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The Impact of Enterohemorrhagic *Escherichia coli* (EHEC) on Ciliate Protozoan Populations in Municipal Sewage

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Abstract

Enterohemorrhagic *Escherichia coli* strains (EHEC) have caused many foodborne outbreaks. Bacterivorous protozoa could remove bacteria from aquatic systems. We analyzed the ciliate protozoan population changes influenced by EHEC co-culture in activated sludge. EHEC and non-EHEC control *E. coli* cells were added to activated sludge samples in microcosms. The ciliate population changes were monitored by terminal restriction fragment length polymorphism (T-RFLP) analysis. EHEC and non-EHEC fed ciliate protozoan populations were different from each other and the no bacteria added controls based on the additive main effects and multiplicative interaction model (AMMI) analysis. Ciliate species were identified by 18S rDNA clone libraries. The 18S rDNA clones from the original sludge sample were identified as *Epistylis wenrichi* (70%) and *Prorodon teres* (30%), while clones from EHEC treated sludge sample were identified as *P. teres* (52%), *Vorticella fusca* (41%), *Dexitrichides pangi* (5%), and *Opisthonecta henneguyi* (2%). This study could provide helpful information about ciliate protozoan population changes caused by different *E. coli* strains in wastewater treatment plants, which could be useful for preventing and tracking *E. coli* outbreaks.

Keywords

18S rDNA, Activated Sludge, Protozoa, Enterohemorrhagic *E. coli*, T-RFLP

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1. Introduction

Enterohemorrhagic *Escherichia coli* strains (EHEC) have caused increased cases of worldwide foodborne *E. coli* outbreaks [1]-[3]. *E. coli* O157:H7 strain EDL 933 was first isolated in 1982 from an *E. coli* outbreak which caused hemorrhagic colitis and hemolytic-uremic syndrome (HUS) [4]. The major virulence factor of EHEC is the Shiga toxin, which is produced by *E. coli* in two forms: Stx1 and Stx2. It was first reported in 1983 that EHEC also made Shiga toxin [5]. The *stx* genes are encoded on lysogenic lambdoid bacteriophages in the *E. coli* genome, which can be expressed in the lytic cycle of the bacteriophage [6]. Stx toxins may have evolved as a bacterial defense against protozoan predators, such as the ciliate *Tetrahymena thermophila* [7] [8]. Steinberg and Levin [7] found that the ratio of Stx^+ to Stx^- *E. coli* increased under the predation of *T. thermophila*. Lainhart *et al.* [8] further confirmed that *T. thermophila* can be killed by either *stx*-carrying EHEC strain EDL 933 or purified Stx protein.

Many EHEC outbreaks have been associated with contaminated food products and water [9] [10]. EHEC can be transmitted from contaminated cattle manure and irrigation water to agricultural products, such as lettuce [11] [12]. Ravva *et al.* [10] showed that *E. coli* O157:H7 failed to proliferate in dairy lagoon wastewater microcosms, indicating that EHEC could be removed from wastewater system. Some ciliate protozoa were found to be resistant to *E. coli* O157:H7 and also able to reduce *E. coli* populations in dairy lagoon wastewater [13].

Pathogens in urban sewage could reflect infections in human populations [14]. *E. coli*, as well as total and fecal coliform bacteria, has been used as indicators for wastewater treatment efficiency [15]. EHEC and other *stx*-positive *E. coli* strains have been found in municipal sewage [16] [17]. Moreover, bacteriophages carrying stx_2 genes were also discovered in municipal sewage [18]. Thus, the sewage systems in urban areas could be potential reservoirs for EHEC evolution.

Protozoa are unicellular eukaryotes and bacterivorous protozoan species are considered to be important in shaping the structure of bacterial communities in planktonic as well as terrestrial ecosystems [19]. Activated sludge from wastewater treatment plants is a known reservoir for both bacterial and protozoan populations [20] [21]. Protozoan populations have been used as indicators of wastewater treatment plant performance [20] [22]. Protozoan population numbers could be as high as 50,000 cells per ml in the activated sludge in wastewater treatment plants [20]. Ciliates are the dominant protozoa in sewage [20] [22] [23]. Ciliate protozoan bacterivory of both pathogenic and non-pathogenic *E. coli* could remove up to 95% of *E. coli* in the activated sludge system of domestic sewage treatment plants [20].

