THE EFFECT OF LACTIC ACID BACTERIA ON THE ACETONE-BUTYL ALCOHOL FERMENTATION[1]

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In the manufacture of solvents by the fermentation process, Thaysen (1921) and Speakman and Phillips (1924) have shown that lactic acid bacteria often cause serious losses. The antagonism between *Granulobacter pectinovorum*[2] and the lactic acid bacteria is usually so marked that within twelve to eighteen hours after inoculation the growth of the acetone-butyl alcohol organism is suppressed.

In previous papers (Fred et al., 1925, and Stiles et al., 1925) the occurrence and the fermentation characteristics of some of these harmful lactic acid bacteria from corn mash have been discussed.

According to their effect on the acetone-butyl alcohol fermentation, these lactic acid bacteria may be divided into three groups: first, long rod forms usually granulated and very injurious to the butyl alcohol organism, second, long and short rod forms which may or may not be granulated, but never with large distinct granules as seen in group one, and which are also harmful to the butyl alcohol organism, but not so injurious as the preceding group; third, rod forms often very small, almost cocci, which are harmless or only slightly injurious to the growth of the butyl alcohol organism. These lactic acid organisms may be divided in other ways according to their fermentation products. For example, nos. 2, 3, 4, 7, 15, 19, 20 and 21, in table 1, produce

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[2] For convenience the term Granulobacter or Granulobacter pectinovorum is used throughout this paper in place of Bacillus granulobacter pectinovorum.

large amounts of lactic acid and very little CO_2 in the breaking down of sugars, while nos. 5, 6, 8, 9, 10, 11, 12, 13, 14, 16, 17 and 18 produce small amounts of acid, mannitol from fructose, ethyl alcohol from aldo-hexoses, and considerable amounts of CO_2 from these sugars.

The present communication deals with the harmful effect of the lactic acid bacteria on the butyl alcohol organism, and also with some of the factors that favor the growth of these lactic acid bacteria in mashes. Two phases of the subject have received special study; the persistence of the various lactic acid bacteria in mashes, and the nature of the agent injurious to the butyl alcohol-producing organism.

EXPERIMENTAL

The cultures of the lactic acid bacteria used in this study were isolated from mash of contaminated fermenters of the acetone-butyl alcohol fermentation. From more than two hundred cultures obtained from mash, fifteen of the more distinctive strains were selected for special study. In addition to the organisms from corn mash, five cultures of lactic acid bacteria isolated from other sources were used. Through the kindness of the Curator of the Lister Institute, London, transfers of the original culture of *Bacterium volutans*, Fleming and Thaysen, were supplied. Although this culture had been kept in the laboratory for several years, it had not lost its ability to injure *Granulobacter pectinovorum*. This culture from the Lister Institute will be discussed under the name, *Bacterium volutans*.

Some of the cultures have been under study for almost three years, and during this time have never shown a permanent loss in toxicity.[3] Occasionally the lactic acid bacteria develop in mash without producing any harmful effect on the growth of *Granulobacter pectinovorum*. Subsequent transfers from this same culture, however, have always shown that this apparent loss of toxicity is only temporary. The so-called non-toxic

[3] The term toxicity as used in this paper relates to the injurious effect of lactic acid bacteria on the butyl-alcohol organism and not to any specific substance elaborated by the former.

strains of the granulated lactic acid organism have not been encountered, although variations in degree of toxicity are not uncommon. If transfers are not made at frequent intervals, many of these high acid-forming bacteria are soon lost. Attempts to carry these cultures on such media as peptone agar or beef peptone agar, with or without sugar, proved unsatisfactory. The best results were obtained from cultures grown on potassium phosphate glucose yeast-water agar.

Effect of lactic acid bacteria on the growth of Granulobacter pectinovorum in corn mash

The inhibiting effect of the lactic acid bacteria on the growth of *Granulobacter pectinovorum* is quickly and easily determined from corn mash cultures inoculated with these two organisms. In a corn meal mash, the growth of *Granulobacter pectinovorum* may easily be detected by its amylolytic activity, as indicated by the decrease in viscosity, and opaqueness of the medium. In addition to these changes, the growth of the butyl alcohol organism results in a vigorous gas production, and also a compact head consisting of protein, fibrous material and slime. This head is forced to the top of the liquid in the early stages of the fermentation, and remains as a thick compact mass. The lactic acid bacteria on the other hand produce very little gas and no head. In the presence of certain strains of lactic acid bacteria, the characteristic gas and head of the butyl alcohol fermentation are lacking, and most of the mash remains as an unfermented residue in the bottom of the flask.

To measure the antagonism between the lactic acid and butyl alcohol bacteria, small Erlenmeyer flasks containing 100 cc. of 8 per cent corn mash were inoculated with 1 cc. of a twenty-four-hour old culture of *Granulobacter pectinovorum* and an equal quantity of a lactic acid culture. The gas and head formation were noted and at the end of three days the titratable acid and pH value determined. The results are shown in table 1. From the figures of this table it is clear that many of the lactic acid bacteria inhibit markedly the growth of the butyl alcohol organism, while others exert very little effect. Those forms commonly found in

water infusions of cereals, such as *L. leichmanni*, *L. mannitopoeum*, *Bact. volutans*, and *L. gracile* are especially harmful to the growth of *Granulobacter pectinovorum*. An exception to this is noted with

TABLE 1

Showing the effect of lactic acid bacteria on the growth of Granulobacter pectinovorum and on the acid produced in corn mash

