Extractable Organic Components and Nutrients in Wastewater from Dairy Lagoons Influence the Growth and Survival of *Escherichia coli* O157:H7

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The influence of nutrients in wastewater from dairy lagoons on the survival of *Escherichia coli* O157:H7 was monitored. Initially, the survival of *E. coli* O157:H7 in wastewater from which the competing native organisms had been removed by filter sterilization or autoclaving was compared with that in wastewater from which competing organisms had not been removed. Numbers of *E. coli* O157:H7 or *E. coli* ONT (O-nontypeable)-H32 cells declined rapidly in filter-sterilized water and exhibited a slower decline in nonsterile water, while the organisms proliferated in autoclaved water. Subsequently, the growth of *E. coli* O157:H7 strains was monitored in 300 μL of Luria-Bertani (LB) broth supplemented with incremental proportions of filter-sterilized wastewater. *E. coli* O157:H7 and *E. coli* ONT-H32 strains failed to grow in filter-sterilized wastewater, and their growth was reduced incrementally with wastewater supplementation of LB broth. Consequently, the influence of organic extracts of wastewater on the growth of *E. coli* O157:H7 and *E. coli* ONT-H32 in reduced-strength LB was monitored, followed by scale-up tests in wastewater. Acidic and basic extracts inhibited growth of both strains, while the neutral aqueous extract improved growth. However, a scale-up with a threefold increase in the acidic components supplementing the wastewater did not result in any additional decline in numbers of *E. coli* O157:H7 cells. When protected inside a 300-kDa dialysis tube and exposed to diffusible components, *E. coli* O157:H7 survived longer, with a decimal reduction time of 18.1 days, compared to 3.5 days when inoculated directly into wastewater. Although wastewater can potentially provide nutrients to naturally occurring human pathogens, the chemical components, protozoa, and coliphages in wastewater can inhibit the growth of freshly introduced pathogens from manure.

The deadly Walkerton (Canada) outbreak of *E. coli* O157:H7 was most likely a result of contamination of drinking water with low levels of pathogen that grew in biofilms (19). The recurrence of outbreaks (9) related to produce suggests that contamination with the pathogen may occur through wastewater or manure applied to the crops. In fact, the pathogenic strain of *E. coli* O157:H7 from the September 2006 outbreak was traced to cattle feces from ranches implicated in the outbreak (6). The same strain was also detected in samples from a stream and from wild pigs that have been in the spinach fields and the ranches. Once contamination occurs, regrowth in nutrient-rich root zones may further enhance the risk of transfer to the above-ground portions of plants (23, 34).

Although *E. coli* O157:H7 can be isolated even after 21 months (24) by selective enrichments from a manure pile from inoculated sheep, the pathogen failed to grow and decline rapidly in manure, manure slurries, and wastewater from dairy lagoons (2, 17, 31). In addition, feeding cattle with a straw-rich diet (12), silage made with bacteriocin-producing bacteria (3), and competitive exclusion products using lactic acid bacteria (7), colicinogenic *E. coli* (8, 33) and O157-specific bacteriophages (35) has the potential to inhibit pathogen replication inside the animals. Nevertheless, releasing even a few cells into environments that favor pathogen regrowth (10) may pose an epidemiological risk. Other studies have not provided any insights into the failure of pathogenic *E. coli* to proliferate in manure or wastewater that are rich in nutrients. In this study, we determined the influence of dairy wastewater components...
on the survival and proliferation of pathogenic strains of \textit{E. coli} O157:H7. The growth and survival of organisms was monitored in microcosms of 300 $\mu$l and scaled up to larger volumes of wastewater supplemented with or without nutrients or organic extracts of wastewater. To do this, wastewater was extracted and fractionated into acidic, basic, and neutral organic components and tested for their influence on pathogen growth. In addition, the influence of diffusible components of wastewater was evaluated by comparing the growth of \textit{E. coli} O157:H7 inside dialysis tubes to growth in wastewater. These comparisons also indirectly highlight the influence of other biotic factors in controlling pathogenic \textit{E. coli} in dairy wastewater.

