. Huss, H.H. (1981) *Clostridium botulinumtype E and botulism.* 58 pp. Lyngby, Denmark, Technical Univ. of Denmark.

38. Huss, H.H., Pedersen, A. and Cann, D.C. (1974). The incidence of *Clostridium botulinum* in Danish trout farms.I. Distribution in fish and their environment. *J. Food Technol*. 9, 445-450.

39. International Commission on Microbiological Specifications for Foods (ICMSF) 1998. *Microorganisms in Foods.* 6. *Microbial Ecology of Food Commodities*. Blackie Academic & Professional, Baltimore.

UNDER PEER REVIEW

40. Islam, M.S., A. Begum, S.I. Khan, M.A. Sadique and M.N.H. Khan *et al*., 2000. Microbiology of pond ecosystems in rural Bangladesh: Its public health implications. Int. J. Environ. Stud., 58: 33-46.
41. Karmas, E. and Harris, R.S.. 1987. Nutritional Evaluation of Food Processing (3rd Edition), Van Nostand Reinhold CO, New York.
42. Kapperud, G. and Jonsson, B. (1976), *Yersinia enterocolitica* in brown trout (*salmo trutta* l.) from norway. Acta Pathologica Microbiologica Scandinavica Section B Microbiology, 84B: 66–68.
43. Kayis, S., Capkin, E.,Balta, F., Altinok, I. 2009. Bacteria in Rainbow Trout (*Oncorhynchus mykiss*) in the Southern Black Sea Region of Turkey - A Survey.The Israeli J Aqua – Bamidgeh 61, 339-344.
44. Kim D-H, Brunt J. and Austin B. 2007. Microbial diversity of intestinal contents and mucus in rainbow trout (*Oncorhynchus mykiss*). *J. Appl.Microbiol.* 102: 1654–1664
45. Kirov S.M**.** (2001). *Aeromonas* and *Plesiomonas*species. In M. Doyle, L. Beuchat & T. Montiville,Food Microbiology: Fundamentals and Frontiers (pp 301-327), Washington, D.C: ASM Press.
46. Koziñska, A., Pêkala, A., 2004. First isolation of Shewanella putrefaciens from freshwater fish—a potential new pathogen of fish. Bull. Eur. Assoc. Fish Pathol. 24, 189–193.
47. Krieg N.R., Holt J.G.(1984) *Bergey's Manual of Systematic Bacteriology*.vol.1. 964pp Baltimore, USA: Williams and Wilkins.
48. Lalitha, K.V. and Surendran, P.K. 2004. Bacterial microflora associated with farmed freshwater Prawn *Macrobrachiumrosenbergii*(de Man) and the aquaculture environment. Aqua. Res. 35: 1-7
49. Lalitha, K.V. and Surendran, P.K. 2006. Microbiological changes in farm reared freshwater prawn (*Macrobrachiumrosenbergii*de Man) in ice. Food Control.17, 802-807.
50. Lalitha,K.V. and K. Gopakumar..(2000).Distribution and ecology of *Clostridium botulinum* in fish and aquatic environments of a tropical region. *Food Microbiology* 17 (5),535-541

UNDER PEER REVIEW

51. Lee S, Kim S, OhY, Lee Y (2000) Characterization of *Aeromonas hydrophila* Isolated from Rainbow Trouts in Korea. J. Microbiol. 38: 1-7.

52. Lyhs U. et al. (2002). Identification of lactic acid bacteria from spoiled, vacuum-packaged 'gravad' rainbow trout using ribotyping. Int J Food Microbiol 72:147-53.

53. McAdams,T.J. Reinhart, R.G, Fernandes,C.F, Flick, G.J, Smith, S.A, Hackney, C.R, Libey,G.S and Granata, L.A.(2005). Incidence of Pathogenic Microorganisms in Aquacultured Rainbow Trout (*Oncorhynchus mykiss*). J Aqua Food Prod Technol 14, 95-105

54. Mead, G. C., 1985. Enumeration of pseudomonads using cephaloridine-fucidin-cetrimide agar (CFC). Int. J. Food Microbiol. 2, 21-26

55. Mexis, S.F., Chouliara, E. and Kontominas, M.G. 2009**.** Combined effect of anoxygen absorber and oregano essential oil on shelf life extension of rainbow trout fillets stored at 4 °C. Food Microbiol, 26, 598-605

56. Molin, G., Ternstrom, A., 1982. Numerical taxonomy of psychrotrophic pseudomonads. J. Gen. Microbiol.128, 1249–1264.

57. Montiville,Food Microbiology : Fundamentals and Frontiers (pp 301-327),

58. Mossel, D.A.A.(1987) de Man, Rogosa and Sharpe agar with sorbic acid (MRS-S agar). Int J Food Microbiol 5, 230 – 232.

59. Nam I.Y, Joh K.. 2007. Rapid detection of virulence factors of Aeromonas isolated from a trout farm by hexaplex-PCR. J Microbiol. 45:297-304.

