Hydrogen Peroxide Formation by Lactobacilli and Its Effect on *Staphylococcus aureus* [1]

R. S. DAHIYA and M. L. SPECK
Department of Food Science, North Carolina State University, Raleigh

Abstract

Culture filtrates of *Lactobacillus lactis* and *Lactobacillus bulgaricus* were observed to contain material inhibitory against *Staphylococcus aureus*. The concentration of inhibitor increased on storage of the lactobacilli at 5 C, and the maximum was attained after five days. A carbohydrate source was necessary for the formation of this inhibitory activity by resting cell suspensions. The inhibitory factor was identified as hydrogen peroxide. Storage of lactobacilli in dextrose at neutral pH and at 5 C resulted in maximum accumulation of hydrogen peroxide.

Inhibitory activity associated with cultures of lactobacilli has been shown previously by several workers (1, 3, 6, 8, 9, 14, 15, 17). In addition to lactic acid, a heat labile compound(s) has been commonly implicated as an inhibitory product (10, 16). Wheater et al. (18) observed inhibition of *Staphylococcus aureus* by a strain of *Lactobacillus lactis*. They proposed that inhibition of *S. aureus* resulted from the formation of hydrogen peroxide by the *L. lactis*. However, they were unable to show the presence of hydrogen peroxide in the lactobacillus growth medium and indicated that viable lactobacilli were necessary for the inhibition of the staphylococcus. During our studies we also observed that inhibition of *S. aureus* by certain strains of *L. lactis* and *L. bulgaricus* was caused by the formation of H_2O_2 by lactobacilli. However, we were able to show the accumulation of H_2O_2 in the lactobacillus growth medium.

This observation prompted further work to obtain information on the factors influencing the hydrogen peroxide formation by the lactobacilli and to clarify the influence of this compound on the growth of *S. aureus*.

Materials and Methods

Organisms. Single strains of lactobacilli were isolated from yogurt starter cultures obtained from commercial supply houses by plating on lactic agar (2). All isolates were transferred in sterile litmus milk and subcultures were made with 1% inoculum; incubation was at 35 C for approximately 16 hr. For inoculation of lactic broth or trypticase soy broth, cultures were transferred in the same medium for at least three transfers before use.

Strains LA 10-65 and DN-1 were identified as *L. lactis* and strains NYL1 and SYL1 as *L. bulgaricus*, according to the scheme described by Sharpe et al. (11).

Staphylococcus aureus MF31, an enterotoxigenic strain, was transferred daily in trypticase soy broth using 1% inoculum and incubation at 35 C for 15 hr.

Between transfers all cultures were stored in the refrigerator.

Cup plate assay. An agar diffusion method was used to determine inhibition of *S. aureus* by lactobacilli. Fifty milliliters of sterile 3% agar and 50 ml of reconstituted nonfat dry milk (20% total solids) were tempered to 45 C, and mixed. To this mixture 10 ml of 20% calcium carbonate solution was added. Again the contents were mixed and 10 ml aliquots were removed to duplicate plates to be used as controls. The remainder of the agar-milk mixture was inoculated with 1% of the lactobacillus test strain. Volumes of 10 ml were placed in duplicate plates. The open end of a sterile tube (inside diameter 22 mm) was pressed on the solidified milk agar. This was first done on the control plates and then repeated on plates containing the lactobacillus. A sterile spatula was used to lift out and discard the agar from the circle. Then to a tube containing 10 ml of trypticase soy agar (tempered to 45 C), 0.1 ml of 0.4% triphenyltetrazolium chloride and 0.1 ml of actively growing *S. aureus* culture were added. After mixing, a portion of the inoculated agar was used to fill the cup inside the solidified milk agar. The plates were then incubated at 35 C for 24 hr and were observed for inhibition of the staphylococcus inside the cup. The width of the clear band was measured and recorded.

The foregoing method allowed certain advantages in detecting inhibitory strains of the lactobacillus: 1) the inhibitory effect of

Received for publication October 20, 1967.

[1] Paper number 2503 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, North Carolina.

acid produced by the growing culture was eliminated by the carbonate; 2) the lactobacillus could be preincubated to allow accumulation of inhibitor before adding the staphylococcus.

