Studies on the Survival of Enterohemorrhagic and Environmental Escherichia coli Strains in Wastewater and in Activated Sludges from Dairy Sewage Treatment Plants

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Abstract
Survival of Escherichia coli O157:H7 strain isolated from milk in Poland and an environmental E. coli strain in wastewater from Garwolin and Łowicz dairies and in activated sludges from dairy sewage treatment plants as well as in dairy wastewater with activated sludges was examined. Environmental materials were contaminated with about 10^8 of target bacteria/ml of sample. The experiments were performed under temperature conditions typical of autumn-winter (6°C) and spring-summer (24°C) seasons. It was found that the non-pathogenic E. coli strain survived longer in all media than the enterohemorrhagic serotype. E. coli O157:H7 bacteria were not detected (in direct plating method) in activated sludges after 21–28 days; in dairy wastewater as well as in wastewater with activated sludges after 21–25 days. These periods for environmental E. coli strain were 35–42 days (activated sludges), 25–28 days (wastewater with activated sludges). At higher temperature environmental E. coli were not detected in wastewater from Łowicz dairy sewage treatment plant after 25 days, but the bacteria were still present in wastewater from Garwolin dairy sewage treatment plant after 34 days.

The obtained results show that the lack of environmental E. coli bacteria (as a indicator bacteria of fecal contamination) in dairy wastewater and in dairy wastewater with activated sludges could indicate the absence of pathogenic E. coli bacteria. Prolonged existence of the enterohemorrhagic serotype in activated sludges shows the need to treat activated sludges prior to the utilization of these materials as fertilizer.

Key words: E. coli O157:H7, environmental E. coli strains, dairy wastewater, activated sludge

Introduction
Escherichia coli strains are present in the group of microorganisms that grow in the intestine of people and warm-blooded animals and aid in digestion. Approximately 0.1% of total bacterial count within an adult’s intestine is represented by E. coli strains. These bacteria are released into the environment in feces; their presence in samples indicates fecal contamination, perhaps accompanied by pathogens. Although most E. coli strains are harmless, some serotypes can cause severe illness. E. coli O157:H7 is one of the pathogenic, verotoxin-producing serotypes that is more significant than other well-recognized foodborne pathogens for reasons that include the severe consequences of infection, low infection dose, acid tolerance, high survival rate in cooling and freezing conditions and relatively low sensitivity to some preservatives used in the food industry (Benjamin and Data, 1995; Buchanan and Doyle, 1997; Kwiatek, 2000; Leyer et al., 1995). Undercooked ground beef, fermented sausages, raw milk and dairy products have been identified as the principal vehicle in most outbreaks (Buchanan and Doyle, 1997).

The main reservoir of E. coli O157:H7 bacteria is the intestinal tract of ruminants. In calves naturally infected with E. coli O157:H7 no clinical signs but only shedding of the bacteria in their feces was noted (Brown et al., 1997; Cray et al., 1995). Published research in many countries on cattle colonized with E. coli O157/E. coli O157:H7 have reported prevalence rates of 1.8–10.8% (Faith et al., 1996; Hancock
et al., 1997; Rice et al., 1997; Van Donkersgoed et al., 1999; Orr, 2000; Zhao et al., 1995). Monitoring studies conducted in the northern region of Poland, indicated that 4 (0.73%) of 551 cattle were positive for serotype E. coli O157 (Uradiusinski, 2001). E. coli O157:H7 from dung can get to a cow’s udders, the hide of animals or milking equipment, this being followed by milk contamination.

In Poland the E. coli O57:H7 serotype has been isolated from food samples. Róžańska and Kwiatek (1996) found that 3 (5.5%) of 54 samples of raw milk as well as 8 (10.7%) of 75 samples of ground beef and minced pork-beef meat were contaminated by pathogenic bacteria. Szteyn et al. (1999) have reported the isolation of four E. coli O157:H7 strains from 141 samples of raw milk originating from cows of the Olsztyn region. Contamination of environmental materials by fecal pathogens is currently monitored by testing them for the presence of indicator bacteria such as E. coli. It is very important to know the period of E. coli O157:H7 survival in the above mentioned environmental materials because if this pathogen can survive longer than other E. coli of fecal origin, the present testing procedure might be inadequate for detecting contamination.

The aim of this study was to determine the survival of the pathogenic E. coli serotype O157:H7 compared to non-pathogenic environmental E. coli strain in dairy wastewater and in activated sludges from dairy sewage treatment plants in the aspect of their possibility of potential environmental contamination as well as public health hazard.

**Experimental**

**Materials and Methods**

**Target bacteria.** In model experiments, the pathogenic E. coli O157:H7 strain isolated from milk by employees of the Veterinary Institute in Puławy and E. coli strain isolated from Czerniakowskie Lake water by employees of the Institute of Agricultural and Food Biotechnology were used. The pathogenicity of E. coli O157:H7 strain (carrying stx 1, stx 2, eae and hly 933 genes) was confirmed by the studies of Łękowska-Kochaniak et al. (2002).