60. Naviner, M., Giraud, E., Le bris, H., Armand,F., Mangion, C. and Ganière, J.P. 2006. Seasonal variability of intestinal microbiota in rainbow trout (*Oncorhynchus mykiss*), with a particular attention to *Aeromonas* spp. as candidate indicator of antimicrobial resistance. Revue Méd. Vét.,157 , 599-604

UNDER PEER REVIEW

61. Nerantzaki, A, Tsiotsias A, Paleologos EK., Savvaidis IN, Bezirtzoglou E, Kontominas MG (2005) Effects of ozonation on microbiological, chemical and sensory attributes of vacuum-packaged rainbow trout stored at 4± 0.5°C. Eur. Food Res. Technol. 221: 675–683.
62. *Niemi*, V.-M., Rahkio, M., Siitonen, A. *2004*(2004). Ruokaturvallisuudenkasikirja, p. 59-63. Art House Oy, Helsinki.
63. George Ninan**,** Lalitha K.V**,** Zynudheen A.A and Jose Joseph**.** 2011.Effect of Chilling on Microbiological, Biochemical and Sensory Attributes of Whole Aquacultured Rainbow Trout (Oncorhynchus mykiss Walbaum, 1792).J Aquac Res Development. 3,Special issue: Fisheries and Aquaculture AdvancementS5-001.doi: 10.4172/2155-9546.S5-001.
64. Olafsen JA (2001). Interactions between fish larvae and bacteria in marine aquaculture. Aquacul. 200: 223-247.
65. Ozogul Y, Ozogul F (2002) Degradation Products of Adenine Nucleotide in Rainbow Trout (*Oncorhynchus mykiss*) Stored in Ice and in Modified Atmosphere Packaging. Turk J.Zool. 26: 127-130.
66. Palumbo, S.E., Maxino, S.A., Williams, F., Buchanan, R.L., Thayer, D.W., 1985. Starch-ampicillin agar for the quantitative detection of *Aeromonas hydrophila*. Appl. Environ. Microbiol. 50, 1027–1030.
67. Pond, M.J., Stone, D.M., Alderman, D.J., 2006. Comparison of conventional and molecular techniques to investigate the intestinal microflora of rainbow trout (*Oncorhynchus mykiss*). Aquac 261, 194–203.
68. Rehulka, J., 2002. Aeromonas causes severe skin lesions in Rainbow Trout (*Oncorhynchus mykiss*): Clinical pathology, haemotology and biochemistry. Acta Vet. Brno, 71: 351-351.
69. Rezaei, M., Montazeri, N., Langrudi, H.E., Mokhayer, B., Parviz, M. and Nazarinia, A. 2007. The biogenic amines and bacterial change of farmed rainbow trout (*Oncorhynchus mykiss*) stored in ice. Food Chem. 31, 150–154.

UNDER PEER REVIEW

70. Rezaei,M., Hosseini, S. F. , Langrudi H.E., Safari, R., Hosseini, S.V. 2008. Effect of delayed icing on quality changes of iced rainbow trout (*Onchorynchus mykiss*). Food Chem, 106, 1161-1165.

71. Ringø, E. Strøm, ,E., J.A. Tabachek, J.A. 1995. Intestinal microflora of salmonids-A review, Aquac. Res. 26: 773–789.

72. Ringø, E., Bendiksen, H.R., Wesmajervi, M.S., Olsen, R.E., Jansen, P.A. and Mikkelsen, H., 2000. Lactic acid bacteria associated with the digestive tract of Atlantic salmon (*Salmon salar L.*). J. Appl. Microbiol. 89, 317-322

73. Ringø, E., Sperstad, S., Myklebust, R., Refstie, S. and Krogdahl, Å. 2006. Characterisation of the microbiota associated with intestine of Atlantic cod (*Gadus morhua* L.): the effect of fish meal, standard soybean meal and a bioprocessed soybean meal. Aquacul, 829–841

74. Salgado-Miranda, C., Palomares, E., Jurado, M., Marín, A., Vega, F., Soriano-Vargas, E. 2010. Isolation and distribution of bacterial flora in farmed rainbow trout from Mexico. J aqua anim health. 22:244-7.

75. Savvaidis I N, Skandamis P, Riganakos K A, Panagiotakis N, Kontominas M G (2002) Control of natural microbial flora and Listeria monocytogenes in vacuum-packaged trout at 4 and 10 degrees C using irradiation. J Food Prot. 65(3) :515-22.

76. Schmidt, A.S., Bruun,M.S., Dalsgaard, I., Pedersen, K.. and Larsen, J.L. 2000. Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. Appl. Environ. Microbiol. 66, 4908–4915.

77. Shigematsu.T., Hayashi.M., Kikuchi. I., Ueno. S., Masaki.H., and T. Fujii. (2009). A culture-dependent bacterial community structure analysis based on liquid cultivation and its application to a marine environment. FEMS Microbiol Lett 293: 240–247

78. Singh, I., Bhatnagar, S. and Virdi, J.S.2003. Isolation and characterization of *Yersinia enterocolitica* from diarrheic human subjects and other sources. Curr. Sci.84, 1353-1355.

