

# Acetoin production as an indicator of growth and metabolic inhibition of *Listeria monocytogenes*

T.L. Romick and H.P. Fleming

Food Fermentation Laboratory, US Department of Agriculture, Agricultural Research Service, and North Carolina Agricultural Research Service, Department of Food Science, North Carolina State University, Raleigh, NC, USA

6039/12/96: received 18 December 1996, revised 9 April 1997 and accepted 10 April 1997

T.L. ROMICK AND H.P. FLEMING. 1998. It has been shown that *Listeria monocytogenes* produces acetoin from glucose under aerobic conditions. A defined medium with glucose as the sole carbon source was used in an aerobic shake flask culture to reliably produce acetoin. Acetoin, the reactive compound in the Voges–Proskauer test, was assayable in the medium and was used to quantify the metabolic response when inhibitors were added to the medium. Inhibitors such as lactic, acetic, propionic and benzoic acids were used to demonstrate the utility of acetoin production as an indicator of metabolic disruption. With increasing levels of inhibitor, the metabolic and growth responses were measured by acetoin production and optical density change, respectively. Both measurements decreased in a similar manner with increasing inhibitor concentrations. The data also showed the apparent mode of action of the inhibitors. A bacteriostatic effect was observed for the protonated organic acids, acetic (4 mmol l<sup>-1</sup>) and propionic (4 mmol l<sup>-1</sup>), whereas protonated lactic (4 mmol l<sup>-1</sup>) and benzoic (0.16 mmol l<sup>-1</sup>) acids gave an irreversible (apparent bacteriocidal) effect. Lactic, acetic, and propionic acids showed stimulation of metabolic activity at low concentrations, but benzoic did not. Acetoin production is a novel method for quantifying and assessing the mode of action of inhibitors against *L. monocytogenes*. This system can be used to screen inhibitors for applications in food safety.

## INTRODUCTION

*Listeria monocytogenes* performs a 2,3-butanediol fermentation in defined medium and accumulates acetoin under aerobic incubation. Under anaerobic conditions, a mixed acid fermentation is apparent (Gottschalk 1986) (see over).

The metabolic pool of pyruvate provides the substrate for acetoin formation where two molecules of pyruvate are converted to  $\alpha$ -acetolactate by the action of acetolactate synthase using thiamine pyrophosphate as a coenzyme (Neidhardt *et al.* 1990; Voet and Voet 1990). The  $\alpha$ -acetolactate is then decar-

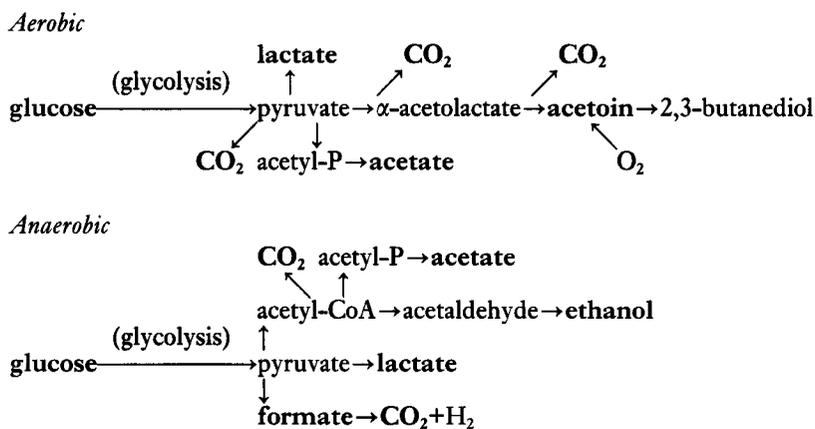
boxylated to form acetoin and further reduced to form 2,3-butanediol. Under aeration, acetoin accumulates, rather than the terminal diol compound (Underkofler and Hickey 1954; Romick *et al.* 1996). The formation of  $\alpha$ -acetolactate is also a precursor in the synthesis of valine and isoleucine (Voet and Voet 1990), both of which are provided in the medium. In actively growing cells, the metabolic flux, with respect to the pyruvate pool, is optimal since pyruvate is rarely measurable in the growth medium, meaning that the fueling reactions of glycolysis are supplying appropriate amounts of pyruvate for all cellular needs. All the major fueling reactions, polymerization and biosynthetic reactions are tightly controlled, with any disruption causing a decline in growth (Neidhardt *et al.* 1990). Thus, by measuring acetoin, it was presumed that any decrease in production represented a proportionate decrease in the pyruvate pool due to the disruption of the fueling reactions and metabolic flux.

The 2,3-butanediol pathway operates at capacity when the

Correspondence to: H.P. Fleming, USDA-ARS, Box 7624, NCSU, Raleigh, NC 27695–7624, USA.

