

Use of pH Gradient Plates for Increasing the Acid Tolerance of Salmonellae

C. N. HUHTANEN

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Philadelphia, Pennsylvania 19118

Received for publication 4 November 1974

Several strains of salmonellae survived higher concentrations of lactic acid after streaking on the surface of pH gradient plates. Most strains increased their acid tolerance by about 0.8 to 1.0 pH unit (9- to 10-fold), with *Salmonella madelia* showing the greatest differential, pH 5.2 in the wild strain and pH 4.2 after conditioning. The increased acid resistance was quickly lost after transferring to normal tryptic soy agar. Tests for survival in a liquid medium at pH values lower than those giving visible growth indicated that these pH values were bactericidal rather than bacteriostatic for both the wild and acid-conditioned strains.

The minimum pH for growth of salmonellae has been studied by a number of workers. Stokes and Bayne (8) did not observe colony formation in trypticase soy agar buffered with phosphate-phosphoric acid at pH 5.0 with any of several dozen strains and serotypes of salmonellae. Even at pH 6.0 *Salmonella gallinarum* and *S. pullorum* grew very poorly. The optimum for all strains was pH 7.0, with many strains growing equally well at pH 8.0. On the other hand, in liquid egg white, Banwart and Ayres (1) found *S. pullorum* and several other strains to grow at a more rapid rate at pH 6.0 than at 7.0 and faster at 7.0 than at 8.0. Reduction in numbers occurred at pH values of 9.0 or above.

Prost and Reimann (6) indicated that pH values above 9.0 and below 4.5 had a killing effect on salmonellae with destruction in a matter of minutes in lemon or lime juice (pH 2.3 to 2.5), whereas tomato juice (pH 4.3 to 4.5) was less bactericidal, with survivors found after 10 to 30 days. Chung and Goepfert (2) showed that the type of acid was important in determining the minimum pH for growth. Hydrochloric or citric acids required a pH of 4.05 to inhibit growth of *S. anatum*, *S. tennessee*, and *S. senftenberg*; lactic acid inhibited at pH 4.40, whereas acetic and propionic acids inhibited at pH 5.40 and 5.50, respectively. Levine and Fellers (5) also showed that the type of acid was important for determining minimum pH for growth, acetic acid being more toxic than lactic or hydrochloric acids. The bactericidal action of volatile fatty acids on *S. typhimurium* was shown to increase when the pH was lowered from 6.0 to 5.0 (3); there also appeared to be a

decrease in bactericidal activity with increasing chain length of the acid.

Many foods are naturally or artificially protected against microbial contamination. Smith and Palumbo (7) recently reported on the microbiology of Lebanon bologna manufacture. They found a considerable population of lactic acid bacteria during the fermentation, with a final pH of from 4.6 to 5.6, due most likely to lactic acid. Lactic acid was demonstrated to be the chief acid produced in Swedish sausage (4). Salmonellae are found in meat products and could be present in the raw materials for sausage manufacture. The study reported here was undertaken to determine if salmonellae could survive the acidity of fermented sausage and become adapted to growing at increasing lactic acid concentrations.

MATERIALS AND METHODS

Gradient plates. The two-layer gradient agar technique of Szybalski and Bryson (9) was used with square plastic petri dishes having bottom dish dimensions of 90 mm with a depth of 14 mm. The bottom layer was 25 ml of tryptic soy agar (TSA; Difco) poured while the lugs of one end of the dish rested on plastic tubing with a diameter of 6 mm. After solidification, the plates were placed on a level surface and a second layer of 27 ml of TSA containing 0.34 or 0.68% lactic acid was poured. The plates were then placed in a refrigerator for 20 to 24 h.

Cultures and media. Salmonella serotypes were received from B. Blackburn of the National Animal Disease Laboratories, U.S. Department of Agriculture, Ames, Iowa, and were maintained on TSA slants. Cultures were streaked on the plates by means of a needle with a semicircular curve of about 2 mm diameter, using several back-and-forth strokes. Incu-

bation was at 35 C for 20 to 24 h.

pH measurements. The pH of the surface of the gradient plates was determined by placing Whatman no. 1 filter paper strips (10 by 35 mm) on appropriate portions of the equilibrated plates for 20 min. The strips were then placed in a 10-ml beaker, and 1.5 ml of distilled water was added.

Minimum pH for growth. The lowest pH for growth in tryptic soy broth was determined by adjusting the pH with 20% lactic acid. The pH values did not change appreciably after autoclaving. The inoculum was 1 drop per 4 ml of a small amount of growth from the gradient plates or slants emulsified in 4 ml of H₂O.

RESULTS

The growth of the salmonellae on pH gradient plates is shown in Table 1. The cultures used as inocula were from 12-day-old TSA slants kept at room temperature. The pH values of 10-mm increments, as measured by the filter paper method, were 4.60, 4.55, 4.65, 4.75, 5.05, 5.35, 5.90, 6.40, and 6.75. The most acid-resistant culture was *S. grumpensis*, growing at a distance of 30 mm from the most acid end. The next most resistant was *S. dublin* (34 mm) followed by *S. saint-paul* and *S. tournai* (39 mm). These were in the 10-mm area, showing a pH of 4.75. All the other strains grew in the area with a pH of 5.05.

