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ORIGINAL ARTICLE

Study on the Density and Identification of Gut Bacteria from Nile Tilapia (*Oreochromis Niloticus*, Linnaeus, 1758) Cultured in Biofloc System at West Bengal, India

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ABSTRACT

Biofloc technology (BFT) is gaining popularity in aquaculture. The primary objective of the current study was to examine intestinal bacterial counts in water and gut (stomach and hindgut) samples obtained from Oreochromis niloticus from different farms of North and South 24 Parganas. The total heterotrophic bacterial counts (THBC) and total bacterial counts have been done following DNA extraction and molecular identification. DNA was sequenced and analyzed at the Genomics Division, Xcelris Labs Ltd, Ahmedabad, India. The total heterotrophic bacterial counts (THBC) and floc development in South 24 Parganas were comparatively better than North 24 Parganas and that the THBC are higher in hindgut samples. Aeromonas veronii was isolated from O. niloticus stomach samples from the Shyamnagar farm and Escherichia coli was identified from another strain of O. niloticus hindgut samples from Panchpota farm by molecular study. The proclaimed THBC in gut and water samples substantiate the suitability of BFT for broader-scale implementation, particularly in West Bengal, where its utilization was previously absent

Keywords: *Biofloc, Bacterial count, Hindgut, O. niloticus, Stomach.*

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INTRODUCTION

Biofloc technology (BFT) is an environmentally friendly and sustainable approach to implementing intensive aquaculture practices with minimal or zero water exchange [7]. Bioflocs are considered as protein-rich "natural food" that forms through the mucus secretion of bacteria and a mixture of filamentous microorganisms. They comprise a diverse range of organisms, including algae, diatoms, phytoplankton, bacteria, zooplankton, protozoans, as well as particulate organic matter such as fecal remnants and unconsumed feed [16]. Nile tilapia (*Oreochromis niloticus*) holds the distinction of being the third most significant fish species for aquaculture, following grass carp and silver carp. Its cultivation has experienced a fourfold increase in the past decade owing to its ease of aquaculture, market demand, and stable pricing [8]. Nile tilapia is known for its ability to directly consume bacteria present in the water column or attached to substrates [2]. This unique characteristic, combined with its filter-feeding, detritivore, and grazing behaviors, enables Nile tilapia to thrive in environments with high solids concentration and suboptimal water quality, making it an excellent choice for Biofloc technology (BFT) implementation, even surpassing other tilapia species within the same genus [4]. The utilization of biofloc technology has gained significant popularity in West Bengal's aquaculture sector [5]. Understanding the

composition of the microbial community in the gut of fish reared using biofloc technology is crucial as it not only impacts water quality but also affects the productivity of the fish. The gut microbiota plays a vital role in maintaining the overall well-being of fish by balancing the presence of native and foreign bacteria. In biofloc systems, the continuous uptake of microbial floc increases the likelihood of bacterial colonization in the gut of tilapia [9]. The microbiota presents in biofloc culture systems and the gut of fish reared in such systems contribute to enhancing the fish's digestive ability. Consequently, there has been growing interest in studying the dynamics of microbial communities in biofloc aquaculture systems and their effects on the performance of the cultured animals [13]. However, there is still a significant knowledge gap in this field of aquaculture that needs to be addressed. Therefore, the objective of this study was to identify and isolate intestinal bacteria from Nile tilapia cultured in biofloc systems at selected farms in the North 24 Parganas and South 24 Parganas districts of West Bengal, India.

MATERIAL AND METHODS

Sampling

The study was conducted over a period of six months, during which the experimental fish (O. niloticus) and water samples were obtained from five distinct biofloc technology (BFT) farms situated in North 24 Parganas, specifically Bongaon, Gaighata, Shyamnagar, Ashoknagar, and Basirhat. Additionally, samples were collected from five different BFT farms located in South 24 Parganas, namely Panchpota, Subhasgram, Narendrapur, Naryanpur, and Baruipur. For the gut bacterial count analysis, five fish specimens were randomly selected from each sampling area, resulting in a total of 50 fish being collected. The live fish and water samples were promptly transported to the laboratory within a span of 2-3 hours from the time of collection to ensure sample integrity.