T. L. Romick is now with Hunt-Wesson, Inc., Research and Development, 1645 W. Valencia Dr, Fullerton, CA 92633, USA.

Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the US Department of Agriculture or North Carolina Agricultural Research Service, nor does it imply approval to the exclusion of other products that may be suitable.



pH is 6.0 or below and where the need for neutral products is induced (Mickelson *et al.* 1938; Mickelson and Werkman 1938; Underkofler and Hickey 1954; Neidhardt *et al.* 1990). Also, it has been suggested that acetolactate synthase is most active at pH less than 6.2 (Underkofler and Hickey 1954; Gunsalus and Stanier 1961). Thus, fully induced cells for the production of acetoin are subcultured in glucose-defined medium (GDM) at pH 6.0 for use as inoculum in the *Listeria* batch culture test. Production of acetoin, a constitutive metabolite of aerobically-grown *L. monocytogenes*, was used as an indication of growth inhibition by additives such as lactic, acetic, propionic and benzoic acids to the growth medium. Acetoin can be reliably produced and easily assayed.

The minimum inhibitory concentration (MIC) of an antimicrobial can be measured in different ways; there is no uniform procedure currently available for food microbiology (Davidson and Parish 1989). *In vitro* screening methods are used to determine end-point MIC and descriptive MIC. Descriptive MIC determinations are designed to give information about the effect the inhibitor has on the growth dynamics of the target micro-organism, whereas end-point screening quantifies the usefulness of the inhibitory compound. There are two general types of descriptive screening methods that measure the viability of the target organism over time. Turbidimetric assays use optical density measurements to show effects on specific growth rate (and lag time) due to the inhibitor, and kill curves measure viable count change due to the inhibitor. End-point screening methods include agar diffusion, agar and broth dilution, gradient plates and disinfectant sanitizer tests to determine the effective concentration of the inhibitor (Davidson and Parish 1989).

Therefore, the objective of this paper was to demonstrate how acetoin production by *L. monocytogenes* can be used as an indication of metabolic flux disruption. The system is novel in that it is an end-point screen and descriptive test combined, since the MIC of the inhibitor is quantified and also described for its bacteriostatic or bacteriocidal effects.

## MATERIALS AND METHODS

### *Listeria* shake flask batch culture

The *L. monocytogenes* strain (F5069) and the formula for GDM at pH 6.0 were described previously (Romick *et al.* 1996). The inoculum was prepared by growth overnight in 20 ml of GDM shaken (180 rev min<sup>-1</sup>) in a 50 ml Erlenmeyer flask, with foam plug, and incubated in a 30 °C water bath (Innova 3000 platform shaker; New Brunswick Scientific, Edison, NJ, USA). A 2 ml sample of the overnight culture was inoculated into 200 ml fresh GDM in a 500 ml Erlenmeyer flask, with foam plug, shaken at 30 °C. The subculture was monitored until an O.D. of 0.2 (630 nm; Novaspec II, Pharmacia LKB Biotechnology Inc., Piscataway, NJ, USA) was obtained, representing early logarithmic growing cells. The inoculum was harvested by centrifugation (10 min at 4000 g; Sorvall GSA rotor, Dupont Co., Wilmington, DE, USA) and was washed twice in fresh GDM and resuspended in 10 ml fresh GDM (5% the original volume). A 1 ml sample of this inoculum was added to 19 ml of GDM, with and without inhibitors, at various concentrations. The initial O.D. was  $\approx$  0.2, which represented  $3.4 \times 10^8$  cells ml<sup>-1</sup> as enumerated on MOX agar (Difco Labs, Detroit, MI, USA). An example test batch culture contained 19 ml of GDM, with inhibitor concentration calculated for a 20 ml culture volume in the 50 ml Erlenmeyer shake flask. After inoculation with 1 ml of inoculum preparation, the batch cultures were shaken for 2 min and sampled for zero time acetoin concentration and initial O.D. After incubation of the shake flask test culture for 4 h, final time samples were taken for acetoin concentration and final O.D. Also, control test batch cultures were set up from which periodic samples were taken, every 30 min over the 4 h period, to generate specific growth rate and acetoin production curves.

