SELECTIVITY OF SORBIC ACID MEDIA FOR THE CATALASE NEGATIVE LACTIC ACID BACTERIA AND CLOSTRIDIA

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A wide variety of media have been used to grow lactic acid bacteria (Ayers and Mudge, 1920; Barber and Frazier, 1945; Kulp, 1927; Kulp and Rettger, 1924; Mickle and Breed, 1925; Mobley, 1937*a,b,c,* Norris *et al.*, 1950; Orla-Jensen, 1919, 1943; Pederson, 1929; Pelczar and Vera, 1949; and Weiss and Rettger, 1934, among others). However, in the experience of the authors, these media have, in many instances, failed when they have been used to enrich, enumerate, or isolate lactic acid bacteria found together with yeasts, molds, and other bacteria in various food product samples. These media do not selectively favor the growth of the lactic acid bacteria; consequently, the other microorganisms also present in the sample may outgrow them. Colonies of yeasts and other bacteria frequently may be mistaken for lactic acid bacteria when plate "counts" are made. Therefore, the advantages to be gained from a medium which would favor the growth of the lactic acid bacteria but inhibits all other microorganisms would be considerable.

Attempts to develop such a medium were made. The use of different substrates and combinations of substrates with required nutrients was tried with alteration of physical conditions (pH, atmosphere, and temperature). These exploratory experiments, made over a period of years, were of little value. On investigation of inhibitory compounds added to conventional media it was noted that sorbic acid selectively favored the growth of *Lactobacillus* and *Leuconostoc* strains and inhibited all of the other test cultures of bacteria, molds, and yeasts (Vaughn and Emard, 1951).[1] This observation confirmed, in part at least, earlier observations made by Gooding (1945) and Phillips and Mundt (1950) concerning the inhibitory properties of sorbic acid. It also stimulated further attempts to develop a medium which would be useful for enrichment and presumptive identification of the lactic acid bacteria. The results of these latter investigations are described in the following pages.

EXPERIMENTAL METHODS AND RESULTS

Previous experience had shown liver (bovine, porcine, or ovine) infusion media prepared as described by Vaughn (1942) to be advantageous for growth of the lactic acid bacteria. Consequently, liver infusion was the first basal medium used to test the selective inhibitory effect of sorbic acid. As the investigation progressed other media were used to carry the acid. These are described in the text.

[1] An approach to this ideal was made by Green and Gray (1950, 1951) who used various antibiotics in media to inhibit yeasts. Their media were not specific for the lactic acid bacteria. Recently, however, Rogosa, Mitchell, and Wiseman (1951) have described a medium which apparently does fulfill this requirement.

A total of 229 different cultures including 2 genera of actinomycetes, 15 genera of bacteria, 19 genera of molds, and 7 genera of yeasts was used for the experiments. The names of these genera together with the number of cultures and species of each genus are listed below:

Genera Tested

15 genera of bacteria
(179 cultures representing 73 species)

Acetobacter	6 cultures,	3 species
Aerobacter	2 cultures,	2 species
Aeromonas	2 cultures,	2 species
Bacillus	20 cultures,	7 species
Clostridium	10 cultures,	4 species
Erwinia	1 culture,	1 species
Escherichia	6 cultures,	1 species
Lactobacillus	65 cultures,	11 species
Leuconostoc	6 cultures,	2 species
Propionibacterium	9 cultures,	9 species
Pseudomonas	11 cultures,	10 species
Salmonella	5 cultures,	5 species
Staphylococcus (*Micrococcus*)	7 cultures,	4 species
Streptococcus	27 cultures,	10 species
Xanthomonas	2 cultures,	2 species

19 genera of molds
(36 cultures representing 33 species)

Absidia	1 culture,	1 species
Aspergillus	8 cultures,	7 species
Botrytis	2 cultures,	1 species
Cephalothecium	1 culture,	1 species
Chaetomium	1 culture,	1 species
Cladosporium	1 culture,	1 species
Curvularia	1 culture,	1 species
Fusarium	4 cultures,	4 species
Gliocladium	1 culture,	1 species
Memnoniella	1 culture,	1 species
Mucor	1 culture,	1 species
Myrothecium	1 culture,	1 species
Neurospora	1 culture,	1 species
Paecilomyces	1 culture,	1 species
Penicillium	7 cultures,	6 species
Rhizopus	2 cultures,	1 species
Scopulariopsis	1 culture,	1 species
Thielavia	1 culture,	1 species
Tricoderma	1 culture,	1 species

7 genera of yeasts
(10 cultures representing 10 species)

Brettanomyces	1 culture,	1 species
Candida	2 cultures,	2 species
Debaryomyces	2 cultures,	2 species
Hansenula	1 culture,	1 species
Pichia	1 culture,	1 species
Saccharomyces	2 cultures,	2 species
Torulaspora	1 culture,	1 species

2 genera of actinomycetes
(3 cultures representing 3 species)

Nocardia	1 culture,	1 species
Streptomyces	2 cultures,	2 species

The cultures which showed a marked degree of resistance to sorbic acid will be identified as to species.