Bacterial counts

The bacterial counts of both the water and fish gut samples were analyzed using a standardized procedure outlined in the APHA [1]. The stomach and hindgut sections of each sampled fish were collected, and serial dilution techniques were employed. The diluted samples were then plated on general enriched media to determine the total bacterial concentration in colony-forming units (CFU) per gram of sample (CFU/g). Similarly, the bacterial concentration in the sampled water was also analyzed.

Bacterial DNA extraction and PCR amplification of 16S rDNA gene

DNA was extracted, using a genomic DNA isolation kit (Macherey- Nagel, Germany) as per the manufacturer's protocol and PCR reaction was performed using Universal Primers of 16S rDNA gene [forward primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'-ACGGCTACCTTGTTACGACTT-3') in a Master cycler Pro S system (Eppendorf, Germany).

Agarose gel electrophoresis

The 1400 bp PCR products were seen on 1.2% agarose gels containing 0.5 µg/ml ethidium bromide in 1X Tris-acetate- EDTA (TAE) buffer.

DNA sequencing and analysis

The PCR amplified products were sequenced and further data analysis and evolutionary trees for nucleotide sequences were drawn by using the Neighbour-Joining method [17]. The PCR amplified products were sequenced at the Genomics Division, Xcelris Labs Ltd, Ahmedabad, India.

Statistical analysis

The results were expressed as the mean±standard deviation and analyzed by one-way analysis of variance (ANOVA) using SPSS (Statistical Package for the Social Sciences) to test the significance of difference among the Total heterotrophic bacterial counts (THBC) and bacterial isolates from BFT reared Nile tilapia stomach, hindgut and water samples after log transformation. Probability level of 0.05 was used to find out the significance in all cases.

RESULTS AND DISCUSSION

Total heterotrophic bacterial counts (THBC)

THBC of stomach and hindgut samples collected from North 24 Parganas differed insignificantly ($p>0.05$) among all the sampling areas (Fig. 1A). The highest counts of stomach samples were obtained in Ashokenagar (log 6.58±0.02 CFU/g) while the lowest counts were obtained in Shyamanagar (log 6.26±0.05 CFU/g) BFT farm and differed significantly ($p<0.05$) with all the other sampling areas. The highest counts of hindgut samples were obtained in Ashokenagar (log 6.62±0.04 CFU/g) and lowest in Shyamanagar (log 6.3±0.03 CFU/g). The THBC of hindgut samples collected from Bongaon (log 6.45±0.03 CFU/g) and Gaighata (log 6.51±0.03 CFU/g) differed significantly ($p<0.05$) between themselves. The counts of water samples fluctuated between Basirhat (log 5.84±0.01 CFU/ml) and Ashokenagar (log 6.01±0.01 CFU/ml) (Fig. 2A). The THBC of water samples collected from Gaighata (log 5.97±0.02 CFU/ml)

and Ashokenagar differed significantly (p<0.035) with all other sampling areas. Total heterotrophic bacterial counts (THBC) of stomach and hindgut samples collected from South 24 Parganas showed significant differences (p<0.05) in four sampling areas except Baruipur (Fig. 1B). The highest THBC of stomach samples were obtained in Baruipur (log 6.47±0.04 CFU/g) while the lowest counts were obtained in Narendrapur (log 6.24±0.03 CFU/g) farm. The highest THBC of hindgut samples were obtained in Panchpota (log 6.64±0.02 CFU/g) and lowest were achieved in Narendrapur (log 6.32±0.05 CFU/g) but it differed significantly (p<0.05) with others. The counts of water samples from Panchpota (log 5.89±0.04 CFU/ml) to Narendrapur (log 6.17±0.01 CFU/ml) showed significant differences (p<0.05) in Panchpota than other sampling areas (Fig 2B).

