ACTA MICROBIOLOGICA BULGARICA Volume 34 / 3 (2018)

Biofilm Formation Potential of Enteropathogenic Bacteria and Their Survival in Drinking Water-Associated Biofilms

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Abstract

The present study aimed to assess the potential of three enteropathogenic bacteria to form single-species drinking water-associated biofilms and their capacity to attach to and survive in the multispecies bacterial biofilms, pre-existing on polypropylene and polyethylene pipe surfaces, in 63-day batch experiments with real drinking water. Dynamics of the biofilm formation process of the tested enteropathogens was studied, and the influence of the inter-species interactions and the type of polymer pipe materials as surface for attachment were clarified.

It was found out, that the biofilm formation potential was a specific characteristic of each tested enteropathogen and was influenced by the pipe material type. The single-species- and multispecies biofilms of each enteropathogen differed in the culturable cells density and dynamics. The pathogen attachment to the bacterially pre-colonized surfaces was more significant compared to the non-colonized ones. The culturable *S. dublin 1953* and *Y. enterocolitica O:8 8081* cells persisted in the biofilms in a lower number than the *E. coli O157* cells. The number of culturable pathogen cells in the single-species biofilms decreased faster than in the mixed bacterial biofilms.

Keywords: biofilm, drinking water, *E. coli O157*, pathogen survival, Salmonella, *Yersinia enterocolitica*

Резюме

Представените изследвания целят да се оцени способността на три щама ентеропатогенни бактерии да образуват моновидови биофилми и да се прикрепят и оцеляват в съществуващи многовидови бактериални биофилми върху материали полиетилен и полипропилен в 63-дневни експерименти с реална питейна вода. Изследвана е динамиката на биофилм-образуващия процес на изпитваните ентеропатогени и е изяснено влиянието на междувидовите взаимодействия и тръбния материал като субстрат за прикрепяне.

Установено е, че биофилм-образуващата способност е специфична характеристика на изследваните ентеропатогенни щамове и се влияе от типа на тръбния материал. Моновидовият и многовидовият биофилми на изследваните ентеропатогени се различават по бактериална плътност и по нейната динамика във времето. Изследваните патогени се прикрепят по-значително към съществуващите многовидови биофилми в сравнение с неколонизираните полимерни тръбни повърхности. В многовидовите биофилми, количеството на *S. dublin 1953* и *Y. enterocolitica O:8 8081* в култивируема форма е по-малко в сравнение с това на *E. coli O157*. В моновидовите биофилми, броят на култивируемите патогенни клетки се редуцира по-бързо отколкото в смесените бактериални биофилми.

Introduction

Biofilm formation is a common phenomenon in drinking water distribution systems (DWDS) (LeChevallier *et al.*, 1987). Attached microbial biomass comprises about 95 % of the active biomass in a DWDS and may cause a number of technological and hygienic problems during drinking water transport and distribution (Flemming *et al.,* 2002). Detachment of bacteria from the biofilm or sloughing of larger pieces may increase bacteria number in drinking water. Specific microenvironments in the biofilm structure can ensure protection against predators and environmental stress, can create

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niches for survival of the pathogens that pass into DWDS as a result of insufficient water treatment/ disinfection or can create conditions for cell recovery of the bacteria injured through disinfection (Le Dantec *et al.,* 2002; Norton *et al.,* 2004; Storey *et al.,* 2004; Lee *et al.*, 2014). In this way biofilms can be a continuous source of drinking water contamination together with accidental fecal contamination and can increase dissemination of waterborne pathogens.

Most obligate pathogens quickly lose their vitality and virulence in water, cannot propagate and decay over time while opportunistic pathogens, e.g. *Mycobacterium avium, Legionella pneumophila*, *Pseudomonas aeruginosa* and *Aeromonas hydrophila,* are able to proliferate in drinking water and in water-associated biofilms and may cause infections and diseases mainly in immuno-compromised persons (Le Dantec *et al.,* 2002; Norton *et al.,* 2004; Storey *et al.,* 2004). The excreted pathogens like *Campylobacter coli, C. jejuni* and *Yersinia enterocolitica* can be detected in the water as a result of contamination by excrements of livestock, wild animals and birds (Buswell *et al.,* 1998; Kot *et al.,* 2005). The zoonotic Shiga toxin-producing *Escherichia coli O157:H7* emerged as an important food- and water-borne pathogen and approximately 10% of reported *E. coli O157:H7* outbreaks were related to the consumption of contaminated water or to the use of surface water for recreation or irrigation (Chekabab *et al.,* 2013). Viable *E. coli O157:H7* cells had been isolated from water sources that met all hygienic requirements for drinking water quality (Schets *et al.,* 2005) and a prolonged survival (from 14 to 64 days) had been determined in private wells (Artz and Killham 2002). There is data showing that *E. coli O157:H7* strains could form biofilms on biotic and abiotic surfaces outside the host (Ryu *et al.,* 2004; Klayman *et al.,* 2009), but little data is available on *Yersinia enterocolitica* survival in water and water-associated biofilms.