The *stx* genes were detected in Pocatello wastewater treatment plant in Pocatello, Idaho, USA [24]. Municipal wastewater is collected from a population of approximately 60,000 people, and treated with an activated sludge system. The effluent is chlorinated and discharged into Portnuef River in Pocatello and solid waste is recycled on local agricultural fields as fertilizer. In order to understand the ciliate protozoan responses to EHEC in the activated sludge of municipal sewage ecosystems, the protozoan population changes were monitored under the influence of EHEC strain EDL 933 by terminal restriction fragment length polymorphism (T-RFLP) analysis. Ciliate protozoa species resistant to EHEC were also identified by 18S rDNA clone libraries.

2. Materials and Methods

2.1. Sample Collection and Microcosms Set Up

The activated sludge samples were collected from the wastewater treatment plant in Pocatello, Idaho, USA. To construct a microcosm, 1 liter of sludge samples was added to an Erlenmeyer flask and kept at 20˚C. Fresh air was pumped into each microcosm at 27.5 ml · $second^{-1}$ to simulate the aeration process in the sewage treatment plant.

E. coli strains used in this study were EHEC strain EDL 933 provided by the National Food Safety & Toxicology Center at Michigan State University, and non-EHEC strain K-12 received from American Type Culture Collection (ATCC). *E. coli* cells were cultured in Luria-Bertani (LB) medium and collected at log phase. *E. coli* cells were kept in 0.1% peptone solution at 4×10^{10} ml^{-1} colony forming unit (CFU) [25]. Ten milliliters of *E. coli* solution was added to each microcosm every 2 days. One set of 3 microcosms included one EHEC added microcosm, one K-12 added microcosm and one control microcosm without any bacteria added. The experiment was repeated in triplicate.

Samples were collected from one set of microcosms at day 0, 2 and 4 for total bacterial counts. The total bac-

terial counts were performed using the serial dilution method on LB agar plates [26]. Sample collection for T-RFLP analysis was done according to Li *et al.* [27]. Briefly, 1 ml of sludge sample was collected as the control sample before the sludge was added to each microcosm at day 0. At day 10 and 25, 1 ml of suspended sludge sample was collected from each microcosm. DNA was extracted using the FastDNA® SPIN Kit for Soil Kit (MP Biomedicals, Santa Ana, CA, USA). Each DNA sample was equally divided into 3 aliquots and stored at −20˚C before PCR amplification.

At day 0, 1 ml of sewage sample was collected from one of the control microcosms for 18S rDNA analysis. At day 53, 1 ml of sewage sample was collected from one of the three EDL 933-added sewage microcosms for 18S rDNA analysis. The FastDNA® SPIN Kit for Soil Kit (MP Biomedicals, Santa Ana, CA, USA) was used to extract total DNA from activated sludge samples.

