Effects of Various Acids on Growth and Survival of Yersinia enterocolitica

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ABSTRACT

The relative effects of several different acids on survival of Yersinia enterocolitica in tryptic soy broth after 24 h of incubation were compared. The acids compared included 25, 50, 75, and 100 mM concentrations of hydrochloric (HCl), citric (CIT), acetic (ACE), lactic (LAC), propionic (PRO), or phosphoric (PHO) acid. The decrease in viable Yersinia cells was compared based on concentration of acid added, pH, and the calculated concentration of the undissociated portion of the monoprotic acids. In addition, data were subjected to analysis of variance procedures to determine significant differences among the antimicrobial activities of the various acids. Results indicated that the relative activity of the acids differed depending on how the data were compared. When based on equal molar concentration of acid added, the overall hierarchy of antimicrobial activity appeared to be CIT > HCl > LAC > PHO > PRO > ACE. The antimicrobial activity based on pH gave the apparent overall hierarchy PRO > LAC > ACE > CIT > PHO > HCl. The antimicrobial activity based on the concentration of the undissociated portion of monoprotic acids appeared to be HCl > LAC > PRO > ACE. Although graphic representation of the data gave the appearance of differences in antimicrobial effects, statistical analysis revealed that often there was no significant difference (p<0.05) between acids. Differences which did appear were concentration-dependent. Statistical analysis also revealed that pH alone did not have a significant effect on survival.

Yersinia enterocolitica is a bacterium which has only in the last decade become established as a foodborne human pathogen. During this time a number of foodborne disease outbreaks have been attributed to this organism (2,3,17). The most common symptom of a Yersinia infection is gastroenteritis although other symptoms can include mesenteric lymphadenitis (18), terminal ileitis (8), and arthritis (18). In addition, this organism is unique among common foodborne pathogens in being able to mimic appendicitis (3).

The ability of foodborne pathogens to survive in acidic environments is important because acidity is often used in foods to control these bacteria. Y. enterocolitica is one pathogen that has shown this ability. In one study (1), viable Y. enterocolitica were isolated from tartar sauce, an acid food. However, Brackett (4) could not recover viable Y. enterocolitica cells from artificially contaminated tartar sauce or spoonable salad dressing at any time, or from mayonnaise after 48 h. Stern and Pierson (15) reported that four separate strains of Y. enterocolitica grew at pH 4.6. Kendall and Gilbert (7) demonstrated that this organism grew at pH 4.4 and above and survived for at least 72 h at pH 4.2. Brackett (4) obtained similar results, but also found viable Y. enterocolitica cells after 21 days at pH 4.0 and 5°C. None of the reports mentioned the effects of different acids on Yersinia.

The purpose of this study was to determine the relative effects of different acids on growth and survival of Y. enterocolitica. In addition, methods of presenting survival data were compared.

MATERIALS AND METHODS

Cultures

Cultures used were Y. enterocolitica strains Y7P (serotype 0:8), C122-76 (serotype 0:3), and 2635 (serotype 0:8). All three cultures were provided by M. P. Doyle of the Food Research Institute, University of Wisconsin. Before use in experiments, cultures were checked for purity and the genus and species were confirmed using the API 20E system (Analytab Products, Plainview, NY). Stock cultures were maintained on tryptic soy agar (TSA; Difco) slants incubated for 24 h at 25°C, and held at 5°C until use.

Preparation of acid solutions

Two molar stock solutions of acetic (ACE), hydrochloric (HCl), lactic (LAC), propionic (PRO), and phosphoric (PHO) acids, and a 1 M stock solution of citric (CIT) acid were prepared and sterilized by autoclaving at 121°C for 15 min as described by Minor and Marth (9). Appropriate amounts of the stock solutions were then aseptically added to sterile tryptic soy broth (TSB; Difco) to give 100 ml each of 0, 25, 50, 75 and 100 mM concentrations of the individual acids in the acidified TSB (TSBA) in 250-ml Erlenmeyer flasks. Duplicate flasks of TSBA solutions were used as growth media for all experiments.
Effects of various acids