The effect of concentration of sorbic acid. Sorbic acid (0.1 per cent) in liver infusion agar was observed to exert a marked inhibitory effect on most of the catalase-positive cultures. However, certain strains of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) grew as well as the catalase negative cultures of *Lactobacillus* which were tested. There was some indication that the pH of the medium might affect the selective inhibitory power of sorbic acid. The basal liver infusion medium was modified by removal of the K_2HPO_4 and retested with 0.1 per cent sorbic acid. The cultures of *S. aureus* still grew, but not as luxuriantly as before, so the effect of the concentration of sorbic acid was tested on the same cultures. Both liver infusion broth and agar (without K_2HPO_4 in either) were

prepared with concentrations of 0.08, 0.10, and 0.12 per cent sorbic acid, respectively. The cultures tested included 4 strains of *Lactobacillus plantarum*, 3 strains of *Clostridium butyricum*, 3 strains of *S. aureus*, 1 strain of *Escherichia coli*, and 1 strain of *Torulaspora rosei*. The two media were inoculated with young cultures of these microorganisms and incubated at 34 C. Observations on growth were recorded after 2, 3, and 5 days. The results are shown in table 1. The sorbic acid was less inhibitory when contained in the liver broth, but with either

TABLE 1

Effect of concentration of sorbic acid on growth of various cultures in liver media

MEDIUM	LIVER BROTH			LIVER AGAR SLANTS		
Days' incubation 34 C	2	3	5	2	3	5
Test cultures	Number growing					
0.08 per cent sorbic acid						
L. plantarum (4 strains)	4	4	4	4	4	4
C. butyricum (3 strains)	3	3	3	—*	—	—
S. aureus (3 strains)	3	3	3	2	2	2
E. coli (1 strain)	1	1	1	0	1	1
T. rosei (1 strain)	1	1	1	0	0	1
0.10 per cent sorbic acid						
L. plantarum (4 strains)	4	4	4	4	4	4
C. butyricum (3 strains)	3	3	3	—*	—	—
S. aureus (3 strains)	2	3	3	0	2	3
E. coli (1 strain)	0	1	1	0	0	0
T. rosei (1 strain)	0	1	1	0	0	0
0.12 per cent sorbic acid						
L. plantarum (4 strains)	4	4	4	4	4	4
C. butyricum (3 strains)	3	3	3	—*	—	—
S. aureus (3 strains)	0	0	0	0	0	0
E. coli (1 strain)	0	0	0	0	0	0
T. rosei (1 strain)	0	0	0	0	0	0

* Not tested as slants were aerobic.

medium, a concentration of 0.12 per cent of the acid was required to inhibit the catalase positive cultures.

The same trend in the selectivity of sorbic acid was noted when a glucose, yeast extract medium consisting of 10 g of glucose and 4 g of Difco yeast extract per liter of water was substituted for the liver infusion. However, as seen in table 2, the concentration of acid required for the suppression of the resistant strains was only 0.07 per cent in the glucose media as compared with 0.12 per cent in the liver infusion media. As observed with the liver infusion media the sorbic acid was less inhibitory when contained in the liquid glucose medium.

The inhibition of catalase positive microorganisms by sorbic acid. It was noted that the catalase positive organisms were more susceptible to the effects of sorbic acid than the catalase negative lactobacilli and clostridia used in the previous experiments. A further test of this selective inhibition was required to determine whether all of the other cultures of lactic acid bacteria and clostridia would be

TABLE 2

Effect of concentration of sorbic acid on growth of various cultures in glucose, yeast extract media

MEDIUM	GLUCOSE BROTH			GLUCOSE AGAR		
Days' incubation 34 C	2	3	5	2	3	5
Test cultures	Number growing					
0.03 per cent sorbic acid						
L. plantarum (3 strains)	3	3	3	3	3	3
C. butyricum (3 strains)	3	3	3	—*	—	—
S. aureus (3 strains)	3	3	3	3	3	3
P. zeae (1 strain)	0	1	1	0	1	1
E. coli (1 strain)	1	1	1	1	1	1
T. rosei (1 strain)	1	1	1	1	1	1
0.05 per cent sorbic acid						
L. plantarum (3 strains)	3	3	3	3	3	3
C. butyricum (3 strains)	3	3	3	—	—	—
S. aureus (3 strains)	3	3	3	0	0	0
P. zeae (1 strain)	0	0	0	0	0	0
E. coli (1 strain)	1	1	1	0	0	0
T. rosei (1 strain)	0	0	0	0	0	0
0.07 per cent sorbic acid						
L. plantarum (3 strains)	3	3	3	3	3	3
C. butyricum (3 strains)	3	3	3	—	—	—
S. aureus (3 strains)	0	0	0	0	0	0
P. zeae (1 strain)	0	0	0	0	0	0
E. coli (1 strain)	0	0	0	0	0	0
T. rosei (1 strain)	0	0	0	0	0	0

* Not tested as slants were aerobic.

as tolerant and whether all of the other catalase positive microorganisms would be as susceptible.