2.2. 18S rDNA Gene Clone Library

PCR amplification of ciliate specific18S rDNA was carried out with the following primers: 384F (YTB GAT GGT AGT GTA TTG GA) and 1147R (GAC GGT ATC TRA TCG TCT TT) [28]. PCR amplification reactions were carried out in 50 µl reactions containing 1 × PCR buffer, dNTPs at 0.8 mM each, 2 mM $MgCl_2$, each primer at 0.4 µM, 1.25 U of Takara Ex *Taq* polymerase (Clonetech, Mountain View, CA, USA), and 0.2 µg non-acetylated BSA (Promega, Madison, WI, USA) in a PCT-200 Peltier Thermal Cycler (MJ Research Inc., Watertown, MA, USA). Amplification conditions were 94˚C for 1 min, held at 80˚C for addition of dNTPs (hot start), followed by 30 cycles of 94˚C for 45 seconds, 55˚C for 1 min, and 72˚C for 1.5 min, followed by a final extension at 72˚C for 10 min. Amplified 18S rDNA gene fragments were cloned into pGEM®-T Easy Vector (Promega, Madison, WI, USA) and transformed into *E. coli* DH5*α* according to the manufacturer's instructions. Recombinant colonies from each source were identified from Ampicillin/X-gal plates. The plasmid DNA was extracted using the alkaline-lysis mini-preparation method [29]. The plasmids were grouped according to restriction fragment length polymorphism patterns following double digestion by *Rsa*I and *Msp*I (Promega, Madison, WI, USA) and visualization by 2% agarose gel electrophoresis [30]. Plasmid inserts from representative clones of each unique RFLP group were sequenced with the SP6 primer (TACGATTTAGGTGACACTATAG). Basic Local Alignment Search Tool (BLAST) was used to identify ciliate species from the 18S rDNA gene sequences based on the highest identity scores. Selected clones with different BLAST search results were used for sequencing with the T7 primer (TAATACGACTCACTATAGGG). Vector NTI Suite 9 (Invitrogen Corporation, Carlsbad, CA, USA) was used to align contiguous sequences. RDP3 was used to detect possible chimeric sequences from clone libraries [31]. Assembled contig sequences were searched against GenBank accessions via BLAST, and the matched ciliate species with highest identity scores were identified. MEGA 5 was used to construct the phylogenetic tree using the maximum likelihood method [32]. The sequences of 18S rDNA clones and the closely related ciliate species sequences obtained from the NCBI database were used in the phylogenetic tree with *Paramecium tetraurelia* as the outgroup.

2.3. T-RFLP Analysis

T-RFLP analysis was conducted according to Liu *et al.* [33] by using fluorescently labeled 18S rDNA primers: 5'FAM-384F and 5'HEX-1147R. PCR conditions were the same as the 18S rDNA clone library experiments. PCR products were purified and washed with 200 µl TE in the Montage® PCR spin columns (Millipore, Bedford, MA, USA) and resuspended in 30 µl TE. Aliquots of 15 µl were digested with *Msp*I and *Rsa*I separately and incubated overnight at 37˚C. The digested mix was amended with 2 µl of 2.5 M sodium acetate, pH 5.2, and precipitated with 60 µl isopropanol overnight at 4˚C. The pellet was recovered after 10 min of centrifugation at 16,100 × g and washed with 70% ethanol, vacuum dried and resuspended in 5 µl TE. T-RFLP analyses of the fluorescently labeled fragments were performed with an ABI 3100 automated capillary DNA-sequencer (Applied Biosystems Instruments, Foster City, CA, USA) at the ISU Molecular Research Core Facility. T-RFLP analysis expedited (T-REX) software [34] was used to analyze the T-RFLP tabulated data exported from Peak Scanner™ software (Applied Biosystems Instruments, Foster City, CA), using the additive main effects and multiplicative interaction model (AMMI) [35]. The terminal restriction fragments (T-RFs) were processed with the following parameters: Noise filtering (peak area standard deviation multiplier = 1), T-RF alignment (clustering threshold = 0.5), T-RFs omitted if they occurred in less than 2% of samples, "sample name" and "day" were chosen as environments. One-way analysis of variance (ANOVA) was used to compare the numbers of

T-RFs among different samples [36].

2.4. Nucleotide Sequence Accession Numbers

Partial 18S rDNA sequences were deposited in Gen Bank under accession numbers JX667685 through JX667704.

3. Results

3.1. Bacterial Counts

The total bacterial cell count from the original activated sludge was 1.7×10^4 ml^{-1} from one set of 3 microcosms. The total bacterial cell count from the K-12-added microcosm changed from 3.4×10^8 ml^{-1} (with K-12 cells added), to 1.2×10^6 ml^{-1} after 2 days, to 1.5×10^4 ml^{-1} after 4 days. The total bacterial cell count of EDL 933-added microcosm changed from 5.8×10^8 ml^{-1} to 1.5×10^6 ml^{-1} after 2 days, to 1.3×10^4 ml^{-1} after 4 days. This could indicate that the EDL 933 and K-12 cells were removed at approximately the same rate in microcosms. Based on this result, fresh *E. coli* solution at 4×10^{10} ml^{-1} CFU was added to each microcosm to reach 10^8 ml^{-1} final concentration every 2 days.