Sodium salts of lactate, acetate, propionate, benzoate, and NaCl (Fisher Scientific Co., Pittsburgh, PA, and J. T. Baker Inc., Phillipsburg, NJ, USA) were used so that the total ionic

strength of the medium did not change when adjusted to pH 6.0 with HCl. After addition of inhibitors and pH adjustment, the stock media were brought to volume and filter-sterilized (0.2  $\mu$  filters, Costar, Cambridge, MA, USA). Stock solutions of inhibitors in GDM at pH 6.0 were 2.69 mol l<sup>-1</sup> lactate (16.5 mmol l<sup>-1</sup> protonated), 0.26 mol l<sup>-1</sup> acetate (14.3 mmol l<sup>-1</sup> protonated), 0.29 mol l<sup>-1</sup> propionate (20 mmol l<sup>-1</sup> protonated), 1.0 mol l<sup>-1</sup> benzoate (15.6 mmol l<sup>-1</sup> protonated) and 1.71 mol l<sup>-1</sup> NaCl (10%), verified by HPLC (McFeeters *et al.* 1984) except for benzoate and NaCl. The stock acetoin-GDM used for standard curves was also verified by HPLC (McFeeters *et al.* 1984). The amount of protonated species was calculated using the Henderson-Hasselbalch equation, assuming pK<sub>a</sub>'s of 3.79 (lactic acid), 4.74 (acetic acid), 4.87 (propionic acid) and 4.20 (benzoic acid).

### Acetoin assay method

Samples taken for the acetoin assay were centrifuged to remove the cells (5 min microfuge, Brinkmann Instrument Co., Westbury, NY, USA) and duplicate 100  $\mu$ l aliquots transferred to the designated microtitre plate wells (96-well, Costar type No. 3598; Cambridge, MA, USA). Initially, the standard acetoin curve was determined by performing 1:1.5 serial dilutions of acetoin-GDM (2.5 mmol l<sup>-1</sup> acetoin, Sigma Chemical Co., St. Louis, MO, USA) with fresh GDM, adding Voges-Proskaur (VP) reagents and gathering absorbance data automatically (see below). This dilution series was carried out to find the valid linear range of acetoin detection. The routine standard acetoin curve series was done by adding acetoin-GDM (2.5 mmol l<sup>-1</sup> acetoin) to a designated well. On 1:2 serial dilution in fresh GDM to the next three wells, the routine acetoin standard series contained 2.5, 1.25, 0.625, and 0.3125 mmol l<sup>-1</sup> acetoin, the last three points of which fall within the valid range of the standard curve. This acetoin standard series was run in triplicate on each assay plate and used to calculate unknown acetoin concentrations. Samples from the test batch cultures (inhibitors added) were not diluted since the maximum amount of acetoin produced in the standard batch culture (no inhibitor) was always within the valid range of the standard curve after the 4 h incubation period. The VP assay was performed by adding, in order, 10  $\mu$ l L-arginine (Sigma, 10 mg ml<sup>-1</sup> distilled water), 10  $\mu$ l  $\alpha$ -naphthol (Fisher, 10 mg ml<sup>-1</sup> 95% ethanol), and 25  $\mu$ l KOH (Fisher, 40% w/v in distilled water) to each well containing 100  $\mu$ l of sample using an 8-channel pipetter (Costar). The reaction was monitored for 50 min to obtain maximum absorbance readings (see below). Thus, the test was defined as a shake flask batch culture (180 rev min<sup>-1</sup>) incubated at 30 °C for 4 h using 10<sup>8</sup> ml<sup>-1</sup> early logarithmically-growing *L. monocytogenes* cells (0.2 O.D.) to convert glucose to acetoin. Under these defined conditions, the maximum amount of acetoin production always occurred within the linear range

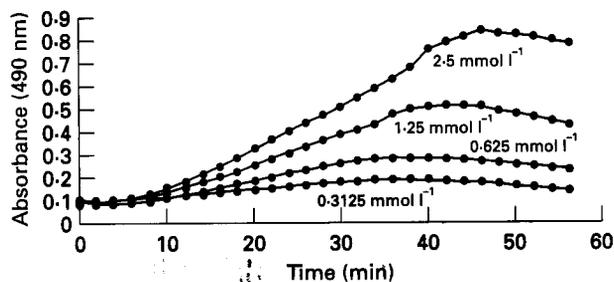
of detection by the VP assay. At the end of the 4 h incubation, samples were taken for final O.D. determinations and acetoin concentration (VP assay).

After addition of VP reagents, the microtitre plate was loaded into the plate reader (model EL 312, Bio-Tek, Winooski, VT, USA) which was programmed to take absorbance readings at 490 nm every 2 min for 50 min at 26 °C. The sample volume in the microtitre plate well was 145  $\mu$ l total (with added reagents) and was automatically mixed for 5 s, preceding each reading, to avoid dissolved oxygen gradients and allow uniform chromophore concentration in the light path. The time for the standard curve assay, performed in triplicate with each plate of samples, was complete within the 50 min test time and temperature, with volume loss measured to be less than 5%. The data were sorted to a commercial spread sheet program (Excel for Windows, Microsoft, Redmond, WA, USA) and calculated for maximum absorbance per well after subtracting blank well maximum absorbance (average of three wells of 100  $\mu$ l sterile GDM with added VP reagents). The concentration of acetoin per well was calculated from the regression equation for the averaged acetoin standard curves.