UNDER PEER REVIEW

79. Sneath P.H.A., Mair N.S., Sharpe M.E. and Holt J.G. (1986) Bergey's Manual of Systematic Bacteriology. vol.2. Baltimore, USA: Williams and Wilkins.

80. Spanggaard, B., Huber, I., Nielsen, J., Nielsen, T., Appel, K.F. and Gram, l. 2000. Microflora of rainbow trout intestine: A comparison of traditional and molecular identification. Aquacul.182, 1–15.

81. Sugita, H., Tsunohara, M., Ohkoshi, T. and Deguchi, Y.1988. The establishment of an intestinal microflora in developing goldfish (*Carassius auratus*) of culture ponds. *Microbiol. Ecol.*, 15 :333–344

82. Surendran, P.K., Thampuran, N. and Gopakumar, K. 1995. Microbial profile of culturedfishes and prawns viz a viz their spoilage and contamination. FAO Fisheries Report No. 514Supplement, FAO, Rome, pp. 1-12.

83. Toor, K.S., Kaur, K. and Dhawan, A. 1991. Utilization of biological wastes in carp culture in: Aquaculture productivity (ed. by V.R.P.Sinha and H.C.Srivastava) New Delhi, Oxford, IBH Public Co. Ltd. 863pp.

84. Tortorello,M.L.(2003)Indicator Organisms for Safety and Quality—Uses and Methods for Detection: Minireview. J. AOAC International, 86, 1208-1217.

85. West P.A. (1989) Human pathogens and public health indicator organisms in shellfish. In: Methods for the Microbiological examination of ish and shellfish (ed. by B. Austin & D.A. Austin), pp. 273-308. Halsted Press, Chichester, UK.

86. WHO **(2007):** Food safety issues associated with products from Aquaculture? Report of a Joint FAO/NACA/WHO study Group, WHO Technical reprint Series: 883 + Geneva.

UNDER PEER REVIEW

Table 1. Mean Microbial count on farmed trout (*Onchorynchusmykis*) from farm located in Idukki district, Kerala (India)

Microbial Parameters		Microbial Count $\log_{10}$ cfu/g *Oncorhynchus mykiss* SM	Int	Feed
TPC	30^0C	4.529 ± 0.109	7.657 ± 0.283	8.282 ± 0.55
	7^0C	3.771 ± 0.128	6.086 ± 0.081	NT*
H2S Producing bacteria		3.964 ± 0.208	5.562 ± 0.159	5.679 ± 0.697
Aeromonas		2.692 ± 0.763	5.601 ± 0.504	NT
Enterobacteriaceae		3.613 ± 0.011	6.226 ± 0.17	5.358 ± 0.316
Pseudomonas		2.34 ± 0.287	4.281 ± 0.232	3.602
Lactic acid bacteria		3.166 ± 0.117	5.764± 0.314	NT
Brochothrixthermosphacta		2.281 ± 0.134	4.775 ± 0.439	4.512 ± 0.47

* Not Tested

UNDER PEER REVIEW

Table 2. Mean Microbial count of water and sediment from farm located in Idukki district, Kerala (India)

Microbial Parameters*		Microbial Count $\log_{10}$ cfu/g		
		Farm water	Feeder canal water	sediment
TPC	30^0C	3.70 ± 0.091	2.849 ± 0.151	7.322 ± 0.009
	7^0C	2.428	NT*	6.085
H2S Producing bacteria		3.756	NT	5.439
Enterobacteriaceae		2.799	2.113	3.519

* Not Tested

UNDER PEER REVIEW

Table 3. Indicator and pathogenic bacteria in water, sediment, feed and trout from farm located in Idukki district, Kerala (India)

Bacteriological parameters	Mean bacterial count (log_{10} cfu g-1)					
				Trout		
	Farm water*	Feedercanal water	Sediment	SM	Intestine	Feed
Total Coliforms [a]	3.041	3.041	3.146	2.041	3.146	3.146
Fecal coliforms[a]	1.653	1.397	3.146	1.397	3.041	3.146
Escherichia coli[a]	1.653	1.176	3.041	0.977	3.041	3.146
Faecal streptococci [b]	ND	ND	2.204	2.216	5.068	5.531
Staphylococcus aureus[b]	ND	ND	2.7	2.389	3.623	2.34
Clostridium perfringens[a]	ND	ND	0.544	ND	1.397	ND
Yersinia enterocolitica[c]	ND	ND	ND	ND	ND	ND
Clostridium botulinum[c]	ND	ND	ND	ND	ND	ND

[a]MPN; [b] cfu/g ; c – present / absent * for water samples MPN/100ml, for sediment, fish and feed MPN/g ; ND not detected

UNDER PEER REVIEW