3.2. 18S rDNA Clone Libraries

Based on the ciliate18S rDNA sequencing results, several ciliate species were identified (**Table 1**). *Epistylis wenrichi* was the most commonly found ciliate in the original sludge sample before the treatment (70% in 79 clones),

Table 1. BLAST search results of ciliate 18S rDNA clones from EDL 933-treated ciliate population after 53 days and no bacteria added control at day 0.

Sequenced Clones (accession number)	Number of clones[a]	GenBank closest match (accession number)	Identity (%)	Class	Family
EDL 933 added microcosm					
1 (JX667699)	1	*Prorodon teres* (X71140)	96	Prostomatea	Prorodontidae
3 (JX667700)	1	*Prorodon teres* (X71140)	96	Prostomatea	Prorodontidae
7 (JX667701)	1	*Prorodon teres* (X71140)	96	Prostomatea	Prorodontidae
8 (JX667702)	2	*Prorodon teres* (X71140)	96	Prostomatea	Prorodontidae
11 (JX667687)	2	*Prorodon teres* (X71140)	96	Prostomatea	Prorodontidae
18 (JX667703)	22	*Prorodon teres* (X71140)	96	Prostomatea	Prorodontidae
2 (JX667685)	3	*Dexitrichides pangi* (AY212805)	95	Oligohymenophorea	Philasteridae
4 ((JX667686)	22	*Vorticella fusca* (JN120230)	98	Oligohymenophorea	Vorticellidae
48 (JX667704)	1	*Vorticella fusca* (JN120230)	98	Oligohymenophorea	Vorticellidae
13 (JX667688)	1	*Opisthonecta henneguyi* (JN120201)	97	Oligohymenophorea	Opisthonectidae
Control microcosm at day 0					
6A (JX667691)	5	*Prorodon teres* (X71140)	96	Prostomatea	Prorodontidae
50A (JX667695)	10	*Prorodon teres* (X71140)	96	Prostomatea	Prorodontidae
51B (JX667698)	6	*Prorodon teres* (X71140)	96	Prostomatea	Prorodontidae
34B (JX667690)	3	*Prorodon teres* (X71140)	96	Prostomatea	Prorodontidae
17A (JX667692)	21	*Epistylis wenrichi* (AF335515)	93	Oligohymenophorea	Epistylidae
27A (JX667693)	19	*Epistylis wenrichi* (AF335515)	97	Oligohymenophorea	Epistylidae
49A (JX667694)	5	*Epistylis wenrichi* (AF335515)	95	Oligohymenophorea	Epistylidae
5B (JX667689)	3	*Epistylis wenrichi* (AF335515)	97	Oligohymenophorea	Epistylidae
7B (JX667696)	4	*Epistylis wenrichi* (AF335515)	96	Oligohymenophorea	Epistylidae
20B (JX667697)	3	*Epistylis wenrichi* (AF335515)	97	Oligohymenophorea	Epistylidae

[a]Total number of clones that shared the same RFLP patterns with the sequenced clones.