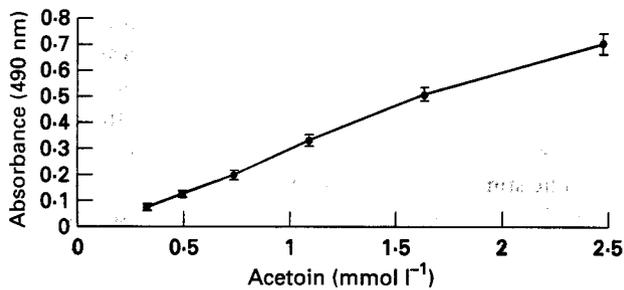
## RESULTS AND DISCUSSION

### The defined *Listeria* acetoin batch culture test

The VP chromophore was scanned to determine the optimal wavelength for detection in the defined medium (data not shown). The wavelength for maximum absorbance of the VP chromophore was 508 nm. Thus, the 490 nm filter available in the microtitre plate reader was used for the VP assays. The kinetics of the chromophore formation is a function of acetoin concentration in the sample. Figure 1 shows how both the rate and maximum absorbance increase with increasing acetoin concentration. The standard acetoin curve represents 1:1.5



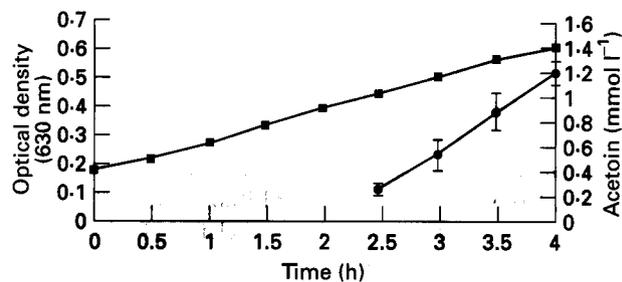
**Fig. 1** Voges-Proskauer chromophore reaction kinetics. Numbers under the maximum height of each curve represent the concentration of acetoin added. The chromophore was developed by addition of reagents and absorbance readings taken every 2 min by the programmable microtitre plate reader (see Materials and Methods). The maximum absorbance reading was used to generate the acetoin standard curve as discussed in the text



**Fig. 2** Acetoin standard curve (bars represent  $\pm 1$  s.d. among eight tests). The  $r^2 = 0.999$  between 0.05 and 0.5 absorbance, representing 0.3–1.5 mmol l<sup>-1</sup> acetoin

serial dilutions of the acetoin in GDM, as described in Materials and Methods, the last five of which fall in the best linear range of the curve (Fig. 2). The standard curve shows the best linear relationship ( $r^2 = 0.999$ ) between 0.05 and 0.5 absorbance, which was 0.3–1.5 mmol l<sup>-1</sup> acetoin in the medium. Thus, the sensitivity of the assay was 0.3 mmol l<sup>-1</sup> of acetoin, which would appear when 0.3 mmol l<sup>-1</sup> of glucose carbon was metabolized. The upper limit of the standard curve was 1.5 mmol l<sup>-1</sup> acetoin. The high  $r^2$  value and low standard deviation among eight replications indicate that using the maximum absorbance value was the appropriate way to determine acetoin concentration in the sample.

The shake flask batch cultures used early logarithmically-growing cells that were resuspended in fresh GDM at  $10^8$  cells ml<sup>-1</sup> and were fully induced for acetoin production. Detectable amounts of acetoin were produced by *L. monocytogenes* within 2.5 h, with a corresponding increase in O.D. (Fig. 3). After 4 h of incubation, the maximum amount of acetoin was produced that was still within the linear range of the standard curve (1.2–1.5 mmol l<sup>-1</sup>). Thus, this was the time set for the batch culture test so that samples for the acetoin assay would always be within the standard curve and