**Inoculum preparation for model experiments.** Suspensions of E. coli O157:H7 serotype as well as the environmental E. coli strain necessary for environmental materials inoculation were prepared according to Czajkowska et al. (2005). Two-stage culture processes were used. In the first stage, one loop of biological material was introduced into 10 ml of bioMérieux Trypcase Soy Broth and incubated for 6 h at 37°C. The entire content of the test tube was then transferred to 250 ml of above-mentioned broth and incubated at the same temperature for 24 h. Subsequently, the bacterial suspensions were centrifuged (6350 × g, 5 min), washed two times with saline solution (0.85% NaCl) and resuspended in 25 ml of the same solution. Plate counts of bacteria were estimated on Trypcase Soy Agar after incubation at 37°C for 24 h. An inoculum contained about 10^10 bacterial cells per 1 ml.

**Model experiments.** Samples of wastewater were taken from Garwolin (A) and Łowicz (B) dairies and samples of activated sludge – from Garwolin and Łowicz dairies sewage treatment plants (A and B, respectively) in September/October, 2003. These materials were characterized with respect to their physicochemical and biological properties and level of natural microflora. Experiments on the survival of pathogenic and non-pathogenic E. coli strains in environmental materials were performed in several variants: (a) E. coli O157:H7 serotype or environmental E. coli strain in activated sludge after 1 h sedimentation process (150 ml of activated sludge in 300 ml Erlenmeyer flasks); incubation in stationary conditions, (b) E. coli O157:H7 serotype or environmental E. coli strain in dairy wastewater (150 ml of wastewater in 300 ml Erlenmeyer flasks); incubation in orbital shaker (80 rpm), (c) E. coli O157:H7 serotype or environmental E. coli strain in dairy wastewater with activated sludge after 1 h sedimentation process (100 ml and 50 ml, respectively in 300 ml Erlenmeyer flasks), incubation in orbital shaker (80 rpm).

In the experiment on the survival of E. coli strains in the variant wastewater with activated sludge, wastewater from Garwolin dairy was water diluted (1:10 vol/vol) to obtain the COD (chemical oxygen demand) and BOD (biochemical oxygen demand) values compared with the COD and BOD (biochemical oxygen demand) values of Łowicz dairy wastewater. In the variant with wastewater from Garwolin dairy alone, initial wastewater was used.

Each Erlenmeyer flask was inoculated with 3.0 mL of E. coli O157:H7 or E. coli bacterial suspension. The flasks were incubated at 6°C or at 24°C. The frequency of analyses on the E. coli strains survival depended on the death rate of bacteria in the specific variant of the experiments. Environmental samples (1 ml) were serially diluted (1/10) in saline solution and assayed for E. coli O157:H7 count or environmental E. coli count by direct plating (0.1 ml or 0.5 ml of initial sample and each dilution) in duplicate on bioMerieux Coli O157:H7 ID Agar with additional selective substances (0.5 g of sodium tellurite and 0.01 g of cefixime per 11 of medium) or Oxoid TBX medium, respectively. Petri dishes were incubated for 24 hours at temperature 37°C or 44°C, respectively. The experiment was terminated when pathogenic bacteria were not detected in three consecutive daily analyses.
Confirmation of the presence of *E. coli* O157:H7 bacteria in the environmental materials during incubation was performed according to Czajkowska et al. (2004). Material from suspect colonies was transferred to Petri dishes with bioMerieux Endo Agar. After 24 h incubation at 37°C, the presence of metallic colonies was checked. These colonies were subject to testing with the use of Merck Singlepath *E. coli* O157 test (immunochromatographic rapid test based on gold-labeled antibodies specific to *E. coli* O157).

**Microbiological analyses of environmental materials.** Total bacterial count was determined on Oxoid Plate Count Agar. Petri dishes were incubated at 20°C for 72–96 hours. Coliform count was determined on Oxoid *E. coli*/Coliform Agar and β-glucuronidase-positive *E. coli* count on Oxoid TBX Agar. Petri dishes were incubated for 24 hours at 37°C or 44°C, respectively.

**Physicochemical and biological analyses of environmental materials.** The following physicochemical and biological methods were used to characterize the environmental materials:

- dry matter of activated sludges was determined by drying 10 ml samples (after centrifugation and washing) to a constant weight at temperature 105°C (Hermanowicz et al., 1967).
- organic substances content in activated sludges was determined as the difference between dry mass content and inorganic substances content determined by burning a dry sample in muffle furnace at 500°C for 1 hour (Hermanowicz et al., 1967).
- chemical oxygen demand (COD) of the wastewater was determined by the dichromate method (Polish Standard 74/C-04578/03),
- biochemical oxygen demand (BOD) of the wastewater was determined by the dilution method (Hermanowicz et al., 1967),
- salinity of wastewater was determined using inoLabCond Level I Tetracon 325 conductometer,
- settling of activated sludge was estimated by the determination of volume of the settleable solids from the 100 ml of activated sludge after 30 minut (Hermanowicz et al., 1967).