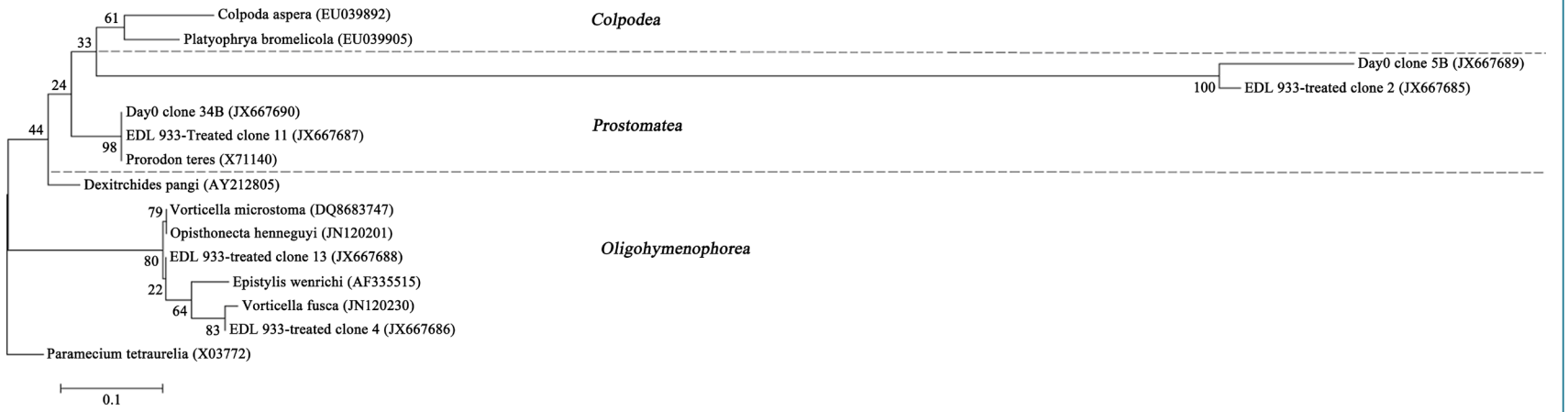

Figure 1. The phylogenetic tree constructed using maximum likelihood method for day 0 control sample and day 53 EDL 933-treated sample 18S rDNA clones and closely related ciliate species from the NCBI database (Gen Bank accession numbers in parentheses). Bootstrap values at the nodes were calculated using 100 replicates. *Paramecium tetraurelia* was used as the outgroup.

but it was not detected in the EDL 933-treated sludge sample. *Prorodon teres* was common in both the EDL 933-treated sludge sample (52% in 56 clones) and the untreated original sludge sample (30% in 79 clones). From the EDL 933-treated sludge sample, 41% were *Vorticella fusca*, 5% were *Dexitrichides pangi* and 2% were *Opisthonecta henneguyi*. Those three species may be resistant to EHEC as they remained at very low numbers in untreated sludge sample. Their phylogenetic relationship is demonstrated in **Figure 1**, along with EHEC resistant ciliates *Vorticella microstoma*, *Platyophyra bromelicola* and *Colpoda aspera* identified by Ravva *et al.* [13].

3.3. T-RFLP

The T-RFLP AMMI analysis of ciliate 18S rDNA genes (**Figure 2**) showed differences between the *E. coli* EDL 933 strain, the K-12 strain, and the no bacteria added control microcosms, based on *Msp*I and *Rsa*I restrictions of triplicate samples. The T-RFLP pattern of original sludge sample at day 0 was different from the patterns of no bacteria added controls at day 10 and day 25, while the patterns from day 10 and day 25 controls were quite close to each other. Without adding new bacteria as food source, the original ciliate population could starve and thus the population would change. At day 10 and day 25, EDL 933-treated ciliate populations were different from K-12-treated ciliate populations (**Figure 2**). At day 25, EDL 933-treated ciliate populations were more similar to no bacteria added controls, than the K-12-added ciliate populations.

The T-RFs average abundance from each T-RFLP file was also analyzed (**Table 2**). Base on the one-way ANOVA test, the numbers of T-RFs from the EDL 933-treated samples and K-12-treated samples, and the no bacteria added controls were not significant different at day 10 in *Rsa*I restricted samples ($F_{2,6}$ = 1.431, p = 0.31). At day 25, the number of T-RFs from the three types of treatments were not significant different from each other in *Msp*I restricted samples ($F_{2,6}$ = 0.54, p = 0.609) and *Rsa*I restricted samples ($F_{2,6}$ = 0.201, p = 0.823). Al-