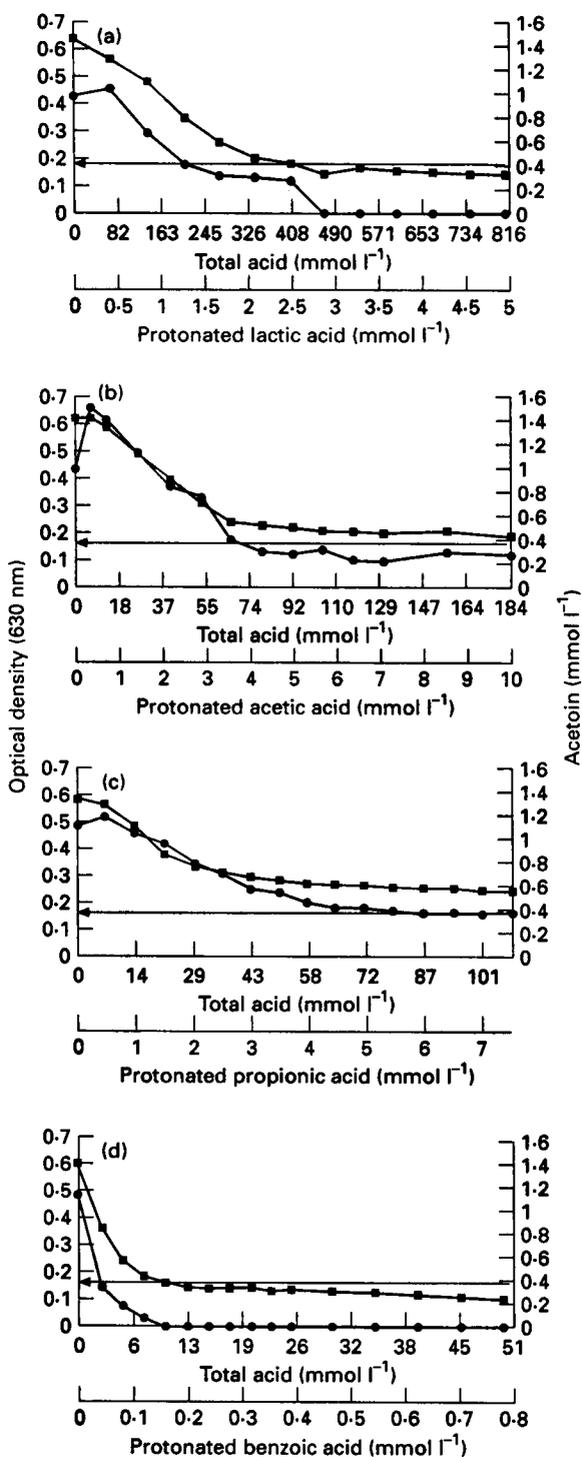
**Fig. 3** *Listeria* standard acetoin batch culture test (bars represent  $\pm 1$  s.d. among five tests). Acetoin is detectable after 2.5 h of incubation, with 4 h of incubation established as the total test time where the total acetoin concentration was within the linear range of the acetoin standard curve. The specific growth rate was 0.29 h<sup>-1</sup>, and the acetoin production rate was 1.01 h<sup>-1</sup>. ●, Acetoin (mmol l<sup>-1</sup>); ■, growth (O.D. 630 nm)

need not be diluted. The growth rate of *L. monocytogenes* in GDM was 0.29 h<sup>-1</sup> (2.39 h doubling time), with an acetoin production rate of 1.01 h<sup>-1</sup> (Fig. 3). The specific production rate of acetoin by *L. monocytogenes* under these defined conditions was calculated at 0.233 mg acetoin mg<sup>-1</sup> cells h<sup>-1</sup> from the growth and acetoin rate curves in Fig. 3, and using a dry weight standard curve (data not shown). This indicates that a 0.2 O.D. ( $\approx 1$  doubling) increase was required before acetoin was detectable in the medium, representing an increase of  $\approx 10^8$  cells ml<sup>-1</sup>. Figure 3 shows that acetoin was detectable after about a 0.2 O.D. increase, corresponding to the doubling time of 2.4 h.

### Determining the MIC of inhibitors by acetoin production

The *Listeria* standard acetoin batch culture test (no inhibitor) was used as the basis for comparison when inhibitors were incorporated into GDM and allowed to affect acetoin production and growth under the defined conditions. When acetoin production was affected relative to the control (no inhibitor), the metabolic flux was assumed to be disrupted (reduced acetoin production). A similar decreasing trend in O.D. was seen after completion of the 4 h test time. The O.D. changes reflected an actual reduction in viable counts, as seen when plated on MOX agar (data not shown). Figure 4 illustrates use of the acetoin batch culture test for quantitating effects of inhibitors (lactic, acetic, propionic, benzoic acids) on the growth and metabolism of *L. monocytogenes*. This method of inhibitor testing against *L. monocytogenes* provides end-point and descriptive measure of the MIC in one test (Davidson and Parish 1989). The data show the apparent MIC for the protonated species of lactic (3 mmol l<sup>-1</sup>), acetic (4 mmol l<sup>-1</sup>), propionic (4 mmol l<sup>-1</sup>), and benzoic (0.16 mmol l<sup>-1</sup>) acids with a description of the mode of action (bacteriostatic *vs* bacteriocidal).