Biofilm formation by pathogens has attracted much attention mainly in the medical and food production fields, where most studies considered single-species biofilms (Giaouris *et al.,* 2015). Since biofilms in nature mostly comprise multiple species, in recent years there has been a shift in focus towards examining the complexity and interactions in multispecies biofilms (Elias and Banin, 2012; Rendueles and Ghigo, 2012). It is now known that the data from the studies of the planktonic cells or single-species biofilms cannot readily be extrapolated to multispecies biofilm consortia (Simoes *et al.,* 2007; Giaouris *et al.,* 2015). Examinations of single- and dual-species biofilms of intergeneric bacteria which were isolated from drinking water found cooperative advantages, neutral interaction or antagonism in the dual-species biofilms (Simoes *et al.,* 2007). It has also been found that intra- and interspecies interactions have a profound influence on the development, structure and physiology of the mixed bacterial biofilms. For example, Wang *et al.* (2013) determined that the participation of an *E. coli O157:H7* strain in the mixed biofilm was dependent on the EPS production of the companion strain *Salmonella enterica*. Uhlich *et al.* (2010) indicated that retention of non-biofilm-forming *E. coli O157:H7* strains on bacterially pre-colonized surfaces was due to biofilm-forming partner strains. Klayman *et al.* (2009) found that a waterborne *E. coli O157:H7* strain required a colonizing partner: its planktonic cells were unable to attach alone, but could co-adhere together with *P. aeruginosa* and best attach to the surfaces pre-colonized with *P. aeruginosa*.

Despite the increased understanding of the biofilm formation process of enteropathogenic *E. coli,* the majority of the data has been received by microtiter plate assays using different culture media. The data on the fate of emerging pathogens, e.g. *Y. enterocolitica* in drinking water and in water-associated biofilms is scarce, despite the striving for provision of safe drinking water to the consumers. Consequently, the present study aimed to establish the biofilm formation potential of some enteropathogenic bacteria in a water environment. The main aim of this study was to assess the individual capacity of the pathogenic bacteria *E. coli O157, S. dublin 1953* and *Y. enterocolitica O:8 8081* to form single-species biofilm on polymer pipe materials in drinking water and attach to bacterially pre-colonized pipe surfaces (i.e. to the multispecies biofilms pre-established on the surfaces).

The present study focused on:

i) the dynamics of the enteropathogen density in both biofilm types;the survival time of the enteropathogens in the drinking water-associated biofilms;

ii) the influence of the pipe material as substratum for biofilm attachment.

Materials and methods

The experimental study included parallel assessments of:

a) the individual potential of the selected enteropathogenic bacteria to form single-species bio-

films on sterile pipe surfaces which were in contact with bacteria-free drinking water, as well as the dynamics of pathogen density and survival time;

b) the pathogen attachment and persistence in the multispecies bacterial biofilms pre-established on the pipe surfaces in contact with protozoa-free drinking water.

Both tests enabled comparisons between:

1) the biofilms of each particular pathogen developed on two polymer pipe materials in bacteria-free and in non-sterile protozoa-free drinking water;

2) the dynamics and survival of each particular pathogen in both the monoculture and in the multispecies biofilms.

Materials

1) Drinking water from the local municipal water supply system, which met all of the quality requirements, was used. It was dechlorinated and filtered for protozoa removal. Bacteria-free tap water was obtained through 0.45 μm membrane filtration.

2) Pathogenic bacteria strains *E. coli O157, S. dublin 1953* and *Y. enterocolitica O:8 8081* used in the study were taken from the collection of the Stephan Angeloff Institute of Microbiology. The pure culture prepared by streaking of a single colony on TSA agar for 24-hour incubation at 35°C was suspended in physiological solution and was used for the inoculation of the water samples.