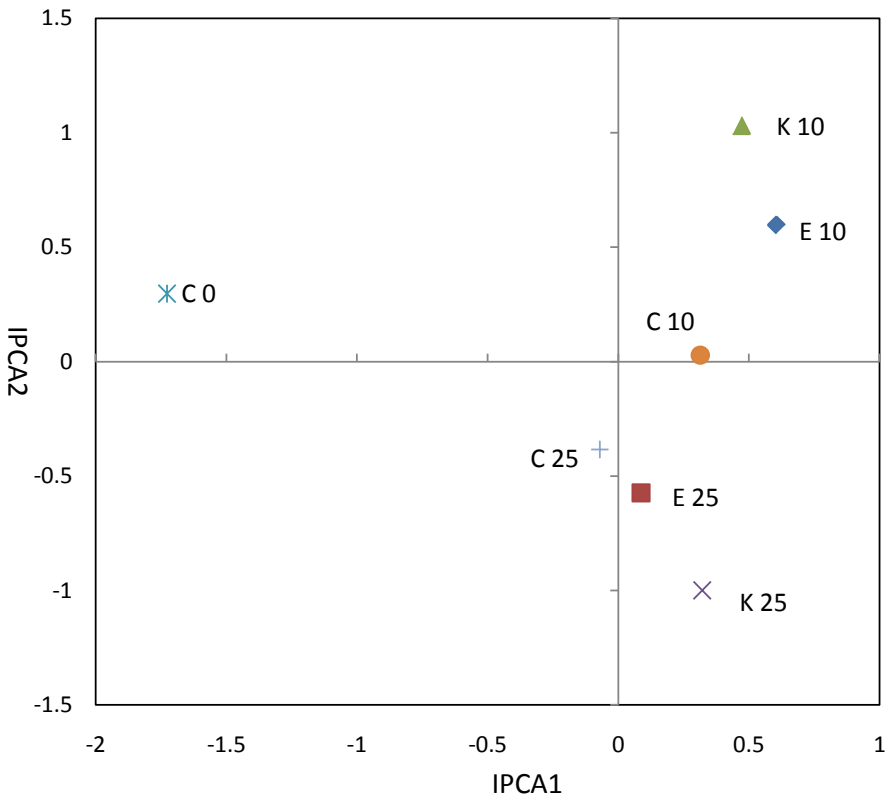

Figure 2. T-RFLP analysis with *Msp*I and *Rsa*I restriction from triplicate samples via AMMI model, which uses analysis of variance (ANOVA) to first partition the variation into main effects and interactions, and then applies PCA to the interactions to create interaction principal components axes (IPCAs). (E10 = EDL 933-treated samples at day 10, E25 = EDL 933-treated samples at day 25; K10 = K-12-treated samples at day 10, K25 = K-12-treated samples at day 25; C0 = control samples at day 0; C10 = control samples at day 10, C25 = control samples at day 25).

Table 2. Average T-RFs from different samples (n = 3, mean ± standard deviation).

	*Msp*I restriction			*Rsa*I restriction		
	EDL 933-treated samples	K-12-treated samples	Control samples	EDL 933-treated samples	K-12-treated samples	Control samples
Day 0	-	-	53[b]	-	-	31[b]
Day 10	59 ± 40	50[a]	33 ± 18	48 ± 25	47 ± 31	20 ± 1
Day 25	46 ± 35	63 ± 55	31 ± 5	30 ± 17	30 ± 19	23 ± 9

[a]Number of T-RFs from K-12-treated samples in day 10 with *Msp*I restriction (n = 2); [b]Number of T-RFs from original sample in day 0 (n = 1).

though the AMMI analysis showed that EDL 933-treated ciliate populations were different from the K-12-treated ones, the numbers of T-RFs were not significantly different from each other.