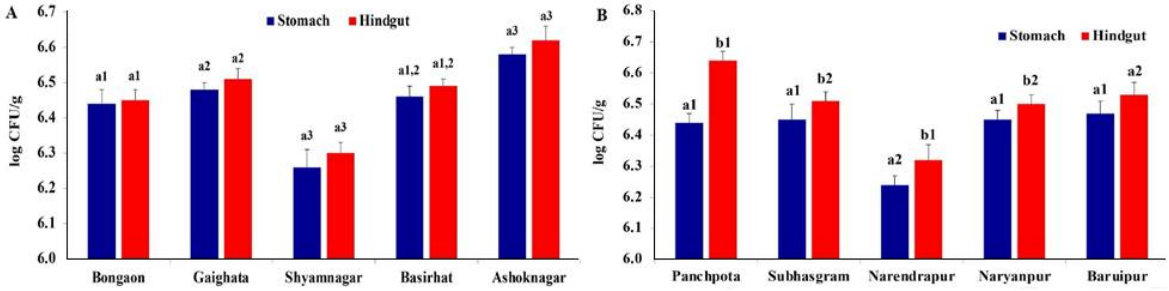

Fig 1.Total heterotrophic bacterial counts in BFT cultured *Oreochromis niloticus* stomach and hindgut samples in North 24 parganas (A) and South 24 Parganas (B). a: Bars sharing common alphabets for a particular sampling area differed insignificantly (p>0.05); 1,2,3: Bars sharing common numerals for a particular sample differed insignificantly (p>0.05).

Total Bacterial counts

The total *Aeromonas* sp. counts (TAC) of hindgut samples were highest in Ashokenagar (log 6.54±0.01 CFU/g) and lowest in the stomach samples collected from Shyamanagar (log 6.05±0.04 CFU/g) (Fig. 3A). Total *E. coli* counts (TEC) in the gut samples varied between log 6.05±0.04 CFU/g and log 6.55±0.01 CFU/g. The TAC in stomach samples collected from Shyamanagar, Basirhat and Ashokenagar differed significantly (p<0.05) from Bongaon and Gaighata BFT farm. The TEC of stomach samples from Gaighata differed significantly (p<0.05) with Shyamanagar and Basirhat. *Klebsiella* sp. counts were only obtained in the stomach samples tested from Panchpota and differed significantly (p<0.05) with the TAC. The TAC and TEC in hindgut samples from Bongaon and Shyamanagar showed significant difference (p<0.05) from Gaighata, Basirhat and Ashokenagar. Total *Klebsiella sp.* counts (TKC) differed significantly among themselves (hindgut samples) from Shyamanagar and Ashokenagar (Fig. 4A). The TAC of water samples in North 24 Parganas ranged from log 5.76±0.01 CFU/ml (Bongaon) to log 5.86±0.01 CFU/ml (Ashokenagar). The TEC fluctuated between Basirhat (log 5.51±0.02 CFU/ml) and Shyamanagar (log 5.58±0.02 CFU/ml). The TAC of water samples collected from Bongaon differed significantly (p<0.05) against all other places. Similarly, the TEC of water samples from Shyamanagar differed significantly (p<0.05) from Bongaon and Basirhat. The TAC and TEC of water samples from Shyamanagar and Bongaon differed significantly (p<0.05) between themselves.

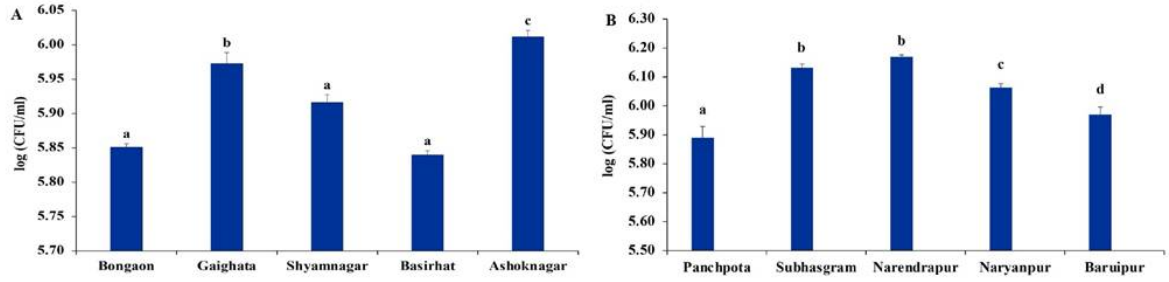

Fig 2. Total heterotrophic bacterial counts in BFT cultured *Oreochromis niloticus* water samples in North 24 parganas (A) and South 24 Parganas (B). a-d: Bars sharing common alphabets for a particular sampling area differed insignificantly (p>0.05).