Acetic and propionic acids were apparently bacteriostatic, whereas lactic and benzoic acids were bacteriocidal. Bacteriocidal action can be defined as deleterious metabolic effects on growing cells that are irreversible, whereas bacteriostatic effects are reversible (Gunsalus and Stanier 1961). The bacteriocidal action is seen in Fig. 4a and 4d (lactic and benzoic acids), where the final O.D. falls below the initial starting O.D. with a cessation of acetoin production (measured after the 4 h test time). Bacteriostatic action is observed in Fig. 4b and 4c (acetic and propionic acids), where final O.D. never falls below initial O.D. with a continuous low level production of acetoin (measured after the 4 h test time). When cells were incubated in the presence of the established inhibitory concentrations of the protonated acids over an extended period of time (7 d), continued decline in O.D. and viable counts were observed for lactic and benzoic acids (with no change in medium pH), but growth resumed for acetic and propionic acids (with a drop in medium pH) after a lag



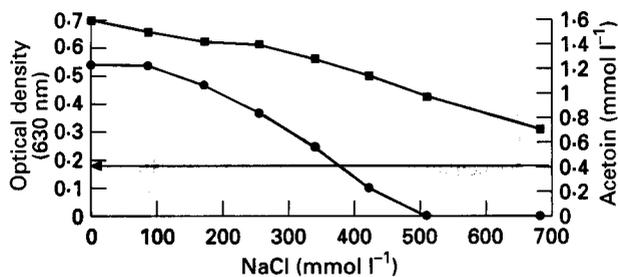
**Fig. 4** Optical density and acetoin production decline by *Listeria monocytogenes* F5069 in GDM shake flask batch cultures due to the added acids: (a) lactic; (b) acetic; (c) propionic; (d) benzoic. Acid concentrations are on different scales, with corresponding total acid concentration noted above the scale. The horizontal arrow represents the initial optical density; other optical densities are final. ●, acetoin (mmol l<sup>-1</sup>); ■, growth (O.D. 630 nm)

time of 1–2 d (data not shown). These findings supported the apparent bacteriocidal and bacteriostatic effects, respectively. Figure 4 also shows how protonated lactic, acetic and propionic acids stimulate acetoin production above the control at very low concentrations. Benzoic acid did not have this effect. The stimulatory effect may be explained by assuming that small concentrations of those protonated acids are metabolized but, as the concentration increases, the bacteriostatic or bacteriocidal effect becomes apparent as the unmetabolized acids lose their proton to the cytosol (Debevere 1988; Davidson and Parish 1989; El-Shenawy and Marth 1989; McDonald *et al.* 1991). Recently, a model was proposed to illustrate how a Gram-positive bacterium (*Clostridium acetobutylicum*) can metabolize acetic acid to prevent internal acidosis (Grupe and Gottschalk 1992). Benzoate apparently was not metabolized by the culture.

Many studies have shown that concentration and type of acid are responsible for antibacterial action, in addition to lowering medium pH (Brackett 1987; Debevere 1988; El-Shenawy and Marth 1989; Sorrells *et al.* 1989; Adams *et al.* 1991; McDonald *et al.* 1991; El-Shenawy and Marth 1992; Passos 1993). The bacteriocidal effect of lactic acid and its MIC have been previously reported (Debevere 1988; El-Shenawy and Marth 1989; Sorrells *et al.* 1989; Adams *et al.* 1991; Kouassi and Shelef 1995; Shelef and Potluri 1995). Still other reports of the MIC for acetic, propionic and benzoic acids against *L. monocytogenes* vary greatly (Debevere 1988; El-Shenawy and Marth 1989, 1992; Sorrells *et al.* 1989; Oh and Marshall 1992; Kouassi and Shelef 1995; Richards *et al.* 1995), which reflects the variety of methods used (Davidson and Parish 1989).

The reported MICs of these acids for *L. monocytogenes* range from 0.5 to 4.0% or 10–40 mmol l<sup>-1</sup>, depending on the pH of the test medium. The pH in this study was kept constant for all acids and MICs, reported as mmol l<sup>-1</sup> of protonated acid species.

The general consensus assumes that decline in internal pH of the cell or direct effect by the protonated species accounts for the inhibitory effect (Debevere 1988; Davidson and Parish 1989; de Wit and Rombouts 1990; Ita and Hutkins 1991; McDonald *et al.* 1991; Miller and Acuff 1994; Richards *et al.* 1995; Shelef and Potluri 1995). Specific antimicrobial action of sodium lactate has been demonstrated against a wide variety of micro-organisms (de Wit and Rombouts 1990; Miller and Acuff 1994; Richards *et al.* 1995; Shelef and Potluri 1995). A more recent theory asserts that acid anion accumulation is responsible for the inhibitory action (Russell 1992). Acid anion accumulation is dependent upon the  $\Delta$ pH (pH outside the cell minus the pH inside the cell). A higher pH inside the cell would result in anion accumulation inside the cell. The external cell pH in these tests was 6.0, where the internal pH of the cells would be expected to be  $\approx$  6.2 in the case of lactic acid with similar  $\Delta$ pH values of 0.2 for the other



**Fig. 5** Optical density and acetoin production decline by *Listeria monocytogenes* F5069 in GDM shake flask batch culture due to total ionic strength (NaCl). The horizontal arrow represents the initial optical density; other optical densities are final. ●, acetoin (mmol l<sup>-1</sup>); ■, growth (O.D. 630 nm)

acids (Ita and Hutkins 1991). This small  $\Delta$ pH would not be sufficient to allow great accumulation of anions needed for inhibition (Russell 1992), but would drive the deprotonation of the protonated species entering the cell. As the cell expends energy to pump out the excess protons, anions continue to accumulate, since they are not freely permeable to the cell membrane (Russell 1992). Thus, at some critical concentration of protonated organic acid species, the energy needed to expel protons is equal to or greater than the energy for overall growth.