\section*{MATERIALS AND METHODS}

\textbf{Survival of \textit{E. coli} O157:H7 in sterile and nonsterile wastewater.} Wastewater used in this study was collected from an aerated manure lagoon from a medium-sized (ca. 800 milking head) dairy in central California (Oakdale, CA) and acclimated overnight at room temperature prior to inoculations. Survival of three strains of \textit{E. coli} O157:H7 (MM149, MM151, and MM158), and one of \textit{E. coli} ONT:H32 (MM158), selected for rifampin and nalidixic acid resistance (Table 1), in wastewater was compared with their fate in 0.2-$\mu$m-filter-sterilized and autoclaved wastewaters. The inoculum was an overnight growth of \textit{E. coli} O157:H7, selected for rifampin and nalidixic acid resistance. All strains, except MM158, were O157:H7.

\textbf{Extraction of organic components from wastewater.} Five hundred milliliters of wastewater was acidified to pH 2 with 6 N HCl and extracted twice with 100-ml portions of ethyl acetate. The aqueous portion was adjusted to pH 7 and extracted again with ethyl acetate. The resulting extract was applied to a 1.65-ml solution in 5 N NaOH and extracted twice with 100-ml portions of ethyl acetate, and the resultant extract was adjusted to 0.3 at 600 nm prior to inoculations. The inoculated plates were constantly shaken at medium speed during incubations at 25°C, and growth was monitored at 10-min intervals by an onboard spectrophotometer equipped with a wide-band filter (420 to 580 nm). A separate study was conducted to monitor growth of native bacteria in wastewater. The above experiment was repeated by adding incremental amounts of nonsterile, coarsely (glass wool) filtered wastewater to LB. As all treatments contained organisms from wastewater, only the wells containing 100% LB broth were inoculated with 10 $\mu$l of wastewater. Two replicate treatments were used for all growth kinetics studies.

\textbf{Influence of extracts of wastewater on growth of \textit{E. coli} O157:H7 and ONT:H32 strains.} The growth of MM149 and MM158 was evaluated in Biosaer wells containing 265 $\mu$l of Murashige and Skoog basal salts (Fisher Scientific, Fair Lawn, NJ) medium with either 5 or 50% LB broth (LB-MS). Since the quantity of solids in each extract was different, the amount of solids in 15 $\mu$l extract and the corresponding amount of wastewater extracted to obtain the solids are shown in Table 3. The pH of the growth medium was also monitored at the termination of the experiment.

\textbf{Influence of acidic extract on survival of \textit{E. coli} ONT:H32 in wastewater.} As cell death cannot be monitored by the optical density-based Bioscreen system, the influence of acidic organic components of wastewater on the growth of MM158 was monitored in 50 ml of nonsterile wastewater supplemented with acidic extract from 137.5 ml of wastewater. The extract applied was an exact scale-up from the Biosaer test box, but this treatment contained wastewater instead of LB-MS. Since the acidic extract was added as a 1.65-ml solution in ethanol, a treatment with the same amount of ethanol was included for growth comparisons. A parallel treatment of wastewater plus acidic extract without ethanol was also included. For this treatment, ethanol from acidic extract was removed by flash evaporation and the residue was reconstituted in 50 ml of nonsterile wastewater. The compositions in all the flasks were inoculated with

\begin{table}[h]
\centering
\caption{\textbf{Chemical properties of filtered and unfiltered wastewater}}
\begin{tabular}{llll}
\hline
Property & Filtered (0.2 $\mu$m) & Unfiltered &
\hline
Concn (ppm) of: & & &
\hline
N & 222 & 484 &
P & 8 & 67 &
K & 351 & 620 &
S & 68 & 72 &
Mg & 77 & 125 &
Ca & 104 & 270 &
Na & 174 & 330 &
Fe & 1 & 8 &
Al & 1 & 3 &
Mn & 0.1 & 1 &
Cu & 0.1 & 1 &
Zn & 0.1 & 2 &
B & 0.6 & 13 &
OM & 303 & 3,295 &
BOD & 158 & 714 &
COD & 400 & 4,600 &
TSS & 90 & 940 &
EC (mS/cm) & 3.8 & 7.2 &
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{\textbf{E. coli O157:H7 strains}}
\begin{tabular}{ll}
\hline
Strain & Source &
\hline
MM100 & Odwalla apple juice outbreak; wt, FDA strain SEA13B88 &
MM149 & Dairy manure, isolated 8/93, northwestern Oregon; wt, RM2543 &
MM151 & Dairy manure, isolated 8/94, western Washington; wt, RM2608 &
MM158 & \textit{E. coli} ONT:H32* isolated from 4-wk-old calf, California veal farm; wt, VMTRC 8051-B &
MM147 & Manure; wt, RM1673 &
MM148 & Manure; wt, RM1695 &
MM150 & Manure, western Washington; wt, RM2563 &
\hline
\end{tabular}
\end{table}
TABLE 3. Details of extracts of wastewater used in Bioscreen treatments