3) Pipe tubes made from both polypropylene (PP) and polyethylene (PE) were used: the PP pipe was produced by Devorex Ltd and the PE version by Filbo Ltd. The tubes were cut into pieces - each piece with a total surface (i.e. inner plus outer) of 50 cm^2. The pieces were autoclaved and then soaked in sterile distillated water for 24 hours.

4) For the development of multispecies biofilm of indigenous aquatic bacteria, half of the pipe pieces were dipped into the dechlorinated protozoa-free tap water and were incubated at 20°C for 20 days. The biofilms formed were analyzed for heterotrophic plate count (HPC) by the pour plate count technique (R2A agar/7 d/22°C).

Experimental procedure

The experimental procedure included incubation of test pieces from a pipe material into enteropathogen inoculated samples of membrane filtered water and into non-sterile protozoa-free drinking water for a certain period of time, as well as regular analysis of the pathogen density of the developed biofilms:

1) Six sterile glass jars were filled with 900 ml of non-sterile protozoa-free water; six other jars were filled with membrane filtered water.

2) Six test pieces from each pipe material were dipped into water in a test jar. Three of the six jars containing PP pieces were filled with the protozoa-free water and the other three - with filtered water. The same procedure was repeated for the PE pieces.

3) Two jars containing filtered tap water (one with dipped sterile PP pieces and one with PE pieces) and two jars containing protozoa-free water (with PP or PE pieces covered by pre-established mixed bacterial biofilm) were inoculated with 1 ml suspension of one of the pathogens to a rough concentration of 1 x 10^6 CFU/ml. The procedure was repeated for each examined pathogen.

The inoculated water samples were analyzed to determine the actual pathogen numbers (MacConkey agar/24 h/35°C). These inoculated water samples contained respectively: *E. coli O157* cells of 9.1 ± 1.2 x 10^5 CFU/ml; *Y. enterocolitica O:8 8081* of 1.0 ± 0.1 x10^6 CFU/ml; *S. dublin 1953* cells of 8.0 ± 2.9 x 10^5 CFU/ml.

All vessels were incubated at 20°C for 63 days. At regular intervals one test piece was taken from each jar and used for biofilm analysis.

4) The biofilm formed on each test piece was washed with 20 ml of sterile physiological solution. Next, the test surface was wiped with a sterile cotton swab dipped in 50 ml physiological solution and treated for 5 min in ultrasonic bath (*Appronex*; 40 kHz). The biofilm suspension was analyzed for enumeration of culturable pathogen cells (MacConkey agar/48 h/35°C). Multispecies biofilm samples were analyzed for HPC (R2A agar/7 d/22°C).

