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ORIGINAL PAPER

Use of Ultrasounds to Reduce the Count of *Campylobacter coli* in Water

MAREK SELWET*

Department of General and Environmental Microbiology, Poznań University of Life Sciences, Poznań, Poland

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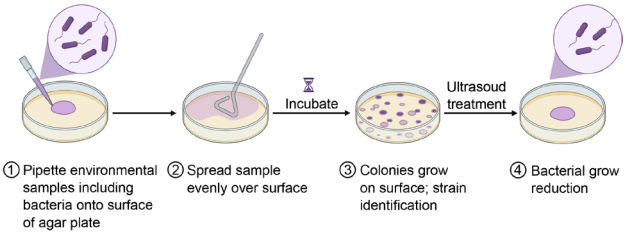
Abstract

The present study aimed to evaluate the effectiveness of low-frequency ultrasounds applied to eliminate *Campylobacter* spp. from water. The strains used in this research were isolated from water contaminated with sewage. *Campylobacter coli* alone was detected in the samples and used for further research. The reference strain *C. coli* ATCC 33559 was simultaneously tested. The isolate was exposed to ultrasounds at frequencies of 37 kHz and 80 kHz in a continuous operation device with ultrapure deionized water. After 5 min of sonication, the count of *C. coli* decreased by 5.78% (37 kHz) and 6.27% (80 kHz), whereas the temperature increased by 3°C (37 kHz), and 6°C (80 kHz). After 30 min of sonication, the death rates of bacterial cells were 40.15% (37 kHz) and 55.10% (80 kHz), whereas the temperature reached the maximum values of 36°C (37 kHz), and 39°C (80 kHz). Sonication at the frequency of 80 kHz reduced the bacterial count from 6.86 log CFU/ml to 3.08 log CFU/ml, whereas the frequency of 37 kHz reduced the bacterial count from 6.75 log CFU/ml to 4.04 log CFU/ml. Despite significant differences ($p < 0.05$) in the number of *C. coli* cells, the cell death rate remained at the same level.



Keywords: *Campylobacter*, sonication, sewage

Introduction

Water plays a significant role in the transmission of infectious diseases. Infections are most commonly transmitted through domestic, farm, and hospital wastewater as well as through rainwater and snowmelt. Large numbers of harmful pathogenic viruses, bacteria, protists, and parasitic worms occur in polluted waters and may pose a direct epidemiological threat to humans and animals (Michałkiewicz et al. 2011). The survival of potentially pathogenic microorganisms in a contaminated environment depends on various synergistic factors, e.g., temperature range, antagonistic interactions occurring in a given ecosystem, and individual characteristics of bacterial strains (Hawrylik 2019). Contaminated water may contain bacteria of the genus *Campylobacter*, which are a severe threat as they cause gastrointestinal infections in humans. In recent years, Europe has witnessed an increased incidence of campylobacteriosis in humans. In 2019, the overall prevalence of infections in the European Union was 22,682 confirmed cases (EFSA and ECDC 2021). In the European Union, campylobacteriosis is listed as a zoonosis and is subject to mandatory registration of all cases. The detection of *Campylobacter* spp., for example in water, should be routinely performed (Selwet 2019). Therefore, increasing efforts are being made to reduce the contamination of surface water with domestic and farm wastewater, and introduce effective water treatment methods (Górka et al. 2018). The application of ultrasounds at a frequency above 20 kHz is a highly efficient water treatment method, which is more effective than other treatment techniques (Li et al. 2019). Ultrasounds can break bacterial cell structures, usually leading to cell death. They can also selectively increase the enzymatic activity of some microorganisms (Subhedar et al. 2014; Marchesini et al. 2015). Thus far, research on the effect of ultrasound for eliminating bacterial cells from the environment has mainly focused on *Escherichia coli*, *Salmonella typhimurium*, and *Listeria monocytogenes*

* Corresponding author: M. Selwet, Department of General and Environmental Microbiology, Poznań University of Life Sciences, Poznań, Poland; **e-mail: marek.selwet@gmail.com**

(Kumar et al. 2014). The present study aimed to determine the effectiveness of ultrasounds of low-frequency on the survival and possible elimination of *Campylobacter* spp. isolated from water, and to compare these results with those for reference strains.