**Results and Discussion**

Wastewater used in these experiments originated from dairies with different production profile. Garwolin dairy (A) manufactures mainly cottage cheese; Łowicz dairy (B) – mainly soft and hard cheeses. This was the reason for differences in wastewater pH (6.67 and 5.93, respectively) (Table I). Moreover, wastewater from Garwolin dairy (A) was characterized by very high COD and BOD levels – over 10-fold higher than wastewater from Łowicz dairy (Table I).

The characteristics of activated sludges from both sewage treatment plants was almost the same. pH values were close to neutral (7.35 – plant A and 6.87 – plant B), settling was 300 and 250 ml/l, dry matter content – 4.6 and 4.1 g/l; organic matter content – 64.2% and 68.3% of dry weight, respectively.

Microbiological analyses of the environmental materials showed that the total bacterial count in dairy wastewater as well as in the activated sludges was almost the same and averaged $10^7$ CFU/ml; coliform count changed in the range between $7.0 \times 10^4$ and $5.4 \times 10^5$ CFU/ml. The number of β-glucuronidase *E. coli* bacteria was higher in activated sludges (about $10^3$ CFU/ml) than in wastewater (about $10^2$ CFU/ml).

In experiments on the survival of enterohemorrhagic and environmental *E. coli* in materials from dairies and from sewage treatment plants it was found that environmental *E. coli* strain survived longer than the

<table>
<thead>
<tr>
<th>Tested parameters</th>
<th>Dairy wastewater A</th>
<th>Activated sludge from treatment plant A</th>
<th>Dairy wastewater B</th>
<th>Activated sludge from treatment plant B</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD mg O₂/l</td>
<td>12960</td>
<td>–</td>
<td>966</td>
<td>–</td>
</tr>
<tr>
<td>BOD mg O₂/l</td>
<td>8750</td>
<td>–</td>
<td>824</td>
<td>–</td>
</tr>
<tr>
<td>pH</td>
<td>5.93</td>
<td>7.35</td>
<td>6.67</td>
<td>6.87</td>
</tr>
<tr>
<td>Salinity g/l</td>
<td>1.2</td>
<td>–</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>Settling ml/l</td>
<td>–</td>
<td>300</td>
<td>–</td>
<td>250</td>
</tr>
<tr>
<td>Dry matter content g/l</td>
<td>–</td>
<td>4.6*</td>
<td>–</td>
<td>4.1*</td>
</tr>
<tr>
<td>Organic matter content (% of d.m.)</td>
<td>–</td>
<td>64.2</td>
<td>–</td>
<td>68.3</td>
</tr>
<tr>
<td>Total bacterial count, CFU/ml</td>
<td>$1.5 \times 10^7$</td>
<td>$9.7 \times 10^6$</td>
<td>$2.2 \times 10^7$</td>
<td>$1.6 \times 10^7$</td>
</tr>
<tr>
<td>Coliform count, CFU/ml</td>
<td>$5.4 \times 10^4$</td>
<td>$7.0 \times 10^5$</td>
<td>$2.5 \times 10^5$</td>
<td>$1.4 \times 10^5$</td>
</tr>
<tr>
<td><em>Escherichia coli</em> count, CFU/ml</td>
<td>$3.6 \times 10^2$</td>
<td>$4.2 \times 10^3$</td>
<td>$5.0 \times 10^2$</td>
<td>$1.6 \times 10^2$</td>
</tr>
</tbody>
</table>

* Dry matter content of activated sludge after 1 h sedimentation
enterohemorrhagic strain (Fig. 1, 2 and 3). Moreover, both strains survived better at 6°C than at 24°C. At 6°C, an undetectable level (<1 CFU/ml) of pathogenic bacteria in activated sludges (as determined by agar plate) was noted within 65 and 78 incubation days, whereas at 24°C after 21–28 days of incubation (Fig. 1). In the above mentioned incubation time, log CFU/ml of environmental *E. coli* bacteria ranged from about 3 to 5 at 6°C and from about 2 to 5 at 24°C. Both *E. coli* strains died faster in the variants: dairy wastewater with activated sludges and dairy wastewater alone than in the variant with activated sludges (Fig. 1, 2 and 3). An undetectable level of *E. coli* O157:H7 serotype in the wastewater with activated sludge was noticed at 6°C after 34–44 days; at this time log CFU/ml of environmental *E. coli* bacteria ranged from 1.0 to 3.0 units; at 24°C, *E. coli* O157:H7 bacteria were not detected after 21–25 days.