<table>
<thead>
<tr>
<th>Extract</th>
<th>Amt (µg)/300 µl medium</th>
<th>Conc (µg/ml) in medium</th>
<th>Wastewater equivalents (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic organic</td>
<td>95</td>
<td>315</td>
<td>3.4</td>
</tr>
<tr>
<td>Basic organic</td>
<td>27</td>
<td>90</td>
<td>6.8</td>
</tr>
<tr>
<td>Neutral organic</td>
<td>35</td>
<td>115</td>
<td>0.8</td>
</tr>
<tr>
<td>Neutral aqueous</td>
<td>1,100</td>
<td>5,500</td>
<td>4.7</td>
</tr>
</tbody>
</table>

* Dry weight of solids in 15 µl of the extract.

**RESULTS**

Survival of *E. coli* O157:H7 in sterile and nonsterile wastewater. Since *E. coli* O157:H7 disappeared rapidly from wastewater in a previous study (31), an attempt was made to determine the reasons for failure of this pathogen in wastewater by eliminating competing organisms by autoclaving or filter sterilization. While autoclaving removes all competing organisms including parasites and predators, filter-sterilized water would contain phages. In addition, filter sterilization also removed most suspended solids along with nutrient-rich organic matter compared to the nonsterile water (Table 2). As a result, two strains of *E. coli* O157:H7 (MM100 and MM151) and a strain of *E. coli* O117:H32 (MM158) completely disappeared from wastewater 4 to 5 days after inoculation, and all four strains disappeared even more rapidly from filter-sterilized wastewater (Fig. 1). These strains survived longer in steam-sterilized wastewater, and two of them (MM149 and MM158) grew during the first 2 days. However, none of them could be recovered from steam-sterilized or other wastewaters after 15 days of incubation (data not shown). The source wastewater used in
this study contained \((1.5 \pm 0.5) \times 10^6\) CFU/ml of aerobic bacteria and \((9.4 \pm 1.5) \times 10^3\) CFU/ml of coliforms. Similar results were obtained a month later in a repeat study with fresh wastewater.

**Growth kinetics of** *E. coli* **O157:H7 and native organisms in LB broth supplemented with wastewater.** The ability of wastewater to supply nutrients and the growth responses of six strains of *E. coli* O157:H7 and a strain of *E. coli* ONT:H32 (Table 1) to chemical components of wastewater were evaluated using the Bioscreen system. Increasing the proportion of filter-sterilized or nonsterile wastewaters in growth medium to \(>50\%\) significantly \((P < 0.01,\) repeated-measures two-way ANOVA) inhibited the growth of all seven strains (Table 1) and native organisms in wastewater (Fig. 2). A further increase in proportion of wastewater to \(>90\%\) significantly \((P < 0.001)\) inhibited their growth. Furthermore, all seven strains and native organisms failed to grow in unsupplemented wastewater. In general, growth patterns shown in Fig. 2A for strain MM100 were similar to those for other strains (data not shown), but the lag time for significant growth to occur at incremental concentrations of wastewater varied among strains. For example, a shortest lag of 10 h was observed for MM100 in LB broth with 50\% wastewater, whereas a 32-h lag was observed with MM147. Although growth was not evident after 7 days in wells with 100\% wastewater, viable pathogens were detected by enumeration on LB-RNC agar plates. An inoculum of \((2 \pm 1) \times 10^6\) CFU per well was used in comparing growth of different strains.