In this experiment the 229 cultures listed previously were grown in the liver infusion and glucose, yeast extract media which contained 0.12 and 0.07 per cent sorbic acid, respectively. Inoculations were made from young cultures of the test organisms with a 2 mm loop. The inoculated media were incubated at 34 C and observed for growth at 2, 3, 5, and 7 day intervals.

The results of this experiment clearly demonstrated the selective inhibitory effect of the sorbic acid, regardless of the medium in which it was carried. All of the catalase positive cultures were inhibited to such a degree that growth was not detected in the test media in 7 days. On the other hand, most of the catalase negative cultures grew without noticeable inhibition. However, as shown in table 3, some of the cultures of *Streptococcus* were inhibited; the inhibition being most striking in the sorbic acid-liver agar. Nevertheless, all of the test cultures of

TABLE 3

Growth of streptococci in sorbic acid liver infusion media

MEDIUM	LIVER INFUSION WITH 0.12 PER CENT SORBIC ACID		
	Liquid	Semi-solid*	Solid†
Test cultures§	Number growing in 5 days at 34 C		
S. bovis (1 strain)	0	0	0
S. durans (1 strain)	1	1	0
S. faecalis (3 strains)	2	3	2
S. lactis (1 strain)	1	1	1
S. liquefaciens (2 strains)	2	2	0
S. mastitidis (1 strain)	1	1	0
S. pyogenes (1 strain)	0	1	0
S. salivarius (2 strains)	1	2	1
S. thermophilus (3 strains)	0‡	0	0
S. zymogenes (3 strains)	2	2	0
Unidentified (9 strains)	9	9	0

* With 0.5 per cent agar.

† With 2.4 per cent agar.

‡ *S. thermophilus* cultures did not grow in the control medium at 34 C.

§ The cultures are named as received.

Lactobacillus, *Leuconostoc*, and *Clostridium* grew well. These cultures included the following species:

Lactobacillus acidophilus	(2 strains)	*Lactobacillus buchneri*	(5 strains)
Lactobacillus bifidus	(4 strains)*	*Lactobacillus fermenti*	(5 strains)
Lactobacillus bulgaricus	(2 strains)	*Lactobacillus hilgardii*	(2 strains)
Lactobacillus casei	(2 strains)	*Leuconostoc mesenteroides*	(6 strains)
Lactobacillus delbrueckii	(1 strain)	*Clostridium butyricum*	(7 strains)*
Lactobacillus leichmannii	(2 strains)	*Clostridium beijerinckii*	(2 strains)*
Lactobacillus plantarum	(25 strains)	*Clostridium pasteurianum*	(1 strain)*
Lactobacillus brevis	(15 strains)		

* Tested under anaerobic conditions.

The possible value of sorbic acid for use in media for enrichment of lactic acid bacteria and sporeforming anaerobes without interference from catalase positive microorganisms is obvious.

The effect of pH on the inhibitory power of sorbic acid. As already stressed, the presence of K_2HPO_4 limited the selectivity of the two sorbic acid-liver infusion

media. This was evidence that the pH of these media influenced the inhibitory power of sorbic acid.

To determine the effect of pH on the selectivity of sorbic acid, portions of the sorbic acid-glucose, yeast extract broth were adjusted to various pH values with McIlvaine's buffer. These portions were sterilized and inoculated with cultures of *L. plantarum*, *S. aureus*, *Propionibacterium pentosaceum* and *Debaryomyces membranaefaciens*, respectively. The cultures then were grown for 48 hours at 34 C. After incubation the relative amounts of growth in the various portions were determined by measuring the turbidity of each with a Klett-Summerson colorimeter. After correction the readings were plotted against the pH values (after sterilization) in a bar graph shown in figure 1.