The TAC, TEC and TKC in stomach and hindgut samples collected from South 24 Parganas BFT farm are displayed in Fig. 3B. The TAC of stomach samples fluctuated between Narendrapur (log 6.15±0.05 CFU/g) and Panchpota (log 6.5±0.02 CFU/g). The least TEC of gut samples was obtained from Narendrapur (log

6.01±0.02 CFU/g) and the highest from Panchpota (log 6.33±0.02 CFU/g) and Baruipur (log 6.33±0.02 CFU/g). The TKC were enumerated in the stomach samples collected from Panchpota (log 6.25±0.04 CFU/g) and hindgut samples collected from Baruipur (log 6.24±0.05 CFU/g) BFT farm. Significant differences ($p<0.05$) were observed in TAC of stomach samples collected from every sampling location. The TEC of stomach samples from Panchpota differed significantly ($p<0.05$) with the collected samples from Subhasgram and Narendrapur. The TAC in fish stomach samples collected from Panchpota farm differed significantly ($p<0.05$) with TEC and TKC. The TAC of hindgut samples collected from Panchpota displayed significant differences ($p<0.05$) from Subhasgram, Narendrapur, Narayanpur and Baruipur. The TEC of samples from Panchpota exhibited significant differences ($p<0.05$) from Subhasgram, Narendrapur, Narayanpur. The TAC, TEC and TKC of hindgut samples collected from Baruipur BFT farm differed significantly ($p<0.05$) among themselves. Similarly, the TAC and TEC of hindgut samples tested from Panchpota, Subhasgram, Narendrapur and Narayanpur differed significantly ($p<0.05$) within themselves. The counts of *Aeromonas sp.*, *Escherichia coli* and *Klebsiella sp.* in water samples are depicted in Fig. 4B. The TAC of water samples in South 24 Parganas ranged from Panchpota (log 5.79±0.01 CFU/ml) to Narendrapur (log 6.01±0.01 CFU/ml). The TEC enumerated from Panchpota was log 5.52±0.03 CFU/ml) and Narayanpur was log 5.71±0.01 CFU/ml. However, only TKC were counted from Narayanpur (log 5.5±0.02 CFU/ml). The TAC from Panchpota differed significantly ($p<0.05$) against others. Similarly, the TEC from Panchpota BFT farm displayed significant differences ($p<0.05$) from Narayanpur. The TAC and TEC of Panchpota exhibited significant differences ($p<0.05$) between themselves. Similarly, the TAC, TEC and TKC from Narayanpur BFT farm differed significantly ($p<0.05$).

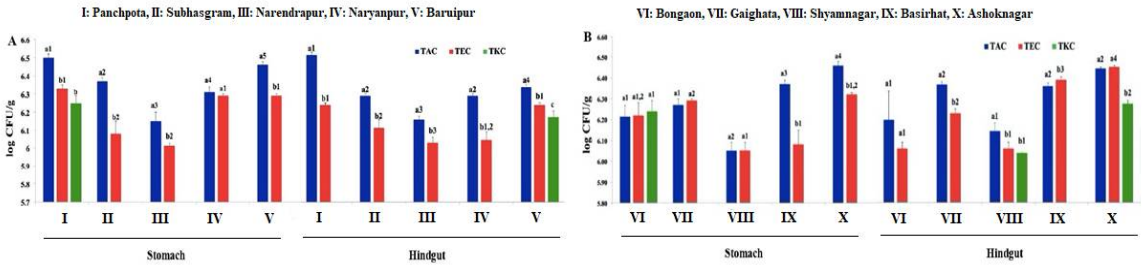

Fig 3. Total *Aeromonas sp.* counts (TAC), Total *E. coli* counts (TEC) and Total *Klebsiella sp.* counts (TKC) of BFT cultured *O. niloticus* stomach and hindgut samples in North 24 Parganas (A) and South 24 Parganas (B). a-b: Bars sharing common alphabets for a particular sampling area differed insignificantly ($p>0.05$) in stomach and hindgut samples; 1,2,3,4,5: Bars sharing common numerals for a particular bacterial species differed insignificantly ($p>0.05$) in stomach and hindgut samples.