4. Discussion

Based on our results, ciliates can consume *E. coli* EHEC and non-EHEC strains in aeration microcosms inoculated with activated sludge samples. The total bacterial density dropped from 10^8 to 10^4 ml^{-1} in 4 days, which is similar to dairy lagoon wastewater experiments where EHEC density dropped from 2×10^7 to 10^4 ml^{-1} [13]. However, the addition of the 10 ml 0.1% peptone solution that contained *E. coli* could possibly bring nutrients to the microcosm and stimulate ciliates to ingest more *E. coli* cells. Similar observations were reported that cereal grass medium enhanced *Platyophyra* sp. to remove EHEC [13].

Our study also indicated that EHEC and non-EHEC *E. coli* food sources could have different impacts on the sewage ciliate populations as seen from the T-RFLP analysis. The T-RFLP patterns from the AMMI model indicated that protozoan populations in no bacteria added control samples, EDL 933-treated samples and the K-12-treated samples changed at day 10 and 25. This could indicate that external food sources could change ciliate populations in sewage systems.

In this study, the diversity of ciliate populations might have been underestimated in activated sludge. Several BLAST searches of Day 0 control clones with the same result (*Epistylis wenrichi*) had different similarity scores (93%, 95%, 96% and 97%) (**Table 1**). This could be caused by lack of adequate identified ciliate 18S rDNA sequences in NCBI database or could represent a high level of strain variation in this species that was present in the ciliate population, which perhaps reflects niche specialization in this organism in the activated sludge. This could also be supported by the fact that 31 T-RFs in *Rsa*I restriction analysis and 53 T-RFs in *Msp*I restriction analysis were found in the day 0 control sample T-RFLP analysis (**Table 2**), while only two species were identified in the same sample (*Epistylis wenrichi* and *Prorodon teres*).

Studies have suggested that some ciliate species could reduce EHEC concentrations in dairy lagoon wastewater. These species include *Vorticella microstoma*, *Platyophyra bromelicola* and *Colpoda aspera* [13]. Similarly, a *Vorticella* species (*Vorticella fusca*) was also found in EHEC treated domestic sewage water in our study. However, this study could not determine which of these EHEC tolerant ciliate species could decrease EHEC from domestic wastewater. In order to do that, further studies would be needed to study individual ciliate species discovered in this study.

This study may also indicate that some ciliate species are more resistant to EHEC than others in the aeration tank of municipal sewage treatment plants. It is known that EHEC strain EDL 933 could kill ciliate *Tetrahymena pyriformis* by using Stx toxins [7] [8]. In our study, *Epistylis wenrichi* clones were only found in original activated sludge samples, but not in EDL 933-treated ones after 53 days. Nevertheless, *Vorticella fusca* and *Opisthonecta henneguyi* clones were found in the EDL 933-treated sludge sample, and were not detected in the untreated ones. These three ciliate species belong to the subclass *Peritrichia*, which is the most important ciliate subclass in the aerobic processes [20]. Thus, *Epistylis wenrichi* might be less tolerant to EHEC than *Vorticella fusca* and *Opisthonecta henneguyi*. *Dexitrichides pangi* belongs to the subclass *Scuticociliatia* and its clones are only found in the EDL 933-treated sludge sample, indicating that it may be more resistant to EHEC. *Prorodon teres* clones were found in both untreated and EDL 933-treated sludge samples, indicating that it might serve as a stable bacterivore ciliate in activated sludge with being less affected by EHEC. The presence of the EDL 933 pathogens might reduce the proportion of ciliates that were able to out-compete EDL 933-resistant ciliates under "normal" conditions, thereby enabling them to become dominant members of the population when EDL 933 was

present. However, further research would be needed to confirm the sensitivity of those identified ciliates to EHEC.

In this study, the impact of EHEC and non-EHEC on ciliate populations were observed in activated sludge from municipal sewage systems. The ciliate population in activated sludge could be affected by EHEC, and also non-EHEC populations. Several potentially EHEC sensitive and resistant ciliate species were also identified. In order to further understand the relationships between bacteria and their ciliate predators in municipal sewage systems, future work could be done to exam the sewage microbial communities under the influence of EHEC and EHEC resistant ciliate protozoan populations.

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