In contrast to the observance of prolonged lag times with *E. coli* O157:H7 or ONT:H32, significant \((P < 0.001)\) growth of native organisms was observed within 3 h in LB broth supplemented with or without 50\% wastewater (Fig. 2B). This treatment contained \(2.3 \times 10^7\) CFU of aerobic bacteria at the beginning of incubations. In addition, a wider range of initial bacterial counts \([1.5 \pm 0.5] \times 10^7\) CFU in 100\% LB broth plus 10 \(\mu\)l wastewater as the inoculum to \(4.5 \times 10^7\) CFU in 100\% wastewater) did not make any difference in lag times to significant growth.

**Influence of organic extracts of wastewater on growth of** *E. coli* **O157:H7 and** *E. coli* **ONT:H32.** As all seven strains failed to grow in wastewater, attempts were made to determine if any organic component of wastewater was inhibitory. Growth of two *E. coli* strains (one each of O157:H7 and ONT:H32) was monitored in reduced-strength LB-MS broth supplemented with organic extracts of neutral, acidic, or alkaline fractions of wastewater (Fig. 3). Since an enhanced effect of organic extracts in nutrient-limited media was expected, we chose 5 and 50\% LB-MS as basal media for these comparisons. The acidic extract completely inhibited the growth of both strains in media containing 5 and 50\% LB broth. The basic extract, on the other hand, inhibited the growth of both strains in 5% LB-MS but not in 50\% LB-MS. The neutral aqueous extracts encouraged the growth of organisms in both media, while the neutral organic fraction slightly inhibited the growth. On a weight basis, neutral aqueous extract added to Bioscreen wells contained the most solids (Table 3). At termination of the experiment, the \(pH\) of all growth media plus extracts was 6.8 \pm 0.2.

**Survival of** *E. coli* **ONT:H32 in wastewater supplemented with acidic wastewater extract.** Since the Bioscreen system monitors optical density and cannot monitor the decline of organisms, the influence of acidic components of wastewater on survival of *E. coli* ONT:H32 in wastewater was monitored by plate counts on LB-RNC agar (Fig. 4). In this scale-up experiment, MM158 declined from \(10^7\) to \(10^4\) CFU/ml during a 10-day incubation in wastewater supplemented with or without an additional 2.75 equivalents of acidic organic fraction from wastewater (Fig. 4). However, the organisms grew in treatments containing ethanol as a carrier for the acidic organic fraction and in wastewater treated with only ethanol. The quantity of ethanol added was 3.5\% (vol/vol) of total volume of wastewater and was a proportional scale-up from the Bioscreen studies (Fig. 3).
Influence of diffusible components of wastewater on the survival of *E. coli*.

To determine the reasons for unexpected decline of *E. coli* ONT:H32 or *E. coli* O157:H7 strains in filter-sterilized wastewater (Fig. 1), the fate of *E. coli* O157:H7 (MM149) in dialysis tubes with filter-sterilized wastewater and exposed to the components of wastewater was monitored (Fig. 5). As coliphages cannot pass through either 1-kDa or 300-kDa dialysis tubes, this test would distinguish nutrient deficiency from native coliphages (if present) in reducing *E. coli* O157:H7 numbers inside the dialysis tubes. A 3-log decline was observed during a 5-day period with MM149 inoculated directly in wastewater compared to a fractional decline from the dialysis tubes exposed to the same amount of wastewater (Fig. 5).

*E. coli* O157:H7 survived better in the presence of diffusible components of 300 kDa than when exposed to components of <1 kDa. However, the components of <300 kDa significantly enhanced the survival of MM149 only after 20 days (*P* < 0.01, repeated-measures two-way ANOVA). In addition, the decline rate of pathogenic *E. coli* in 300-kDa...
dialysis tubes was comparable to that of native aerobic bacteria. The rates were 0.055 and 0.044 log CFU ml$^{-1}$ day$^{-1}$, respectively, for *E. coli* O157:H7 and native bacteria, based on plate counts for days 13 to 15. Meanwhile, the pathogen directly inoculated into wastewater declined at 0.283 log CFU ml$^{-1}$ day$^{-1}$ in other words, the directly inoculated pathogen, pathogen inside the 300-kDa dialysis tube, and native aerobic bacteria declined with decimal reduction times (time for 1-log decline) of 3.5, 18.1, and 22.5 days, respectively.