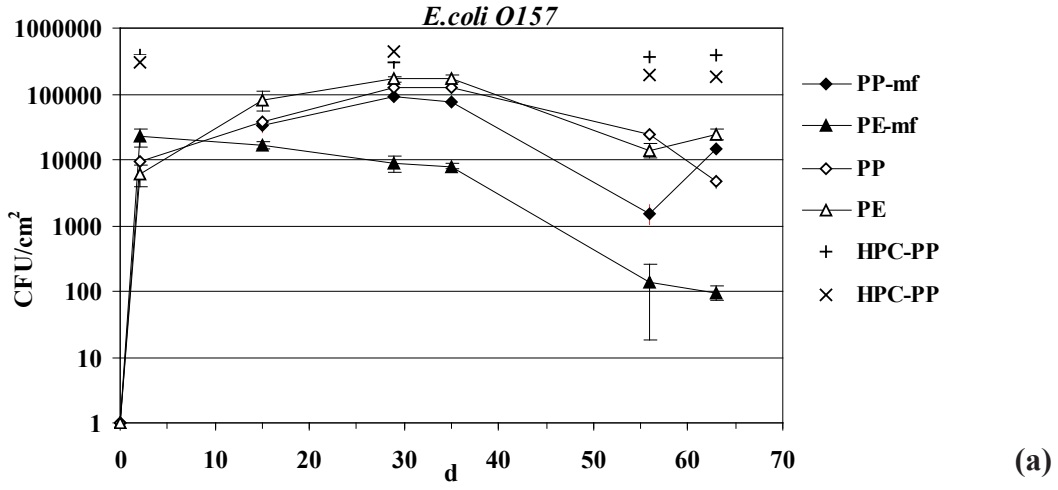
Statistical tools

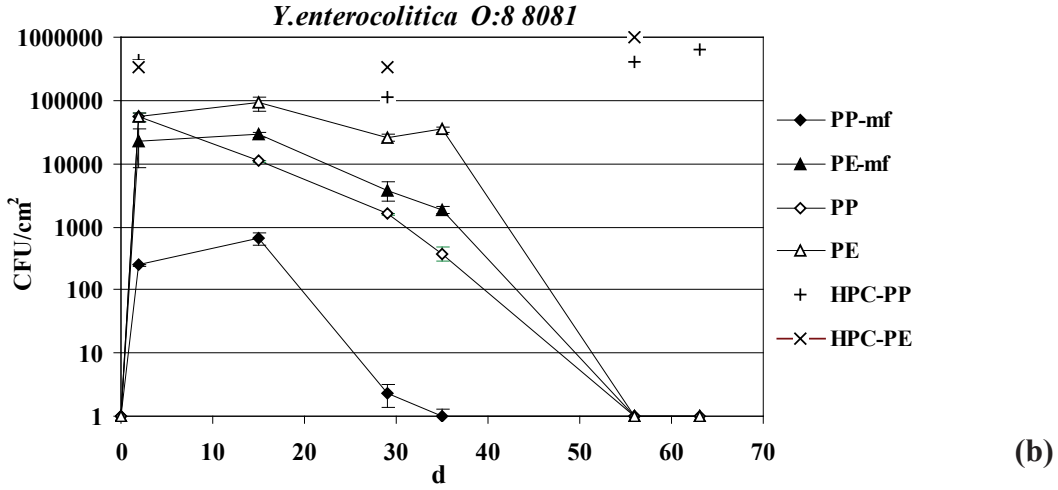
A *t*-test for comparison between biofilm samples was used to show whether the difference between the logarithms of two compared values was significant at level $\alpha = 0.05$. One-way analysis of variance was used to analyze the effects of the presence/absence of preexisting bacterial colonization on the pipe surfaces, the enteropathogen species, and the pipe material type on the biofilm formation potentials.

Results and Discussion

Dynamics of the single-species- and multispecies biofilms

The data presented in Fig. 1. shows the dynamics of the single-species biofilm formation process on the PE and PP pipes and the enteropathogens' capabilities to attach to the pre-established





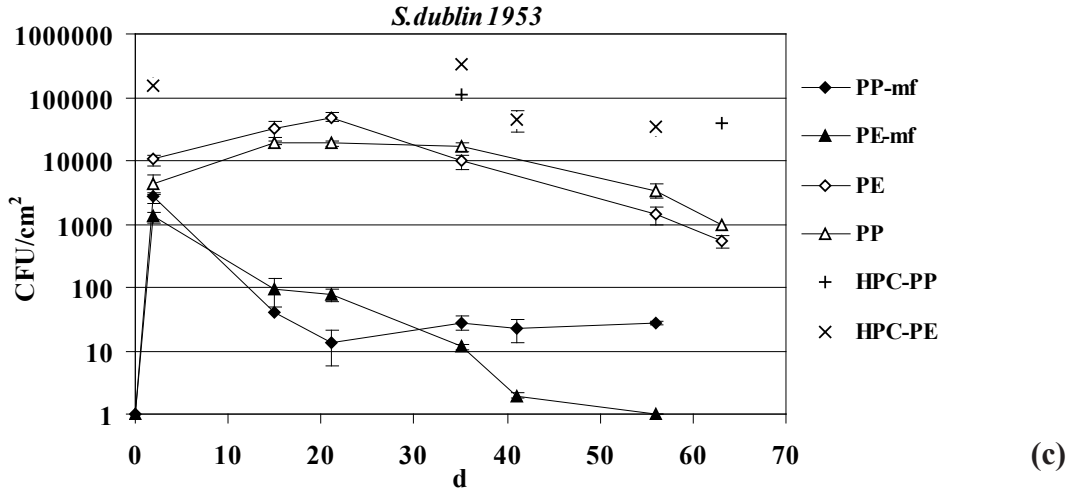


Fig. 1. Dynamics of enteropathogen density of the single-species biofilms formed on the PE and PP pipes in inoculated membrane filtered drinking water (PE-mf and PP-mf) or in preexisting multispecies biofilms in inoculated non-sterile water with: **(a)** *E. coli O157*; **(b)** *Y. enterocolitica*; **(c)** *S. dublin*; d – days; HPC of the multispecies biofilms.

multispecies bacterial biofilms in the drinking water samples (which had been inoculated with culture of a particular pathogen).