Eleven of the salmonellae were further studied. Fresh slants were prepared from the original cultures used for the experiment of Table 1 and these (wild) cultures were compared with inocula taken directly off the gradient plates at the point of growth nearest the acid end (acid conditioned) (Table 2). There was an appreciable increase in acid resistance with all cultures after growing on the gradient plates. The greatest differences of growth termini, indicating the greatest degree of acid conditioning, were with *S. madelia* (32 mm), *S. braenderup* (31 mm), *S. cerro* (29 mm), *S. meleagridis* (28 mm), and *S. havana* (25 mm). The least acid conditioning noted was with *S. montevideo* (7 mm). The change in growth-limiting pH of *S. madelia* appeared to be from 5.20 (50 to 60 mm) to 4.60 (20 to 30 mm), using the filter paper strip method of measuring surface pH values.

A more accurate determination of the minimum pH values permitting initiation of growth of several of these salmonellae and *S. typhimurium* (similarly acid conditioned) was made in tryptic soy broth adjusted with 20% lactic acid to pH values of 4.2 to 5.3, in increments of 0.1 pH units. The pH values remained constant when measured after autoclaving and cooling (Table 3). *S. madelia*, which showed the great-

TABLE 1. Growth of salmonellae and other Enterobacteriaceae on pH gradient plates^a

Culture	Distance ^b (mm)
<i>S. tennessee</i>	43
<i>S. newington</i>	44
<i>S. indiana</i>	41
<i>S. meleagridis</i>	40
<i>S. anatum</i>	42
<i>S. madelia</i>	47
<i>S. dublin</i>	34
<i>S. cerro</i>	42
<i>S. muenchen</i>	41
<i>S. montevideo</i>	42
<i>S. binza</i>	46
<i>S. san-diego</i>	45
<i>S. havana</i>	41
<i>S. thompson</i>	41
<i>S. saint-paul</i>	39
<i>S. eimsbuettel</i>	42
<i>S. infantis</i>	41
<i>S. alachua</i>	46
<i>S. gaminara</i>	43
<i>S. java</i>	41
<i>S. braenderup</i>	42
<i>S. californica</i>	42
<i>S. oranienberg</i>	47
<i>S. derby</i>	43
<i>S. ohio</i>	43
<i>S. reading</i>	46
<i>S. senftenberg 775W</i>	40
<i>S. enteritidis</i>	45
<i>S. tournai</i>	39
<i>S. typhimurium</i>	45
<i>S. grumpensis</i>	30
<i>S. paratyphi B</i>	40

^a Surface pH values of uninoculated plates, as measured by the filter paper strip method, were: 4.60 (0 to 10 mm from most acid end), 4.55 (10 to 20), 4.65 (20 to 30), 4.75 (30 to 40), 5.05 (40 to 50), 5.35 (50 to 60), 5.90 (60 to 70), 6.40 (70 to 80); and 6.75 (80 to 90). The bottom layer was 25 ml of TSA; the top layer was 27 ml of TSA with 0.34% lactic acid.

^b Distance from most acid end.

est increase in acid resistance in agar, was also the most acid resistant in broth, growing at pH 4.2; however, 2 days were required for growth to be evident. *S. montevideo*, which apparently showed the least acid conditioning in agar, showed an appreciable increase in acid tolerance in broth, with growth at pH 4.4 for the conditioned strain and 5.3 for the wild strain. Most of the salmonellae tested showed increases in acid tolerance of about 0.8 pH units. *S. dublin* (wild type) failed to grow at pH 5.3, the highest tested, although in another test it grew at 5.2 (wild) and 4.6 (acid conditioned). The conditioned strains quickly lost their acid

resistance. After transferring two times on TSA slants, the conditioned strains were no more resistant than the wild.

Tests for survival of the original inoculum in

TABLE 2. Growth of wild and acid-conditioned salmonellae on pH gradient plates^a

Culture	Growth terminus from most acid end (mm)		
	Wild	Acid conditioned	Δ
<i>S. havana</i>	50	25	25
<i>S. meleagridis</i>	51	23	28
<i>S. bredeney</i>	55	37	18
<i>S. dublin</i>	50	35	15
<i>S. anatum</i>	53	30	23
<i>S. madelia</i>	52	20	32
<i>S. californica</i>	46	30	16
<i>S. montevideo</i>	41	34	7
<i>S. cerro</i>	55	26	29
<i>S. muenchen</i>	46	28	18
<i>S. braenderup</i>	58	27	31