When correcting the apparent inhibitory concentrations by assessing the total ionic strength effect on the cells (Fig. 5), the organic acids all gave an equivalent effective concentration of protonated form except benzoic acid (Table 1). This apparently indicates that the organic acids act as proton transporters by simple diffusion across the membrane to acidify the inside of the cell. The additional bacteriocidal effect of lactic acid may be attributed to acid anion accumulation relative to acetic and propionic acids, since its  $pK_a$  value

means it would accumulate about a 10-fold higher concentration inside the cell. Figure 5 also shows that acetoin produced by *L. monocytogenes* (acetoin production) is more sensitive to ionic strength than growth which is affected in a linear fashion,  $r^2 = 0.967$ . Apparently, growth was sustained while acetoin production was altered, indicating a possible transport phenomenon disruption or reduced dissolved oxygen concentrations due to NaCl. The combined effect of internal acidification and anion accumulation may account for the bacteriocidal effect of lactic acid, whereas acetic and propionic acids only exert an internal acidification effect that is bacteriostatic until the cells can adapt.

Apart from refrigeration, certain minimally processed, refrigerated foods rely on acidification as an additional hurdle to stabilize the product in the event of temperature abuse (Adams *et al.* 1991; Kraft 1992). Determining the pH and acidulant MIC, alone or in combination, gives conflicting results whether by end-point or descriptive (mode of action) testing. By expressing acidulant inhibitor in terms of protonated species present, a more accurate comparison can be made. The advantage of this method is the ability to measure end-point and descriptive MIC in the same test using a sensitive indicator of metabolism disruption (acetoin production) and a standard O.D. change measurement. The disadvantage is that it is exclusively for *L. monocytogenes* and other organisms that can produce defined amounts of acetoin. Other types of organisms would require initial study to find a constitutive metabolite that could be reliably produced, easily assayed, and quantified in response to inhibitors added to the medium. This system can be used to assess the inhibitory effect of any isolated compound against *L. monocytogenes* that can be incorporated into the medium. Finally, it should be noted that determinations of the current study were made under defined conditions (30 °C, aerobic shake flask, defined

**Table 1** Corrected effective inhibitory concentration of protonated lactic, acetic, propionic and benzoic acids due to the total ionic strength of the test medium

	Protonated acids			
	Lactate	Acetate	Propionate	Benzoate
Apparent inhibitory concentration*	3 mmol l <sup>-1</sup>	4 mmol l <sup>-1</sup>	4 mmol l <sup>-1</sup>	0.16 mmol l <sup>-1</sup>
Total ionic strength†	490 mmol l <sup>-1</sup>	74 mmol l <sup>-1</sup>	58 mmol l <sup>-1</sup>	10 mmol l <sup>-1</sup>
Per cent inhibition due to ionic strength‡	36%	3%	1%	0%
Effective inhibitory concentration§	4.08 mmol l <sup>-1</sup>	4.12 mmol l <sup>-1</sup>	4.05 mmol l <sup>-1</sup>	0.16 mmol l <sup>-1</sup>

\*Determined by inspection of inhibition curves in Figure 4.

†Total sodium salt acid concentration at each protonated inhibitor level.

‡Calculated by estimating the percentage decline due to ionic strength (NaCl) using the linear regression of the optical density line in Figure 5 ( $r^2 = 0.967$ ).

§Calculated by adding back the percent inhibition due to ionic strength alone.

medium), so the MIC values should be validated in specific food applications where pH, temperature, substrate and oxygen may vary.

## ACKNOWLEDGEMENT

This investigation was supported, in part, by a research grant from Pickle Packers International Inc., St Charles, IL.