For each acid and concentration tested, duplicate 20-ml samples of TSB were aseptically placed in sterile 30-ml centrifuge tubes. One loopful of test culture grown in TSB at 25°C was inoculated into each tube and the tubes were incubated for 18 h at 25°C. After incubation, the tubes were centrifuged at 10,800 \( \times g \) for 15 min in a Sorvall RC2-B centrifuge equipped with a SS-34 rotor (Ivan Sorvall, Inc., Newtown, CN) to sediment the cells. The supernatant fluid in each tube was then decanted, replaced with 30 ml of the appropriate TSBA, and the tube was vortexed to resuspend the cell pellet. Duplicate 10-ml samples were then withdrawn and placed into sterile centrifuge tubes; the remaining 10 ml was used to determine initial pH as described below. Cell populations in the centrifuge tubes were determined as described below using 1-ml subsamples from each tube. The resulting 9-ml samples were incubated at 25°C for 24 h, after which cell populations and pH were again determined.

Cell populations were determined by making serial dilutions of a 1-ml sample of broth in peptone buffer (pH 7.0), surface plating 0.1 ml of each dilution on duplicate TSA plates, and counting colonies from plates having between 30 and 300 colonies. All cell populations are expressed as mean logs of colony forming units (CFU) of two replicates. Portions of samples used for pH measurements were first steamed for 20 min to eliminate viable cells. The samples were then allowed to cool to room temperature and the pH was measured with a pH meter and combination pH electrode.

Determination of undissociated portions of acids

Determination of undissociated portions of monoprotic acids at 0 h was done using a computer program written for that purpose. The program was written in BASIC language and was based upon the equilibrium expression, pH = pKa + log [HAc]/[Ac'], where HAc and Ac' are the undissociated and dissociated forms of the acid, respectively. The input variables used in the program were pH, pKa, and concentration of added acid. The main algorithm used was:

\[
\text{HAc} = \text{acid } - \left( (10^{\text{pH}-\text{pKa}}) - \text{acid} \right) \left( 1 - (10^{\text{pH}-\text{pKa}}) \right)
\]

where 'acid' is the concentration (mM) of the acid in the test solution, pH is the pH of the solution, and pKa is the pKa for the particular acid used. Similar calculations were not done for polyprotic acids since several degrees of protonation would exist simultaneously for each acid at the pH values tested.

Statistical analysis

All data were analyzed using analysis of variance (ANOVA) and Duncan’s multiple range test (14) to determine statistically significant differences (p<0.05).

RESULTS

Inactivation as a function of concentration of added acid

Figure 1 depicts inactivation of Y. enterocolitica after 24 h of incubation at 25°C in TSB acidified with acetic, hydrochloric, phosphoric, citric, lactic, or propionic acids vs concentration of acid added. Initial cell population was about 8 Log_{10} CFU/ml. A = C122-76; B = Y7P.

Figure 1. Inactivation of Y. enterocolitica after 24 h of incubation at 25°C in TSB acidified with acetic, hydrochloric, phosphoric, citric, lactic, or propionic acids vs concentration of acid added. Initial cell population was about 8 Log_{10} CFU/ml. A = C122-76; B = Y7P.

the least antimicrobial activity is comprised of PRO and ACE. The apparent hierarchy of antimicrobial activity for strain Y7P is CIT \( \geq \) HCL \( \geq \) LAC \( \geq \) PHO \( \geq \) PRO \( \geq \) ACE. The degree of antimicrobial activity against strain C122-76 is similar to that for strain Y7P except that the apparent hierarchy is CIT \( \geq \) LAC \( \geq \) HCL \( \geq \) PHO \( \geq \) PRO \( \geq \) ACE. These rankings were arbitrarily assigned after comparing the lowest concentration of acid at which most inhibition occurred.