To check the effect of pre-incubation of lactobacillus on the inhibitory zone size, different plates were incubated at 35 C for 2 hr, 4 hr and 6 hr. The cups were then prepared and filled with trypticase soy agar inoculated with the staphylococcus. The plates were incubated for 24 hr after which width of the band of inhibition was recorded.

Measurement of activity of spent medium. Several bottles containing 100 ml of sterilized trypticase soy broth were inoculated using 1% of *L. lactis* LA 10-65 and incubated at 35 C. The cultures were removed at intervals, tempered and stored at 5 C. For assay 22.5 ml from each bottle was adjusted to pH 7.0 and the final volume of each was made to 25 ml. Each sample was sterilized by Seitz filtration. The cell free culture filtrates (1 ml, 2.5 ml and 5 ml) were dispensed in sterile tubes. The volume of each tube was adjusted to 5 ml with sterile water. For control, 5 ml of water was used. Double strength trypticase soy broth was inoculated with 0.05% of *S. aureus*. After mixing, 5 ml of this inoculated broth was transferred to each tube. The tubes were inverted three times and incubated at 35 C for about six hours (Control = approx. 30% transmittance). The per cent light transmission of each tube was determined at 600 mμ.

Preparation of cell suspensions. The lactobacillus culture was grown in trypticase soy broth at 32 C until the medium reached pH 5.5. The culture was cooled and centrifuged at 5000 g for 20 min. The supernatant was discarded and the cells were washed twice with 0.05 M sodium phosphate buffer (pH 7.0). Cells were resuspended in phosphate buffer and distributed equally in sterile solutions of 0.25% dextrose, 0.3% phytone, 1.7% trypticase and 0.05 M phosphate buffer (pH 7.0). The cell suspensions were stored at 5 C. Cell free filtrates of the above suspensions were then tested for the presence of inhibitory material after various periods of storage according to the method described for spent medium.

Measurement of hydrogen peroxide. Hydrogen peroxide accumulated in the cell suspensions was measured by the method of Oram and Reiter (7).

Results

In the preliminary survey various strains of lactobacilli isolated from yogurt starter cultures were tested by the cup plate assay (Fig. 1) for the inhibition of *S. aureus*. The data (Table 1) indicated preincubation of the lactobacillus up to six hours increased inhibition of the staphylococcus. Strain LA 10-65 was the most potent inhibitor producer and was used in further studies.

Similar results were obtained when milk agar base was replaced by lactic agar or trypticase soy broth agar in the above experiment for the cup plate assay. This feature permitted the use of trypticase soy broth for determination of the inhibitory factor produced by strain LA 10-65. The lactobacillus was then grown in trypticase soy broth for various periods of time and the spent broth was tested for inhibition of *S. aureus*. The results (Table 2) indicated that incubation of the lactobacillus culture for 12 hr produced the maximum inhibition. However, when the experiment was repeated using spent broth of a fresh 12 hr culture, very little inhibition was observed. At this point it was realized that in the earlier experiment the cultures were removed from the incubator at different intervals and were stored at 5 C until all samples were tested. Subsequently it was observed that storage of the lactobacillus culture for five days at 5 C resulted in maximum inhibitory activity (Table 3).

Further experiments indicated that suspension of lactobacillus cells in fresh trypticase soy broth or in one of its components produced a similar effect on storage at 5 C (Table 4). The results also indicated that possibly the presence of a carbohydrate source was necessary for formation of this inhibitor, as no activity was observed when cells were suspended in phosphate buffer or in 1% acid hydrolyzed casein.

The inhibitory compound that accumulated in the culture and in the cell suspensions was

TABLE 1. Inhibitory activity of lactobacilli against *Staphylococcus aureus*.

Preincubation of lactobacillus[a]	Lactobacillus strains			
	LA 10-65	DN-1	NYL1	SYL1
(Hr)	(width of inhibited band in mm)[b]			
0	0.7	0.5	0.0	0.0
2	2.5	1.7	1.0	0.5
4	4.0	2.7	3.5	3.0
6	4.7	3.2	4.2	4.0

[a] Incubation at 35 C.
[b] Inhibition of staphylococcus after 24 hr at 35 C.