^a Surface pH values (by the filter paper strip method) were 4.55 (0 to 10 mm from most acid end), 4.55 (10 to 20 mm), 4.60 (20 to 30 mm), 4.65 (30 to 40 mm), 4.95 (40 to 50 mm), 5.20 (50 to 60 mm), 5.80 (60 to 70 mm), 6.25 (70 to 80 mm), and 6.50 (80 to 90 mm).

the tryptic soy broth were performed after 1 day of incubation on the two tubes, with pH values immediately below those showing visible turbidity. For these tests, 0.25 ml of the tryptic soy broth, previously inoculated with either wild or acid-conditioned strains, was placed into 4.5 ml of new broth and the tubes were reincubated for 3 days. The pH values of the new tubes, without further adjustment, were 5.40 for the original pH 4.20, 5.65 for the 4.40, 5.85 for the 4.60, and 6.05 for the 4.80. These pH values were high enough to permit growth of any of the salmonellae. All tests were negative, indicating that the effect of these pH values was bactericidal rather than bacteriostatic.

DISCUSSION

The results reported here indicate that salmonellae can become adapted to growing under more acid conditions, although this does not appear to be a genetic change, the characteristic being lost very soon after transferring to a neutral agar medium. Fermented foods such as Lebanon bologna ordinarily require several days for the pH to reach low enough levels to inhibit salmonellae. During this time salmonellae, if present, could conceivably become adapted to the acidity and persist in the finished product.

TABLE 3. Limiting pH values for growth of wild and acid-conditioned salmonellae in tryptic soy broth

Culture	pH ^a											
	4.2	4.3	4.4	4.5	4.6	4.7	4.8	4.9	5.0	5.1	5.2	5.3
<i>S. cerro</i>												
wild	-	-	-	-	-	-	-	-	-	-	+	+
conditioned	-	+	+	+	+	+	+	+	+	+	+	+
<i>S. muenchen</i>												
wild	-	-	-	-	-	-	-	-	-	-	+	+
conditioned	-	+ ^b	+	+	+	+	+	+	+	+	+	+
<i>S. dublin</i>												
wild	-	-	-	-	-	-	-	-	-	-	-	-
conditioned	-	-	+	+	+	+	+	+	+	+	+	+
<i>S. typhimurium</i>												
wild	-	-	-	-	-	-	-	-	-	-	+	+
conditioned	-	-	+ ^b	+	+	+	+	+	+	+	+	+
<i>S. anatum</i>												
wild	-	-	-	-	-	-	-	-	-	-	+	+
conditioned	-	-	+	+	+	+	+	+	+	+	+	+
<i>S. madelia</i>												
wild	-	-	-	-	-	-	-	-	-	-	+	+
conditioned	+ ^b	+	+	+	+	+	+	+	+	+	+	+
<i>S. montevideo</i>												
wild	-	-	-	-	-	-	-	-	-	-	-	+
conditioned	-	-	+	+	+	+	+	+	+	+	+	+

^a -, No growth; +, growth.

^b These tubes showed growth on the second day of incubation.

A more rapid increase in acidity, such as that produced by adding starter lactic cultures, could possibly prevent such adaptation, although Chung and Goepfert (2) could not demonstrate any degree of increased acid tolerance in liquid media. This may indicate that the acid conditioning, as reported here, takes place only during aerobic growth as on the surface of pH gradient plates. This may be a reflection of the difference in metabolism of these cultures under aerobic conditions (tricarboxylic acid cycle) and in liquid, nonaerated systems (Meyerhof-Embden-Parnas system).

LITERATURE CITED

1. Banwart, G. J., and J. C. Ayres. 1957. The effect of pH on the growth of *Salmonella* and functional properties of liquid egg white. *Food Technol.* **11**:244-246.
2. Chung, K. C., and J. M. Goepfert. 1970. Growth of salmonella at low pH. *J. Food Sci.* **35**:326-328.
3. Goepfert, J. M., and R. Hicks. 1969. Effect of volatile fatty acids on *Salmonella typhimurium*. *J. Bacteriol.* **97**:956-958.
4. Halvorson, H. 1973. Formation of lactic acid, volatile fatty acids and neutral, volatile monocarbonyl compounds in Swedish fermented sausage. *J. Food Sci.* **38**:310-312.
5. Levine, A. S., and C. R. Fellers. 1940. Action of acetic acid on food spoilage microorganisms. *J. Bacteriol.* **39**:499-515.
6. Prost, E., and H. Reimann. 1967. Food-borne salmonellosis. *Annu. Rev. Microbiol.* **21**:495-528.
7. Smith, J. L., and S. A. Palumbo. 1973. Microbiology of Lebanon bologna. *Appl. Microbiol.* **26**:489-496.
8. Stokes, J. L., and H. G. Bayne. 1957. Growth rates of *Salmonella* colonies. *J. Bacteriol.* **74**:200-206.
9. Szybalski, W., and V. Bryson. 1952. Genetic studies on microbial cross resistance to toxic agents. I. Cross resistance of *Escherichia coli* to fifteen antibiotics. *J. Bacteriol.* **64**:489-499.