Inactivation as a function of pH

Figure 2 depicts the same data as presented in Fig. 1 except that the decrease in viable population is plotted against pH. In this instance, results appear very different from those given above. Although the individual acids can again be placed into one of two categories of antimicrobial activity, the members of these categories differ from those in Fig. 1. The category containing the most active acids contains PRO, LAC, and ACE, whereas the category containing the least effective acids is comprised of HCL, CIT, and PHO. The apparent hierarchy of antimicrobial activity for both strains is PRO \( \geq \) LAC \( \geq \) ACE \( \geq \) CIT \( \geq \) PHO \( \geq \) HCL. In this instance, the rank-
tions were arbitrarily assigned after comparing the highest pH at which appreciable inhibition occurred.

**Inactivation based on undissociated portion of monoprotic acids**

Figure 3 illustrates the decrease of viable population plotted versus calculated concentrations of the undissociated portions of monoprotic acids used in the study. This form of presentation was used because of the general belief that it is the undissociated form of organic acids that is responsible for antimicrobial activity (5). When presented in this manner, the ranking appeared to be HCL > LAC > PRO > ACE.

**Results of ANOVA**

ANOVA revealed (Table 1) a significant interaction between acid and concentration. This means that the relative difference in antimicrobial effect between two acids will depend on the concentration at which they are compared. Examples of such situations are shown in Table 2 for 25 and 75 mM concentrations. One can see from these data that the relative rankings of various acids at 25 mM differ from the rankings at 75 mM. Thus it is not possible to say that one acid will always be more effective at inactivating *Y. enterocolitica* cells than another acid. However, it is apparent that some acids, such as CIT, are always among the most potent in antimicrobial effect.

A separate ANOVA using pH as the dependent variable (not shown) revealed that pH was dependent on concentration, acid, and the interaction between acid and concentration. Therefore, pH alone would not have a significant effect on the decrease in viable organisms. Rather, the antimicrobial effect was due primarily to the combination of the type of acid and concentration.

**Figure 2.** Inactivation of *Y. enterocolitica* after 24 h of incubation at 25°C in TSB acidified with acetic, hydrochloric, lactic, or propionic acids vs initial pH. Initial cell population was about 8 Log<sub>10</sub> CFU/ml. A = C122-76; B = Y7P.

**Figure 3.** Inactivation of *Y. enterocolitica* after 24 h of incubation at 25°C in TSB acidified with acetic, hydrochloric, lactic, or propionic acids vs concentration of undissociated form of acid. Initial cell population was about 8 Log<sub>10</sub> CFU/ml. A = C122-76; B = Y7P.

**TABLE 1.** Results of analysis of variance procedures comparing effects of type of acid, concentration and pH on mean changes in viable *Y. enterocolitica* grown in acidified TSB at 25°C.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sum of squares</th>
<th>F-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid</td>
<td>5</td>
<td>321.04</td>
<td>7142.21*</td>
</tr>
<tr>
<td>Concentrations</td>
<td>4</td>
<td>1768.32</td>
<td>49175.02*</td>
</tr>
<tr>
<td>Acid × concentration</td>
<td>20</td>
<td>254.86</td>
<td>1417.49*</td>
</tr>
</tbody>
</table>

<sup>a</sup>Degrees of freedom.

<sup>b</sup>Significant at p<0.05.
TABLE 2. Mean changes in concentrations\(^1\) of Yersinia enterocolitica C122-76 and Y7P in tryptic soy broth containing 25 and 75 mM concentrations of various acids at 25°C for 24 h.

<table>
<thead>
<tr>
<th>Acid</th>
<th>C122-76 25 mM</th>
<th>C122-76 75 mM</th>
<th>Y7P 25 mM</th>
<th>Y7P 75 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic</td>
<td>-0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-2.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-2.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Citric</td>
<td>-0.79&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-1.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-8.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-8.19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hydrochloric</td>
<td>-0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-8.39&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-8.35&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lactic</td>
<td>-0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-7.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-8.66&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phosphoric</td>
<td>0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-8.49&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>-8.34&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Propionic</td>
<td>-0.18&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-3.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-2.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Starting concentrations were about 8 Log\(_{10}\) CFU/ml.
<sup>2</sup>Concentrations within columns followed by different letters are significantly different (p<0.05).