The data on the *E.coli* O157 dynamics in the biofilms (Fig. 1a) revealed rapid colonization on the PP and PE surfaces (registered on the 2nd day) and a trend towards initial increase in pathogen densities - except for the monoculture biofilm on PE. On the 30th day after inoculation, the pathogen density of the mixed biofilms reached its highest level. Subsequently, the pathogen numbers decreased, especially in the monospecies biofilms, which decreased by 2 logs. Culturable *E. coli* cells still persisted within all biofilms at the end of the test (on the 63rd day).

The dynamics of *Y. enterocolitica O:8 8081* biofilm formation showed fast pathogen attachment except for the monospecies biofilm on PP (Fig. 1b). The pathogen density of the biofilms quickly decreased over time with the multispecies biofilm on PE undergoing a lower rate up to the 35th day. The *Y. enterocolitica* number decreased most quickly in the monospecies biofilm on PP, where culturable cells were absent on the 35th day in contrast to the other biofilms.

The single-species *S. dublin 1953* biofilms (Fig. 1c) also initially showed a significant cell density followed by a rapid reduction of up to 20 CFU/cm^2 and pathogen absence in the biofilm on the PE pipe after the 56th day. In the multispecies biofilms, the *S. dublin 1953* cells increased up to 6 x 10^4 CFU/cm^2, then slightly decreased; however, up to 10^3 CFU/cm^2 still remained within the biofilms at the end of the test.

Biofilm formation potential

In order to rank the individual biofilm formation capability of the examined enteropathogens on each material, the data on both the single-species- and multispecies biofilms was compared separately. The resulting data demonstrated the specific biofilm formation potential of each pathogen and a different pathogen persistence time in culturable form within the biofilms.

The comparison between *the single-species biofilms* (made separately for each particular material) revealed significant differences between the biofilm densities of the pathogens ($p < 0.01$), except for the biofilms of *E. coli O157* and *Y. Enterocolitica O:8 8081* on PE and the biofilms of *Y. enterocolitica O:8 8081* and *S. dublin 1953* on PP ($p > 0.05$).

The *E. coli O157* biofilms had the highest bacterial density while the *Y. enterocolitica O:8 8081* showed a higher biofilm formation potential than the *S. dublin 1953* on PE, but predominately lower one on PP. The single-species biofilms of the *Y. enterocolitica 8081* were significantly dependent on pipe material ($p<0.05$) with higher biofilm density on PE than on PP.

The comparison between the multispecies biofilms of the examined pathogens on each material determined significant differences between their pathogen densities ($p<0.05$). The *E. coli O157* biofilm had the highest density. Although the initial attachment of the *Y. enterocolitica O:8 8081* cells on both materials showed the highest values, it was followed by rapid reduction of their culturability. Similarly, initially, the percentage of the *Y. enterocolitica O:8 8081* cells among the HPC of the mixed biofilms peaked (14% in the biofilm on PP and 19% on PE) on the 2nd day, but soon afterwards, the *E. coli O157* part rose to 40-73% as a result of the increased attachment and remained the highest, whereas the *Y. enterocolitica O:8 8081* part reduced to 1-8%.

Single-species - versus multispecies biofilm

Comparisons between the densities of each particular pathogen in both the single-species- and multispecies biofilms showed better pathogen attachment to the preexisting mixed bacterial biofilms than on the non-colonized abiotic surfaces. The biofilm production was different - higher pathogen density was found in the multispecies biofilms. The difference between the single-species- and multispecies biofilms of each tested pathogen was statistically confirmed: the pathogen densities in the multispecies biofilms on both materials were significantly higher than in the single-species biofilms ($p<0.05$). The only exception was the *E. coli O157* biofilms on PP ($p>0.05$); an insignificant difference between the single-species and multispecies biofilm samples was determined by the *t*-test only for two sampling points - on the 15th day ($|t|$ of $1.5<t_{(0.05;3)}$ of 4.3) and on the 36th day ($|t|$ of $2.0<t_{(0.05;3)}$ of 3.2).