Fig. 1. Survival of enterohemorrhagic and environmental *E. coli* bacteria in activated sludges from dairy sewage treatment plants at 6°C and 24°C.

Fig. 2. Survival of enterohemorrhagic and environmental *E. coli* bacteria in dairy wastewater containing activated sludges at 6°C and 24°C.
Survival of E. coli strains in dairy wastewater and in activated sludges

The differences between the survival of both E. coli strains in dairy wastewater at 6°C and 24°C were less distinct (Fig. 3). At 6°C, neither E. coli strain was detected after 28–34 days of incubation; at temperature 24°C, pathogenic bacteria were not detected after 21–25 days but environmental E. coli bacteria – after 25 days (wastewater from dairy B) or their count was about 10 CFU/ml (wastewater from dairy A) after 34 days of incubation. It should be emphasized that high values of BOD and COD of wastewater from dairy A did not effect the survival of pathogenic and environmental E. coli bacteria (Fig. 3).

The effect of temperature on the survival of pathogenic and indicator bacteria in the environment has been reported by numerous authors. Czajkowska et al. (2004 and 2005) have found that E. coli O157:H7 serotype survived in cultivable and meadow soils for 60 and 158 incubation days (5°C) and for 48 and 90 days
(20°C); in water for 32 and 51 days (6°C) and for 21 and 32 days (24°C); in water sediments for 73 and 100 days (6°C) and for 30 and 60 days (24°C). Wang and Doyle (1998) found that populations of E. coli O157:H7 bacteria decreased in water by 1 to 2 log unit by 91 days at 8°C, whereas the bacteria were not detected (≥3 log unit decrease) after 49 to 84 days at 25°C. According to Paluszak et al. (2003), fecal streptococci survived at 4°C in peat soil with addition of cattle slurry 18.2 weeks longer and E. coli 3.2 weeks longer than at 20°C. This phenomenon has also been observed by Rhodes and Kator (1988). In frames of around 12 days long experiment the number of bacteria E. coli and Salmonella decreased at high temperature from the level of 6 log CFU/ml respectively to the level <1 log CFU and around 2 log CFU/ml, whereas at lower temperature to the level of around 3.5 and 4.5 log CFU/ml.

Incubation of dairy wastewater and dairy wastewater with activated sludge samples at both temperatures caused an increase of pH values to the level of 8.0–8.5. In the variant – wastewater from dairy B and temperature 24°C, pH increased to even 9.2–9.3 at the end of incubation (data not presented). These pH values were beyond the maximum pH value for E. coli strains (Roberts et al., 1996). This could cause the faster death of E. coli strains in wastewater from dairy B than in wastewater from dairy A. No regularities were observed with respect to changes in total count of bacteria in the tested media at the final periods of sample incubation. An increase or decrease in the quantity of bacterial population, irrespective of the incubation temperature used, rarely exceeded one logarithmic unit (data not presented). There was a distinct tendency for the number of coliform bacteria to decline. This phenomenon has also been observed by Czajkowska et al. (2005) in experiments on the survival E. coli O157:H7 serotype in water and water sediments.

After incubation of wastewater and wastewater with activated sludge at both temperatures, a reduction of COD values was noted. In the samples with wastewater and pathogenic bacteria, the COD value dropped at the final stages of incubation from the beginning values of about 12000 O2/l (dairy A) to about 880 O2/l (6°C) and 1100 O2/l (24°C) and from about 970 O2/l (dairy B) to about 400 O2/l and 200 O2/l (Fig. 4). In experimental variants with dairy wastewater containing activated sludge, the reduction of COD values in the range of several-fold was noted. With regard to the COD results presented above, the wastewater treatment process should still be continued after the death of pathogenic bacteria. It can be assumed that the likelihood of the presence of E. coli O157:H7 bacteria in wastewater from dairy after processing is very low. Moreover, the lack of indicative E. coli bacteria in wastewater can be judged as the absence of dangerous pathogens. On the other hand, a very significant risk exists resulting from the relatively long survival of pathogenic bacteria in activated sludges. The principle should be the application of the composting, pasteurization, alkaline treatment processes before utilization of activated sludge on lands.

In this studies only the effect of physical and chemical factors of wastewater and activated sludges on the survival of pathogenic and environmental E. coli strains has been tested. Experiments should be continued to evaluate how selected biocenotic factors affect this process, because Wcis³o and Chròst (2000) found that the major factor that responsible for mortality of E. coli in water was autochtonic microflora, especially microflagellate.

Acknowledgements

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Literature


Survival of E. coli strains in dairy wastewater and in activated sludges


Polish Standard PN-74/C-04578/03. Determination of the chemical oxygen demand (COD) by dichromate method (in Polish).