Experimental

Materials and Methods

Water samples (n = 50) for isolating *Campylobacter* spp. were collected from a lake contaminated with sewage according to the procedure specified in Polish standards PN-EN ISO 5667-3:2013 (2013), and PN-EN ISO 5667-4:2003 (2003). The samples were pre-grown in Preston Broth No. 2 (product No. CM067, with lysed horse blood product No. SR0048), Preston Campylobacter Selective Supplement (product No. SR0117), and Campylobacter Growth Supplement (product No. SR0232, Oxoid) for 22–26 h at 41.5°C in an atmosphere of a gas mixture (5% O_2, 10% CO_2, 85% N_2, CampyGen, product No. CN0025, Oxoid). Next, the samples were screened on the mCCD selective medium (product No. CM0739, Oxoid), and incubated for 40–48 h at 41.5°C in a microaerobic atmosphere. The bacterial growth on the agar was identified based on differences in colony morphology and motility examination under a microscope (Axio Imager-A2 Zeiss). The following tests were performed: oxidase (OXI detection strip, product No. 2001, Diagnostics Inc., Slovak Republic), catalase (API ID color catalase, product No. 55561, Biomérieux), and hydrolyzation of hippurate and indoxyl acetate (HIP, product No. 2006 and HIP reagent, product No. 3006; INDOXYL, product No. 2007, Diagnostics Inc.). *Campylobacter* spp. was also differentiated from other Gram-negative bacteria by using an O.B.I.S. Campy test (product No. ID0800M, Oxoid). *Campylobacter* species were identified using real-time PCR with the BAX System Real-Time PCR Assay for *Campylobacter* (product No. D12683449 KIT2018, Hygiena). The same procedure was used for the reference strains: *Campylobacter jejuni* ATCC 33291, *Campylobacter coli* ATCC 33559, and *Campylobacter lari* ATCC 35221. The standardized suspensions with turbidity corresponding to 0.5 McF in the McFarland scale (bacterial concentration 1.5×10^8/ml at an optical density of 550 nm) were prepared from the isolates obtained and reference strains. The suspensions were used to prepare a series of 10-fold dilutions (in NaCl). Next, 0.1 ml of each suspension was plated on the mCCD medium and incubated for 40–48 h at 41.5°C under a microaerobic environment. The colonies grown on plates were counted, and the counts ranged from 10 to 150 CFU (the number of replicates for each dilution was 5). Next, the effect of ultrasounds on the survival of *Campylobacter* was determined. For this purpose, 50 cm^3 of the bacterial suspension with a density of 0.5 McF (1.5×10^8 CFU/ml) was added to 5 l of ultrapure deionized water. The bacterial suspension was placed in an Elmasonic P300H sonicator (Elma) and subjected to ultrasounds at 37 and 80 kHz for 0–30 min. Next, the samples were collected at 5-min intervals and cultured with appropriate dilutions to determine the bacterial count.

Statistical calculations were based on two factors: (1) the test was performed for two groups of microorganisms *C. coli* and *C. coli* ATCC33559, and (2) the sonication effect was assessed at six-time points: 5, 10, 15, 20, 25, and 30 min. All the combinations were carried out in triplicates with temperature measurements. The experimental design corresponded to a randomized block; hence, the one-way analysis of variance (ANOVA, $\alpha = 0.05$) was used to compare the results.

Results and Discussion

Out of 50 water samples, *C. coli* was found in 21 samples, which was 42%. It is also noteworthy that the species diversity among the *Campylobacter* genus isolated from water largely depends on the sources of its contamination. According to Hokajärvi et al. (2013), *C. jejuni* is the commonly detected species in contaminated water. In the present study only, *C. coli* was isolated from the samples.