The experimental data revealed differing survival times of the pathogens in the biofilm types. In the single-species biofilms, the culturable cells persisted to a different extent for different periods of time: at the end of the test, 10^2-10^4 CFU/cm^2 of the *E. coli O157* was detected on both materials, but only 20 CFU/cm^2 of the *S. dublin 1953* on PP. The number of culturable *Y. enterocolitica O:8 8081* cells decreased faster than the other two pathogens, especially on PP. In the multispecies biofilms, the culturable *E. coli O157* cells of 10^4 CFU/cm^2 or the *S. dublin 1953* cells of 10^3 CFU/cm^2 were detected at the end of the test, while the *Y. enterocolitica O:8 8081* cells were absent by the 55th day.

The examined pathogens attached more successfully and survived longer in the mixed bacterial biofilms than in the single-species ones, however, there is insufficient data to assess the full survival time. The observed prolonged enteropathogen existence in the mixed biofilms supported the understanding that the multispecies biofilm community could play a crucial role in pathogen survival under unfavorable environmental conditions. The better attachment, higher biofilm formation potential and prolonged survival of the enteropathogens could be associated with the contribution of colonization partners and with the spatial and metabolic interactions between species within the biofilm community that could drive ecological advantages by providing improved nutrient entrapment and environmental stress protection (Simoes *et al.,* 2007; Elias and Banin 2012; Lee *et al.,* 2014; Giaouris *et al.,* 2015). The higher pathogen density in the multispecies biofilms (especially in either *Y. enterocolitica O:8 8081* or *S. dublin 1953*) when compared to the single-species biofilms, demonstrated interspecies interactions of co-operation. Although that co-operative effect was somewhat less pronounced for the *E. coli O157*, it was in line with the established contribution of colonization partners to more effective attachment of *E. coli O157:H7* cells and coexistence in the multispecies community (Klayman *et al.,* 2009; Uhlich *et al.,* 2010; Wang *et al.,* 2013).

Specific attachment and survival of the examined enteropathogens in the preexisting mixed biofilms was in conformity with the research findings on other pathogens. There is data showing that the biofilm potential of *C. jejuni* was strain specific and relied on the mixed biofilm population (Buswell *et al.,* 1998; Teh *et al.,* 2010), and that its secondary attachment to biofilms could prolong survival in external environments and condition different structures and activities, such as preserved culturable state in the mixed biofilm versus the viable but not culturable state in the monoculture biofilm (Ica *et al.,* 2012).

In addition, the derived data demonstrated an influence of the type of pipe material. The data showed that the attachment of *Y. enterocolitica O:8 8081* cells was significantly influenced by the type of pipe material, probably as a result of the strain specific surface characteristics of the bacterial cells in addition to the pipe surface properties. Although the *S. dublin 1953* and *E. coli* O157 biofilms were less affected by material than *Y. enterocolitica O:8 8081*, the observed effect of the pipe material supported the findings for material-dependent attachment and biofilm formation of other pathogens (Armon *et al.,* 1997; Reeser *et al.,* 2007; Yu *et al.,* 2010).

Conclusions

It could be summarized that in a drinking water environment, the examined enteropathogenic bacteria were capable of forming single-species biofilms or co-existing in the multispecies biofilm consortia of preexisting mixed bacterial biofilms. Both types of biofilms of each enteropathogen tested differed in the culturable cells' density and dynamics.

The biofilm formation potential was a specific characteristic of each tested pathogenic bacteria and was influenced by the type of pipe material. The studied enteropathogens had different biofilm formation potential with the highest being the *E. coli O157* strain.

The enteropathogen attachment to bacterially colonized pipe surfaces was more efficient than the attachment to the non-colonized surfaces: the pathogen density of the multispecies biofilms was higher and the pathogen survival was longer than in the monospecies biofilms.

The data on the prolonged survival times of enteropathogens in drinking water-associated biofilms revealed a concern for the safety of drinking water and a potential health risk to consumers in cases of insufficient water disinfection or accidental fecal contamination. The study provides new information about the survival of three enteropathogenic bacteria outside the host and shows that drinking water-associated biofilms may have an important role in enteropathogen transmission.

Acknowledgement

This study has been supported by the Project BG05M2OP001-1.002-0019: 'Clean Technologies for Sustainable Environment - Waters, Waste, Energy for a Circular Economy' (Clean&Circle), financed by the Operational Program 'Science and Education for Smart Growth' 2014-2020, co-financed by the European Union through the European structural and investment funds.

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