TABLE 2. Inhibitory activity of spent medium of culture LA 10-65.

Age of LA 10-65[a]	pH of LA 10-65	Growth of staphylococcus[b] in: Spent medium %		
		10	25	50
(Hr)		(% transmittance)		
0	7.0	35	35	35
6	6.0	37	58	70
12	5.5	42	71	98
24	5.0	35	37	50
48	5.0	35	38	44

[a] Culture LA 10-65 grown in trypticase soy broth at 35 C.
[b] Staphylococcus incubated for 6 hr at 35 C.

identified as H_2O_2. Catalase, when incorporated in the assay medium, relieved inhibition by the cell-free filtrates (Table 5). Under the conditions of the assay, this strain of *S. aureus* was observed to be sensitive to the presence of 6 μg/ml of H_2O_2 (Table 6). However, other results presented in Table 6 indicated that this concentration was only bacteriostatic. The bactericidal concentration for this strain was observed to be 20 μg/ml when tested under static conditions and 22 μg/ml when tested under shake conditions. The bactericidal concentration for three other strains of staphylococci tested was 25-35 μg/ml.

The data presented in Table 7 indicate the amount of H_2O_2 accumulated by LA 10-65 cells on storage at 5 C in dextrose + phosphate buffer adjusted to different pH. At all pH values most of the H_2O_2 was formed within 24 hr. However, the final concentration of H_2O_2 on storage for 15 days was highest at pH 7.0. With the decrease or increase in pH from neutrality, the concentration of H_2O_2 also decreased markedly. These results indicate that even in an acid medium the lactobacilli were able to form H_2O_2. Furthermore, the H_2O_2 formation was dependent on temperature of storage of cell suspensions. At higher temperatures, the concentration was much lower than at 5 C (Table 8).

TABLE 3. Effect of storage of LA 10-65 at 5 C on inhibitory activity of spent medium.

Storage of LA 10-65	Growth of staphylococcus in: Spent medium %		
	0	25	50
(Day)	(% transmittance)		
0	30	39	46
1	30	40	52
3	30	49	68
5	30	60	87
7	30	60	85

TABLE 4. Effect of storage of LA 10-65 cells at 5 C in trypticase soy broth and its different components on inhibition of *Staphylococcus aureus*.

Storage of cells[a]	Control	Growth of staphylococcus in: Cell free filtrates[b] of cell suspension from			
		TSB	Dextrose	Phytone	Trypticase
(Day)		(% transmittance)			
1	29	52	52	43	46
3	29	65	60	48	56
7	39	68	84	94	49
14	30	73	90	100	41

[a] Initial viable population 30×10^7/ml.
[b] 25% cell free filtrate added.

Discussion

Certain lactobacilli have been shown to produce inhibitory products against other organisms normally considered as objectionable in foods. Hydrogen peroxide formation seems to be one way in which these bacteria repress the growth of organisms such as *S. aureus*. Our studies did not indicate the presence of any inhibitor other than lactic acid and H_2O_2. The results of others (6, 9) would suggest that different species of lactobacilli may also produce other types of inhibitors against various undesirable microorganisms. In our study it was shown that even at refrigeration and low pH temperature, lactobacilli isolated from yogurt produced H_2O_2. To what extent H_2O_2 plays its role during storage of fermented foods such as cheese is not yet clearly understood. From these studies it would appear to be an important factor in repressing the growth of certain undesirable bacteria if present in the food.

It has been reported that some strains of lactic acid bacteria can oxidize certain substances with a high rate of oxygen consumption (4, 12). However, the pathways of electron transport in the course of these oxidations are not yet clearly understood. Strittmatter (12) observed that heme-linked enzymes were

TABLE 5. Effect of catalase on antistaphylococcus activity of LA 10-65 filtrate.

Filtrate LA 10-65	Control	Catalase[a]
(%)	(% transmittance)	
0	30	22
10	39	23
20	53	22
30	72	22
40	90	22

[a] Catalase—3,000 units.