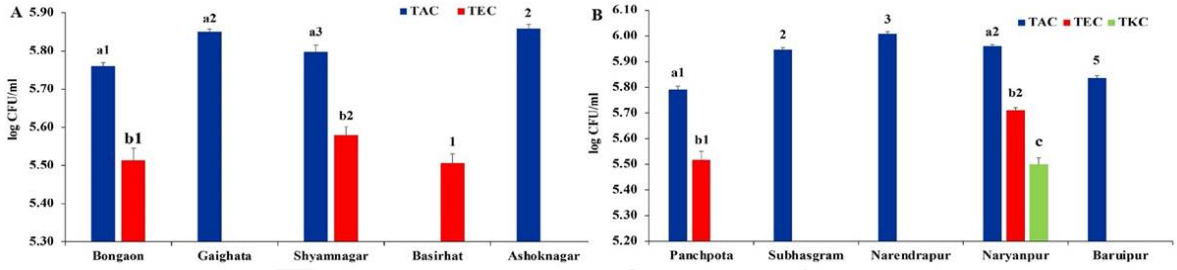

Fig 4. Total *Aeromonas sp.* counts (TAC), Total *E. coli* counts (TEC) and Total *Klebsiella sp.* counts (TKC) in BFT cultured water samples in North 24 parganas (A) and South 24 Parganas (B). a-c: Bars sharing common alphabets for a particular sampling area differed insignificantly ($p>0.05$); 1,2,3,4,5: Bars sharing common numerals for a particular bacterial species differed insignificantly ($p>0.05$).

16S rDNA gene analysis

Randomly selected 2 bacterial strains were further characterized and identified through 16S rDNA gene analysis (Fig. 5). Among the 2 bacterial strains, one strain was identified as *Aeromonas veronii* and other strain as *Escherichia coli*. The phylogenetic analyses (Fig. 6) revealed that the 16s rDNA gene sequence of SSA1 had 100% homology with *Aeromonas sp.* and *Aeromonas veronii*. They were clustered in a phylogenetic tree with highest bootstrap value. Another phylogenetic analysis (Fig. 7) revealed that the 16s rDNA gene sequence of PHE1 was clustered together with other *Escherichia coli* and *Escherichia fergusonii* with 64 base differences.

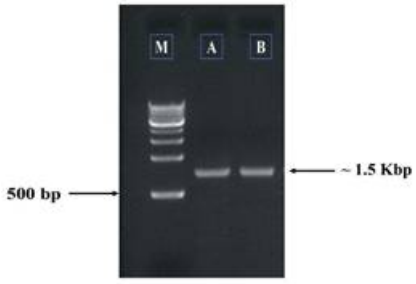

Fig. 5. Agarose gel (1.2%) showing 16S rDNA gene amplicons of bacterial strains in *O. niloticus* gut samples. DNA bands (black arrow), (M) molecular marker; (A) Sample code: SSA1; (B) Sample code: PHE1 at 1500bp