Sewage-contaminated water can show significant diversity of potentially pathogenic microorganisms. The presence of such pathogenic microbial species may entail a high epidemiological risk. *E. coli* is commonly considered as a primary indicator of the sanitary quality of water, sewage, and sewage precipitate (Naidoo and Olaniran 2014). For the present study, we chose the genus *Campylobacter* intentionally for indicating water quality. These bacteria cause campylobacteriosis, which is a zoonotic disease. They are Gram-negative, microaerophilic, motile bacilli belonging to the family *Campylobacteriaceae* (Rokosz et al. 2014). *C. coli* is one of the most common bacterial species isolated from patients with digestive system disorders (Toledo et al. 2017). For a long time, water sources were not considered as the main vector for transmission *Campylobacter* spp. It was widely believed that these bacteria were dormant in this environment and were referred to as VBNC (viable but not culturable) (Karkari et al. 2016). The *C. coli* isolated in this study and the reference *C. coli* ATCC 33559 strain were used for further research. Table I shows the variation in temperature and the count of *C. coli* and *C. coli* ATCC 33559, which were treated with ultrasounds generated by a continuous

Table I
Influence of the sonication process on temperature changes and the number of *Campylobacter coli* [log CFU/ml].

Time [min]	37 kHz					80 kHz				
	C. coli	SD	*C. coli* ATCC 33559	SD	Temperature [°C]	*C. coli*	SD	*C. coli* ATCC 33559	SD	Temperature [°C]
0	6.75	±0.7	6.78	±1.0	20	6.86	±0.9	6.83	±1.0	21
5	6.36	±0.5	6.38	±0.8	23	6.43	±0.6	6.38	±0.8	27
10	5.25	±0.5	5.18	±0.4	24	4.18	±0.5	4.11	±0.4	31
15	5.00	±0.4	5.04	±0.7	28	3.95	±0.7	3.84	±0.5	33
20	4.90	±0.6	4.84	±0.6	30	3.84	±0.7	3.60	±0.4	36
25	4.48	±0.4	4.30	±0.5	34	3.48	±0.4	3.00	±0.3	37
30	4.04	±0.3	3.95	±0.4	36	3.08	±0.5	2.48	±0.5	39

$p < 0.05$

operation device at frequencies of 37 kHz and 80 kHz. Both the ultrasound frequencies and the duration of treatment significantly reduced the count of *Campylobacter*. After 5 min of treatment at 37 kHz, the number of *C. coli* decreased by 5.78%, whereas the temperature increased slightly, i.e., by 3°C. During the sonication process, the number of *C. coli* decreased by 22.22% within the 10th minute of the process. At 30 min of the experiment, the number of bacteria decreased by 40.15%. Throughout the measurement period, the temperature reached a maximum of 36°C, whereas the initial temperature was 20°C. The reference strain demonstrated a similar sequence of variation in the bacterial count and temperature values. The treatment with 80 kHz frequency showed that after 5 min of sonication, the count of *C. coli* decreased by 6.27%, whereas the temperature increased by 6°C. At the 10th min of the process, the bacterial count decreased by 30.07%. After 30 min of sonication, the number of *C. coli* decreased by 55.10%. The temperature range during the entire process increased to 39°C, whereas the initial temperature was 21°C. For the reference strain, similar values of variation were noted in the bacterial count and temperature. Ultrasounds at both frequencies 37 kHz and 80 kHz caused significant changes in the count (log CFU/ml) of *C. coli* isolates and the reference strain at the 10th minute of operation of the sonicator. An important finding is that the frequency of 80 kHz reduced the bacterial count from 6.86 log CFU/ml to 3.08 log CFU/ml,whereas the frequency of 37 kHz reduced the bacterial count from 6.75 log CFU/ml to 4.04 log CFU/ml. Despite significant differences in bacterial numbers after treatments with the selected ultrasound frequencies, the percentage of dead bacterial cells was similar.

Previous studies on the effect of ultrasounds on the disintegration of bacterial cells mainly focused on *E. coli*, *Salmonella enteritidis*, *Bacillus subtilis*, *Enterococcus faecalis*, and *Sarcina* spp. The results of our present study on *Campylobacter* revealed some analogies with the previously published results. Bień et al. (1995) also proved that low-frequency ultrasound eliminated bacteria from sewage-contaminated water. The authors noted that sonication with 21 kHz waves led to the death of 90% of *E. coli* cells.