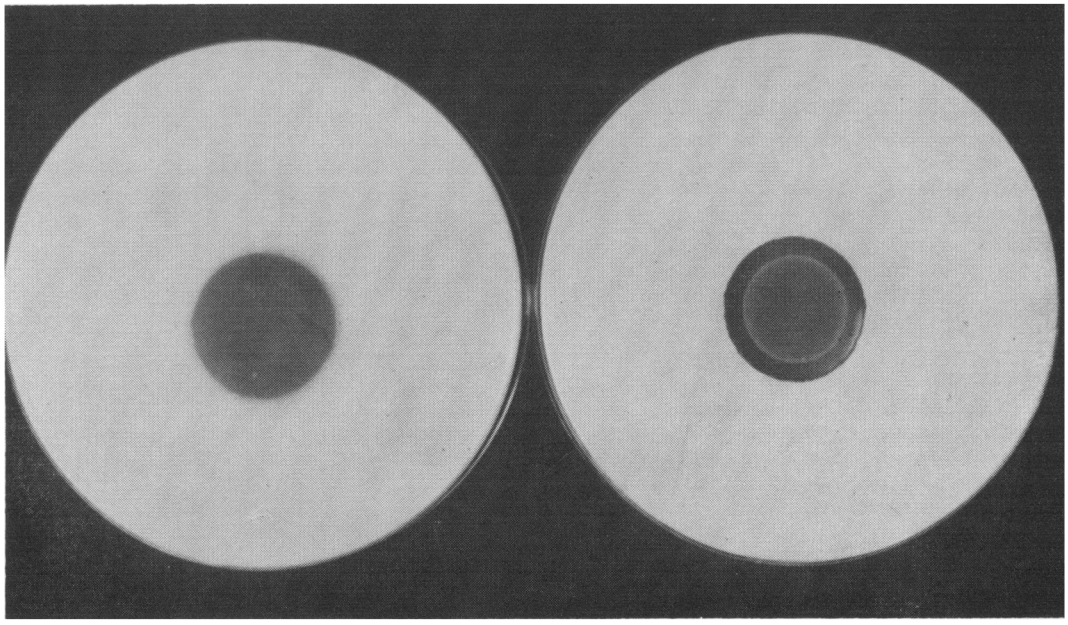

FIG. 1. Left plate containing milk-agar base with the inner cup filled with trypticase soy agar seeded with *S. aureus* (control). Right plate seeded with lactobacillus on the outside of the cup and the inner cup seeded with *S. aureus*. The band of inhibition is clearly visible.

not involved in the high capacities of various lactobacilli for electron transport from a variety of oxidizable substrates to oxygen; however, specific flavin components were present in high concentration in each of these organisms. Accumulation of H_2O_2 is characteristic of flavo-protein respiration and has been observed during some oxidative activities by certain strains (5, 12, 13, 19). A similar system might be responsible for accumulation of H_2O_2 by the lactobacilli used in this study. Preliminary investigations have shown that the cell-free extracts of *L. lactis* LA 10-65 contain an oxidase enzyme system which also inhibits the growth of *S. aureus*. Further studies to characterize this system are in progress.

TABLE 6. Effect of hydrogen peroxide on the growth of *Staphylococcus aureus*.

Concentration of H_2O_2	Growth of staphylococcus[a]			
	Static		Shake	
	6 hr	24 hr	5 hr	24 hr
(μg/ml)	(% transmittance)			
0	30	12	29	4
2	71	12	70	4
4	94	12	95	4
5	98	12	100	4
6	100	12	100	4
10	100	14	100	5
16	100	83	100	16
18	100	85	100	31
20	100	100	100	70
22	100	100	100	100
25	100	100	100	100

[a] Incubation at 35 C.

TABLE 7. The influence of pH on accumulation of hydrogen peroxide by cells of LA 10-65.[a]

Storage at 5 C	pH of cell suspension[b]				
	8.0	7.0	6.0	5.0	4.0
(Day)	(μg H_2O_2/ml)				
1	5.5	7.6	7.3	6.4	6.25
4	6.1	10.2	9.2	7.6	6.6
8	7.1	10.8	10.4	8.4	6.8
12	7.2	12.2	11.0	8.4	7.0
15	7.7	12.5	11.0	8.6	7.0

[a] Initial viable population 12×10^7/ml.
[b] Cells suspended in 0.25% dextrose + 0.15 M phosphate-citrate buffer at pH 4.0 to 7.0. For pH 8.0 phosphate buffer substituted for phosphate-citrate buffer.