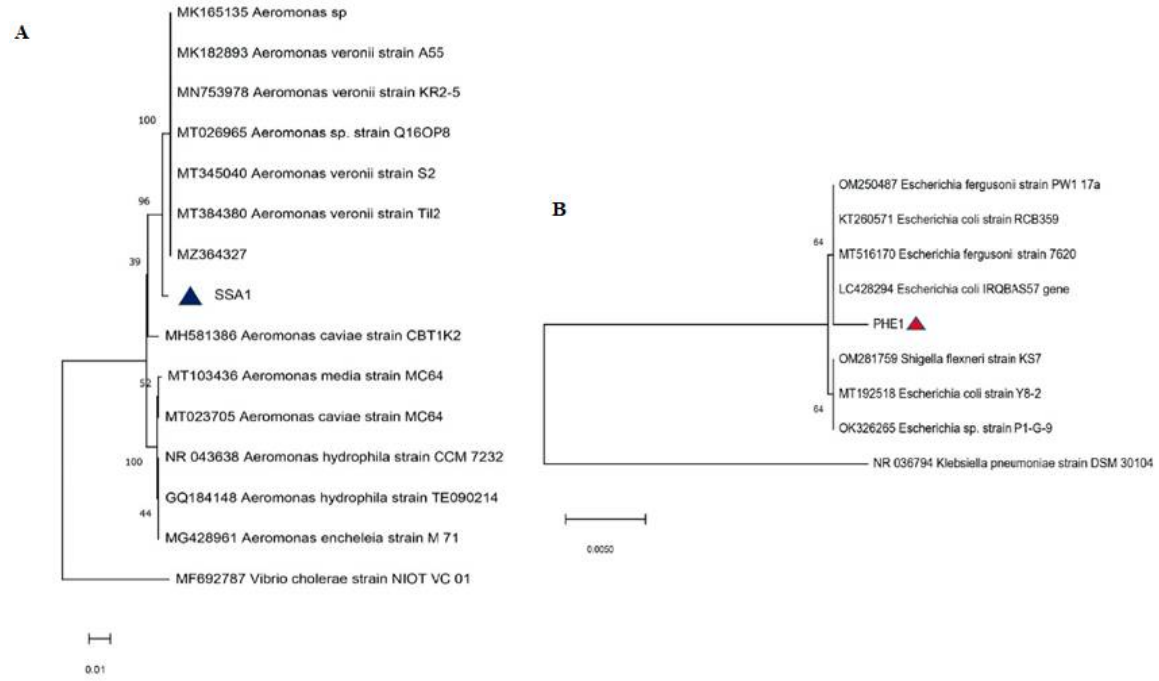

Fig. 6. Phylogenetic tree generated by Neighbour-Joining Kimura-2 parameter of the 16S rDNA sequence of bacterial strains isolated from BFT cultured (A) *O. niloticus* stomach and (B) *O. niloticus* hindgut samples. The isolates SSA1 from stomach and PHE1 from hindgut samples identified in this study are indicated by the blue and red shaded triangles respectively.

The presence and impact of biofloc bacteria on the bacterial community in the digestive tract of Nile tilapia, particularly in the intestine, can be significant due to the ability of biofloc technology (BFT) systems to create and maintain high bacterial concentrations [3]. Although the counts were lower in stomach samples in sampling regions of North 24 parganas, the difference were insignificant ($p>0.05$). Biofloc rearing fishers often use molasses and carbohydrate sources to increase the heterotrophic bacterial counts in culture water as a source of feed supplementation [19]. Similarly, the insignificant differences can be attributed to such external supplementation. Total heterotrophic bacterial counts in stomach and hindgut samples collected from Shyamnagar were significantly lower than other sampling areas. The variation in counts in different regions can also be attributed to culture period, as THBC tend to increase with days of culture [12]. The THBC of water samples collected from Narendrapur, Narayanpur and Subhasgram biofloc farm were comparable to those of the reports of [11]. The study found more colonization of bacteria in the stomach and hindgut samples than the BFT cultured water samples similar to [22]. The total counts of bacterial species were much higher in hindgut samples followed by stomach and water samples. Similar result was reported by [6]. The study concluded that the counts of both *Aeromonas sp.*, and *E. coli* were higher than *Klebsiella sp.* The present results showed that the counts were in the similar range of previous studies [18]. However, the current study showed higher values of *Aeromonas* (10^6) loads in fish gut samples which are similar to the reports of [21]. Since *E. coli* and *Klebsiella sp.* are known to propagate among the members of the Enterobacteriaceae, there is a high probability of transmission from aquatic animals to humans and vice-versa [20]. The *E. coli* counts of water samples sampled from Shyamnagar may be higher due to the BFT water fertilized with the animal

faecal matter and influents of domestic wastes [15]. It was reported that heterotrophic microbial biomass has a controlling effect on pathogenic bacteria *A. hydrophila* [14]. The count of *Aeromonas sp*., *E. coli* and *Klebsiella sp*. in hindgut samples were higher than the stomach samples as the hindgut offers favorable conditions for the proliferation of bacteria that have transit through the foregut [10]. Whereas most of the farmers in North and South 24 Parganas used molasses, sugarcane, hence the count was much higher. Very few reports are available on the biofloc management practices in North and South 24 Parganas districts of West Bengal. The current study documented the intestinal bacterial composition, THBC, total count and prevalence of the isolated bacteria from BFT cultured *O. niloticus* gut and water samples from both districts. The present study displayed mean heterotrophic bacterial count and floc development in South 24 Parganas were comparatively better than North 24 Parganas. The proclaimed THBC in gut and water samples prove the suitability of BFT for a broader scale.

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