Foladori et al. (2007) also observed that sonication at a frequency of 20 kHz reduced the count of *E. coli* and *E. faecalis* in sewage-contaminated water. The authors additionally noted an increased loss of cell membrane integrity in the reference strains of bacteria subjected to sonication. Amabilis-Sosa et al. (2018) examined the effect of ultrasound at a frequency of 20 kHz on the inactivation of *E. coli* and *B. subtilis* in municipal sewage (exposure periods of 15, 30, and 45 min). The authors found that the counts of these bacteria decreased within 15 min of sonication. After 45 min, they observed that the bacterial counts were reduced by more than 99%. Some studies have used two ultrasound frequencies for cavitation. Rusin and Machnicka (2011) studied the *Enterobacteriaceae* family and *Staphylococcus* genus, and noted that sonic waves at frequencies of 25 kHz and 40 kHz reduced the counts of these bacteria. Hawrylik (2018, 2019) studied the effect of ultrasound frequencies of 20–22 kHz and 40 kHz on the disintegration of bacterial cells of *E. faecalis* and *Sarcina* isolated from sewage-contaminated water. The author observed that ultrasounds and sonication time significantly reduced the count of *Sarcina* spp. in the tested samples. After 10 min of sonication with waves at a frequency of 20 kHz, the count of *Sarcina* spp. decreased from 10^6 CFU/ml to 10^5 CFU/ml, whereas the temperature increased by 5°C. These research findings were supported by the results of a study on *Campylobacter*. The author did not observe a more significant influence of ultrasound waves at a frequency of 40 kHz. The results of our *Campylobacter* study showed a much stronger inhibition of the growth of these bacteria when sonicated at 80 kHz frequency. The author noted that at the 5 min of sonication, the death rate of

Sarcina spp. was 62.22%, whereas at the 10 min, it was 97.56%. These results agree with the values observed in our study on *Campylobacter*. The author also noted that the death rate of *Sarcina* spp. remained at the level of 99.71% until the end of sonication (30 min), whereas the temperature increased to 33°C (20 kHz) and 38°C (40 kHz). Our own research results on *Campylobacter* confirmed this observation. Kumar et al. (2014) also observed that ultrasounds effectively treated sewage-contaminated water. The authors noted that the count of *E. coli* tended to decrease as the ultrasound frequency increased (35 and 130 kHz) and the time of exposure progressed (5, 10, 20, and 30 min). They observed that ultrasounds at a frequency of 130 kHz were more effective than at frequency 35 kHz. Because *Campylobacter* is a Gram-negative bacterium with an outer membrane composed of phospholipids, it is important to note the role of temperature. An increase in temperature can modify the membrane's permeability, disrupt the transport of nutrients, and change its composition (cellular components). Moreover, hydroxyl ions and free radicals generated during the sonication treatment oxidize essential chemical components of bacteria (lipids, proteins, and nucleic acids) and generate hydrogen peroxide (Lefebvre and Moletta 2006). As water contaminated with sewage, for example, from secondary settling tanks may contain dissolved organic compounds from various aggregates and floccules, a longer sonication time is recommended (Amabilis-Sosa 2018). Cavitation causes the rupture of the bacterial cell wall, as higher water temperature increases the permeability of the outer membrane (Ashokkumar 2011).

The length of time for sonication to inhibit bacterial growth may depend on biofilms' formation in an aqueous environment. Bacterial consortia may be more resistant to the cavitation process than a single species observed in pure cultures (Ramalho 2012). The results of our study indicate that treatment of *Campylobacter* with ultrasound of a higher frequency, i.e., 80 kHz, resulted in a higher percentage of inactivated bacteria in a shorter time. Similar observations were reported by Jyoti and Pandit (2004) in studies on *E. coli*. Apart from bacterial inactivation percentage, the purpose of disinfection of wastewater is to protect public health by complying with the accepted maximum allowed limits. The absence of *C. coli* from treated wastewater indicates that it is safe to use for irrigation in green areas. The use of ultrasounds would distinctly diminish the demand for using drinking water for irrigation.

The results of other studies and the findings of our present research on *Campylobacter* may contribute to the implementation of ultrasounds as a technique to disinfect water and wastewater. Some limitations of this method should also be noted. Although the growth of *C. coli* cells was inhibited by ultrasound, their numbers remained high. The present research was performed on planktonic monoculture in pure water; however, the water will contain multispecies aggregates, biofilms, and compounds that could protect the cells from the ultrasound.

ORCID
Marek Selwet https://orcid.org/0000-0002-1004-7254

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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