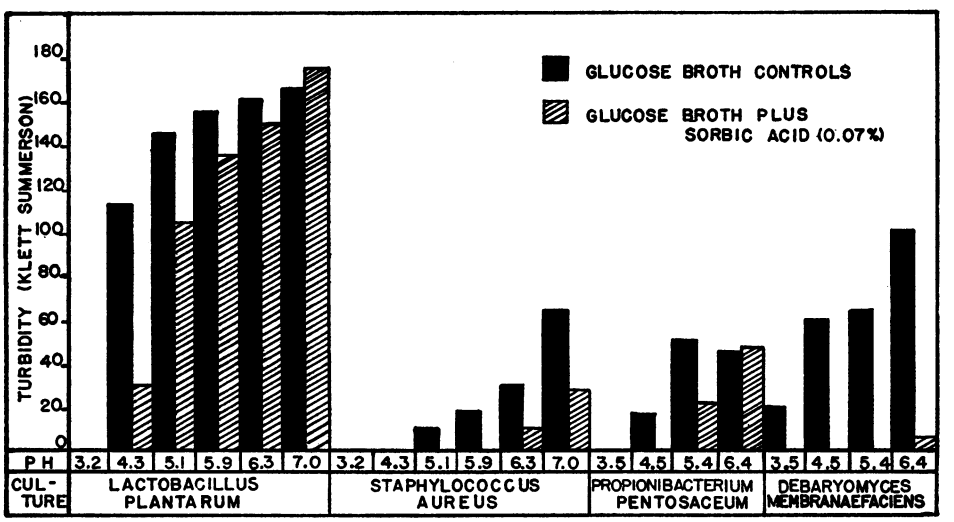

Figure 1. The effect of pH on the inhibitory power of sorbic acid

The pH of the medium did influence the selectivity of the sorbic acid. It is evident too that all of the test cultures were inhibited but not to the same extent. Even the culture of *L. plantarum* was inhibited 27.5 per cent in the sorbic acid medium at pH 5.1. Additional investigation has shown the inhibition of strains of *Lactobacillus* by sorbic acid to vary widely. Some were inhibited as much as 35 per cent; others showed no significant retardation when grown in either the glucose or liver infusion media with sorbic acid. Despite the demonstrated inhibition of the lactic acid bacteria it is to be stressed that under the same conditions none of the catalase positive cultures grew significantly. Therefore, it is felt that the reaction of the sorbic acid media should be adjusted so that after sterilization the pH is between 5.0 and 5.5.

Selectivity in mixed cultures. Further proof of the value of the sorbic acid media was obtained by investigation of their selectivity for isolation of lactic acid bacteria from mixed cultures. In the first experiments a loopful of a suspension of from 5 to 15 different catalase positive cultures, representing different genera

mixed with a culture of the desired lactic acid bacterium was streaked on glucose agar with 0.7 per cent sorbic acid and liver infusion with 0.12 per cent sorbic acid and corresponding control media without the acid. After as little as 48 hours at 30 C, lactic acid bacteria were observed to be growing without discernible contamination on the sorbic acid media whereas the populations of the control media were badly mixed. Similar results were obtained when the experiments were repeated with liquid media.

The value of the sorbic acid media was demonstrated in a limited way by isolation of lactobacilli (gram positive, nonsporeforming, nonmotile, catalase negative rods) from a few samples of olive brines, feces, and oral swabs without interference from catalase positive contaminants. These experiments are continuing.

DISCUSSION

It has been demonstrated that the selective inhibitory properties of sorbic acid can be used to advantage for the isolation of lactic acid bacteria, particularly those belonging to the genus *Lactobacillus*. It also is to be emphasized that the experiments reported here do not preclude the possibility that sorbic acid in the same or in some other medium would not be as useful for the isolation of the catalase negative cocci (*Leuconostoc* and *Streptococcus*). The limited number of cultures of these latter genera used for the tests have demonstrated the possibilities.

On the basis of the results obtained with 10 cultures of the genus *Clostridium*, it is believed that sorbic acid may be useful for selective enrichment of these catalase negative, sporeforming bacteria.

It is hoped that the observations reported here will stimulate other investigations. There is a need for selective media which will permit rapid enrichment and presumptive identification of these catalase negative bacteria. Neither of the two basal media used in this study fulfills all of the requirements of an ideal selective medium. The liver infusion, although very productive, is laborious to prepare. The glucose, yeast extract is not productive enough. Experiments with various dehydrated media indicate that these commercial preparations may be substituted for the freshly prepared liver media. The requirements are that the concentration of sorbic acid most effective at a pH of 5.0 to 5.5 be determined and that the resultant medium be productive.

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SUMMARY

The use of sorbic acid in media to selectively favor the growth of the catalase negative lactic acid bacteria has been described. The effectiveness of sorbic acid was found to be dependent upon the concentration, the type of basal medium, and the pH of that medium.

It was found that 0.12 per cent sorbic acid contained in liver broth or agar permitted the growth of lactic acid bacteria and clostridia but inhibited the catalase positive actinomycetes, bacteria, molds, and yeasts when the initial pH values of the media were in the range 5.0 to 5.5 and the media contained no phosphate salts. The use of sorbic acid in liver infusion or some equally efficient basal medium is suggested as a means for enrichment and presumptive isolation of the catalase negative bacteria, particularly species of *Lactobacillus*.

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