## REFERENCES

- Adams, M.R., Little, C.L. and Easter, M.C. (1991) Modelling the effect of pH, acidulant and temperature on the growth rate of *Yersinia enterocolitica*. *Journal of Applied Bacteriology* **71**, 65–71.
- Brackett, R.E. (1987) Effect of various acids on growth and survival of *Yersinia enterocolitica*. *Journal of Food Protection* **50**, 598–601.
- Davidson, P.M. and Parish, M.E. (1989) Methods for testing the efficacy of food antimicrobials. *Food Technology* **43**, 148–155.
- Debevere, J.M. (1988) Effect of buffered acidulant systems on the survival of some food poisoning bacteria in medium acid media. *Food Microbiology* **5**, 135–139.
- de Wit, J.C. and Rombouts, F.M. (1990) Antimicrobial activity of sodium lactate. *Food Microbiology* **7**, 113–120.
- El-Shenawy, M.A. and Marth, E.H. (1989) Inhibition or inactivation of *Listeria monocytogenes* by sodium benzoate together with some organic acids. *Journal of Food Protection* **52**, 771–776.
- El-Shenawy, M.A. and Marth, E.H. (1992) Behavior of *Listeria monocytogenes* in the presence of sodium propionate together with food acids. *Journal of Food Protection* **55**, 241–245.
- Gottschalk, G. (1986) *Bacterial Metabolism*, 2nd edn, pp. 237–242. New York: Springer-Verlag.
- Grupe, H. and Gottschalk, G. (1992) Physiological events in *Clostridium acetobutylicum* during the shift from acidogenesis to solventogenesis in continuous culture and presentation of a model for shift induction. *Applied and Environmental Microbiology* **58**, 3896–3902.
- Gunsalus, I.C. and Stanier, R.Y. (eds) (1961) *The Bacteria, Volume II: Metabolism*. pp. 86–149. New York: Academic Press.
- Ita, P.S. and Hutkins, R.W. (1991) Intracellular pH and survival of *Listeria monocytogenes* Scott A in tryptic soy broth containing acetic, lactic, citric, and hydrochloric acids. *Journal of Food Protection* **54**, 15–19.
- Kouassi, Y. and Shelef, L.A. (1995) Listeriolysin O secretion by *Listeria monocytogenes* in broth containing salts of organic acids. *Journal of Food Protection* **58**, 1314–1319.
- Kraft, A.A. (1992) Health hazards vs. food spoilage. In *Psychrotrophic Bacteria in Foods: Disease and Spoilage*. Boca Raton: CRC Press, Inc.
- McDonald, L.C., Fleming, H.P. and Daeschel, M.A. (1991) Acidification effects on microbial populations during initiation of cucumber fermentation. *Journal of Food Science* **56**, 1353–1356.
- McFeeters, R.F., Thompson, R.L. and Fleming, H.P. (1984) Liquid chromatographic analysis of sugars, acids, and ethanol in lactic acid vegetable fermentations. *Journal of the Association of Official Analytical Chemists* **67**, 710–714.
- Mickelson, M., Reynolds, H. and Werkman, C.H. (1938) Behavior of added hydrogen acceptors on the metabolism of *Aerobacter indologenes*. *Journal of Bacteriology* **36**, 657–658.
- Mickelson, M. and Werkman, C.H. (1938) Influence of pH on the dissimilation of glucose by *Aerobacter indologenes*. *Journal of Bacteriology* **36**, 67–76.
- Miller, R.K. and Acuff, G.R. (1994) Sodium lactate affects pathogens in cooked beef. *Journal of Food Science* **59**, 15–19.
- Neidhardt, F.C., Ingraham, J.L. and Schaechter, M. (eds) (1990) *Physiology of the Bacterial Cell*. pp. 161–168. Sunderland: Sinauer Associates, Inc.
- Oh, D.H. and Marshall, D.L. (1992) Effects of pH on the minimum inhibitory concentration of monolaurin against *Listeria monocytogenes*. *Journal of Food Protection* **55**, 449–450.
- Passos, F.V. (1993) Modeling the cucumber fermentation. Ph. D. thesis, North Carolina State University, Raleigh.
- Richards, R.M.E., Xing, D.K.L. and King, T.P. (1995) Activity of p-aminobenzoic acid compared with other organic acids against selected bacteria. *Journal of Applied Bacteriology* **78**, 209–215.
- Romick, T.R., Fleming, H.P. and McFeeters, R.F. (1996) Aerobic and anaerobic metabolism of *Listeria monocytogenes* in defined glucose medium. *Applied and Environmental Microbiology* **62**, 304–307.
- Russell, J.B. (1992) Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. *Journal of Applied Bacteriology* **73**, 363–370.
- Shelef, L.A. and Potluri, V. (1995) Behaviour of foodborne pathogens in cooked liver sausage containing lactates. *Food Microbiology* **12**, 221–227.
- Sorrells, K.M., Enigl, D.C. and Hatfield, J.R. (1989) Effect of pH, acidulant, time, and temperature on the growth and survival of *Listeria monocytogenes*. *Journal of Food Protection* **52**, 571–573.
- Underkofler, L.A. and Hickey, R.J. (eds) (1954) *Industrial Fermentations*. Volume II. pp. 27–93. New York: Chemical Publishing Co., Inc.
- Voet, D. and Voet, J.G. (eds) (1990) *Biochemistry*, pp. 400–404. New York: John Wiley and Sons.