DISCUSSION

Results of this investigation revealed that there were apparent differences in the antimicrobial activity of various acids on \(Y.\) enterocolitica. In this way these results support those found in similar studies with other microorganisms (9,16). In addition, the amount of decrease in populations of \(Y.\) enterocolitica caused by a given concentration of acid was similar to that for \(S.\) aureus tested with the same acids and concentrations (9).

The specific hierarchies of activities by the acids obtained in studies such as this one are difficult to compare, largely because various researchers report experimental data in different ways. For example, measurements of the amount of acid used in similar experiments includes percent (6), molarity (9,13), unspecified amounts (16), ml added (11,12), and ml necessary to cause a specified pH (10) or level of microbial inhibition (11). Likewise, results of antimicrobial effect are reported versus pH attained (9,16), concentration (9), and time (12). It is therefore important that these parameters be considered when one compares results of different researchers. Perhaps an effort to encourage the reporting of such data in a standardized manner would help avoid such confusion. Reports should at least provide populations of initial and surviving cells, concentrations of acids added, and pH values attained at each concentration of each acid. This would allow readers the flexibility to plot the data in any of the ways illustrated here.

Another difficulty in comparing results of past research such as this is the lack of proper statistical analysis to determine real differences in antimicrobial effect. Statistical analysis of data from this investigation revealed lack of significant differences where one might have perceived a difference by simply observing graphical representations of survival curves. Such a perception could lead one to believe that the use of one acid might give a greater margin of safety over the use of another acid when no real difference exist. The increased availability of computers should make statistical analysis more common in future research.

The finding that pH is less important in affecting survival of microorganisms than acid concentration supports the early claim by Fabian and Wadsorth (6). More recently, Young-Perkins and Merson (19) suggested that titratable acidity was better than pH in defining the limits of toxin production by \(C.\) botulinum. It is likely that many external factors and interactions, whose effects have not hereto been quantified, work together to influence growth and survival of microorganisms (5).

The conclusions of this study are that different acids have different antimicrobial activity on \(Y.\) enterocolitica and that the degree of this activity is similar to at least one other bacterium (\(S.\) aureus). The particular rankings of antimicrobial activity depend on both the manner in which they are presented and the concentrations at which they are compared. An additional conclusion was that future research would be more valuable to other researchers if results were presented in a standard form and subjected to statistical analysis as well as graphical interpretations.

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REFERENCES

Salt uptake and retention by herring

Reproducibility in determining salt content in herring, using QUANTAB, ICP, SIE, and the potentiometer can be seen by examining Table 3. Analysis of variance of the data for the raw 11.6-cm herring shown in Fig. 2 revealed no significant difference among QUANTAB, ICP, and SIE. A similar examination of the data for the raw 20.0-cm fish reveals that QUANTAB values are significantly lower from the ICP and SIE values at the 5% level. Analysis of variance for the raw 25.1-cm herring detected significant difference at the 5% level among all four methods of analysis (QUANTAB, ICP, SIE, and Potentiometer). It is important to point out that the numerical differences among the four methods are smaller than such differences among the three methods in the 11.6- and the 20.0-cm fish. When the data for three schools were pooled (QUANTAB, ICP, and SIE data) and subjected to analysis of variance, significant difference at the 5% level due to the method of analysis was not detected.

The potentiometric data for the 25.1-cm fish appear to be significantly lower from those generated by SIE (Table 3); however, the potentiometric data fall between that generated using SIE and ICP. Since it was shown above that SIE and ICP data were not significantly different at the 5% level, using pooled data of several runs, it is considered valid to assume that data generated by any of the four analytical methods tested in this study are not significantly different from one another.

Analysis of variance on salt content for canned product of 11.6- and 20.0-cm herring indicates some significant differences due to method of analysis at the 5% level (Table 3). Significant differences among the three methods of salt determination are retained when pooling the data for the two different sized fish. It is perhaps surprising that while no differences were detected among methods of salt determination when testing raw fish, significant differences were detected with cooked fish. This observation should be kept in mind when comparing data on cooked fish when different methods of analysis are used, although it should be pointed out the observed differences were at <10% of the salt concentration detected (Table 3).

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