In a separate study, the capacity of wastewater to maintain MM149 in dialysis microcosms was determined indirectly by exposing the pathogen to dilutions of wastewater (Fig. 6). MM149 declined rapidly in 10% wastewater, while the population remained at a constant level even when the nutrients were diluted by half.

**DISCUSSION**

Manure and wastewater from dairy lagoons are an excellent source of nutrients for agricultural crops, but they are also known to be a potential source of contamination with human pathogens like *E. coli* O157:H7 (30). Although *E. coli* O157:H7 occurs at low prevalence (13, 14, 25), conditions that favor regrowth (10) may increase the risk of contamination of crops fertilized with manure or wastewater. Furthermore, *E. coli* O157:H7 persists longer in manure and wastewater at low temperatures (24, 31), in subsurface soils (1), and in dairy wastewater wetlands (22). However, the pathogen fails to establish in manure, manure slurries, and wastewater from dairy holding lagoons (17, 18, 21, 31). We found that the pathogen fails to establish during repeat inoculations that simulate continuous fecal input into lagoons through lane flushing (31). In this study, *E. coli* O157:H7 populations also declined rapidly in wastewater, while they proliferated in autoclaved wastewater. Pathogen regrowth in autoclaved wastewater may be a result of nutrient release from organic matter (16) and elimination of competing organisms (26, 39) during steam sterilization. It is surprising, however, that pathogens survived slightly longer in nonsterile wastewater than in filter-sterilized water, which did not contain any competing organisms. A higher level of inorganic nutrients in unfiltered water may be responsible for the improved survival in unfiltered nonsterile wastewater. Nonetheless, pathogens declined to undetectable levels after 2 weeks in either sterile or nonsterile wastewater. In addition to low nutrient levels, coliphages (35) that pass through 0.2-µm filters were also suspected of being responsible for the enhanced decline of *E. coli* O157:H7 or *E. coli* ONT:H32 strains in filter-sterilized wastewater. An earlier observation (31) of wastewater sustaining native aerobic bacteria but not native coliforms or inoculated *E. coli* O157:H7 also implies that pathogen survival in dairy lagoons could be regulated by factors other than nutrient availability.

Even though the failure of pathogens to establish in wastewater as a result of competition from native organisms is expected, dialysis tube incubations resulted in unpredictable results. Although pathogen numbers declined rapidly in directly inoculated wastewater, they persisted at high levels inside both the dialysis tubes. Surprisingly, the prolonged survival of pathogens in dialysis tubes was comparable to maintenance of native aerobic bacteria in wastewater outside the tube. Therefore, native organisms appear to have a limited influence, if any, on the nutrient supply to pathogens inside the dialysis tube. This assumption is further confirmed by the maintenance of pathogens inside the dialysis tube even at 50% reduction of nutrients in wastewater. Since there were sufficient nutrients to prolong growth, pathogen decline in wastewater is suspected to be a result of antagonistic interactions other than competition for nutrients by native organisms or nutrient deficiency in wastewater. As bacteriocins and antibiotics can pass through the high-molecular-weight cutoff dialysis tube, protection from
pathogen decline in wastewater may be increased by limiting nutrients through removal of manure solids. Thus, the practice of separating manure solids to recycle wastewater may have the added benefit of on-farm pathogen control. However, daily washing of the lanes in dairy barns replenishes the lagoon with nutrients and possibly pathogens through fresh manure. Although nutrients are available, the failure of freshly introduced pathogens to survive in this study and others (18, 21, 31) suggests that the pathogens have difficulty competing for the available nutrients with the more acclimated native organisms.

Overall, these results demonstrate the possibility of controlling pathogenic E. coli by altering the chemistry and nutrients in wastewaters, and such modifications of chemistry should be explored in combination with other promising techniques that minimize pathogen replication and release from cattle (3, 7, 8, 12, 33, 35). An equally significant finding of prolonged survival of E. coli O157:H7 when protected in dialysis tubes suggests that coliphages (35) and protozoan predators may be responsible for the rapid decline of pathogenic E. coli in wastewaters from dairy lagoons. Nonetheless, the environmental significance of coliphages and protozoa in controlling pathogenic E. coli requires further scrutiny.

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