Acknowledgment

This study was supported in part by a research grant (No. 67-1-A) from the American Dairy Association.

References

(1) De Klerk, H. C., and J. N. Coetzee. 1961. Antibiosis among lactobacilli. Nature, 192: 340.

TABLE 8. Accumulation of hydrogen peroxide by LA10-65 cells[a] at different temperatures.

Storage time	Storage temperature (C)					
	5	21	26	32	35	37
(Days)	(μg H_2O_2/ml)					
1	6.6	4.0	2.6	2.0	1.8	1.4
2	8.6	6.0	3.2	1.7	0.7	0.4
4	9.5	8.2	3.5	1.8	0.7	0.42
6	9.65	8.7	3.6	1.95	0.73	0.45

[a] Cells suspended in 0.25% dextrose + 0.2 M PO_4 buffer (pH 7.0). Initial viable population 12×10^7/ml.

(2) Elliker, P. R., A. W. Anderson, and G. Hannesson. 1956. An agar culture medium for lactic acid streptococci and lactobacilli. J. Dairy Sci., 39: 1611.

(3) Grosowics, N., D. Kaplan, and S. Schneerson. 1947. Production of an antibiotic substance by a lactobacillus. Proc. IV. Intern. Congr. Microbiol., Copenhagen, 137.

(4) Gunsalus, I. C., and J. M. Sherman. 1943. The fermentation of glycerol by streptococci. J. Bacteriol., 45: 155.

(5) Gunsalus, I. C., and W. W. Umbreit. 1945. The oxidation of glycerol by *Streptococcus faecalis*. J. Bacteriol., 49: 347.

(6) Kodama, R. 1952. Studies on lactic acid bacteria. II. Lactolin, a new antibiotic substance produced by lactic acid bacteria. J. Antibiotics, 5: 72.

(7) Oram, J. D., and B. Reiter. 1966. The inhibition of streptococci by lactoperoxidase, thiocyanate and hydrogen peroxide. The effect of inhibitory system on susceptible and resistant strains of group N streptococci. Biochem. J., 100: 373.

(8) Neri, A. 1961. Bactericidal properties of some strains of lactobacilli. Latte, 35: 518.

(9) Ritter, P. 1956. The antagonism between lactic acid bacteria and bacteria of the coliaerogenes group. II. The development of specific antibiotic substances by lactic acid bacteria. Schweiz. Milchz. 82 (Sci. Suppl. No. 35), 273.

(10) Sabine, D. B. 1963. An antibiotic effect of *Lactobacillus acidophilus*. Nature, 199: 811.

(11) Sharpe, M. E., T. F. Fryer, and D. G. Smith. 1966. Identification of the lactic acid bacteria. In Identification methods for microbiologists (ed. Gibbs, B. M., and F. A. Skinner), London, Academic Press.

(12) Strittmatter, C. F. 1959. Electron transport to oxygen in lactobacilli. J. Biol. Chem., 234: 2789.

(13) Strittmatter, C. F. 1959. Flavin-linked oxidative enzymes of *Lactobacillus casei*. J. Biol. Chem., 234: 2794.

(14) Tramer, J. 1966. Inhibitory effect of *Lactobacillus acidophilus*. Nature, 211: 204.

(15) Vakil, J. R., and K. M. Shahani. 1965. Partial purification of antibacterial activity of *Lactobacillus acidophilus*. Bacteriol. Proc., p. 9.

(16) Vincent, J. G., R. C. Veomett, and R. F. Riley. 1959. Antibacterial activity associated with *Lactobacillus acidophilus*. J. Bacteriol., 78: 477.

(17) Wheater, D. M., A. Hirsch, and A. T. R. Mattick. 1951. "Lactobacillin", an antibiotic from lactobacilli. Nature, 168: 659.

(18) Wheater, D. M., A. Hirsch, and A. T. R. Mattick. 1952. Possible identity of "Lactobacillin" with hydrogen peroxide produced by lactobacilli. Nature, 170: 623

(19) Whittenbury, R. 1964. Hydrogen peroxide formation and catalase activity in the lactic acid bacteria. J. Gen. Microbiol., 35: 13.