Eight per cent corn mash

NUMBER	CULTURE NAME OR NUMBER	GROWTH OF GRANULOBACTER IN THE PRESENCE OF VARIOUS STRAINS OF LACTIC ACID BACTERIA		REACTION AFTER THREE DAYS	
		Growth	Head	0.1 N acid in 10 cc. of culture	H ion
				cc.	*pH*
1	Control*	Good	Good	3.5	5.4
2	*L. leichmanni*	Poor	None	12.2	3.6
3	*L. leichmanni*	Poor	None	11.9	3.6
4	*L. leichmanni*	Poor	None	11.3	3.7
5	*L. mannitopoeum*	Poor	None	10.9	3.7
6	*L. mannitopoeum*	Poor	None	10.0	3.8
7	*Bact. volutans*†	Poor	None	10.0	3.7
8	*L. mannitopoeum*	Poor	None	9.7	3.7
9	*L. mannitopoeum*	Poor	None	9.0	3.8
10	*L. gracile*	Poor	None	9.0	3.8
11	*L. gracile*	Poor	None	8.8	3.8
12	*L. intermedium*	Fair	Fair	7.9	3.8
13	*L. intermedium*	Fair	Fair	5.6	3.9
14	Culture 19	Fair	Good	4.7	5.0
15	Culture 60	Good	Good	4.7	5.2
16	Culture 7	Good	Good	4.5	5.2
17	Culture 4	Good	Good	3.8	5.2
18	*L. pentoaceticus*	Good	Good	3.8	5.2
19	*Streptococcus lactis*	Good	Good	3.7	5.5
20	*L. bulgaricus*	Good	Good	3.5	5.4
21	*L. acidophilus*	Good	Good	3.4	5.4

* *Granulobacter pectinovorum* alone.

† *Bacterium volutans* probably identical with *L. leichmanni*.

L. pentoaceticus and cultures nos. 4, 7 and 19. Culture no. 60, a streptococcus, has no effect on the growth of *Granulobacter pectinovorum*. The results suggest that the inhibition of the solvent-forming organism is related to the amount of acid produced.

Relation between the size of inoculum and the amount of acid formed

Five hundred cc. portions of corn mash in Erlenmeyer flasks were inoculated with an active *Granulobacter pectinovorum* culture and also with varying amounts of the lactic acid organism. At regular intervals samples were drawn from these flasks and the reaction measured.

TABLE 2

Effect of Lactobacillus leichmanni on acid production in corn mash inoculated with Granulobacter pectinovorum

NUMBER	AGE	GRANULOBACTER ALONE		GRANULOBACTER PLUS 1 CC. OF L. LEICHMANNI		GRANULOBACTER PLUS 5 CC. OF L. LEICHMANNI		GRANULOBACTER PLUS 10 CC. OF L. LEICHMANNI	
		Acid*	pH	Acid*	pH	Acid*	pH	Acid*	pH
		cc.		cc.		cc.		cc.	
	After								
1	Beginning	0.2	6.6	0.2	6.6	0.3	6.5	0.3	6.4
2	3 hours	0.4	6.0	0.5	5.6	0.6	5.4	0.8	5.0
3	6 hours	0.8	5.3	1.3	4.8	1.3	4.6	1.5	4.4
4	9 hours	2.0	5.0	2.4	4.8	2.4	4.4	2.3	4.4
5	12 hours	3.5	4.8	4.1	4.4	3.9	4.0	3.1	4.0
6	15 hours	3.9	4.6	4.8	4.4	4.1	4.0	4.2	4.0
7	18 hours	4.2	4.4	5.1	4.2	4.5	3.8	4.4	3.8
8	21 hours	2.9	4.2	7.6	3.7	4.7	3.6	4.9	3.6
9	24 hours	2.5	4.6	8.4	3.6	5.0	3.6	5.0	3.6
10	27 hours	2.2	4.8	9.0	3.6	5.2	3.6	5.1	3.6
11	30 hours	2.0	4.8	9.3	3.4	5.8	3.6	5.4	3.6
12	3 days	3.2		11.4		10.0		9.8	
13	7 days	4.1		13.0		10.8		10.4	
14	14 days					11.7		11.4	
15	30 days	4.4		13.5		11.7		11.4	

* 0.1 N acid in 10 cc. of culture.

The formation of titratable acid and change in hydrogen ion concentration during the fermentation are given in table 2. Within three hours after inoculation, the flasks which received lactic acid bacteria showed a great increase in the hydrogen ion concentration. This gain in active acidity was especially noticeable in the cultures which received the largest amounts of lactic acid culture. By the end of six hours this rapid increase in the dissociated acid reached a pH of 4.8 or below while the

control containing *Granulobacter pectinovorum* alone did not exceed pH 5.3. The low buffer content of corn mash and consequent high dissociation of acids in all probability soon renders the mash unsuitable for the growth of the solvent-forming organism. The highest titratable acid occurred where small rather than large inocula of the lactic acid bacteria were used. High production of titratable acid is the result of a fine balance between the growth of the acetone-butyl alcohol organism and the lactic acid bacteria in the early stages of fermentation. If one or the other

TABLE 3

Relation between age of lactic acid cultures and their inhibiting effect on Granulobacter pectinovorum

In 5 per cent corn mash

NUMBER	ORIGINAL CULTURE	KIND OF GRANULOBACTER FERMENTATION				
		Age in days of the original culture added to corn mash				
		2	7	14	21	42
1	*Granulobacter* plus *L. leichmanni*	None	Poor	Poor	Fair	Good
2	*Granulobacter* plus *L. leichmanni*	None	Poor	Fair	Fair	Good
3	*Granulobacter* plus *L. intermedium*	Poor	Fair	Fair	Fair	Good
4	*Granulobacter* plus *L. intermedium*	Poor	Fair	Fair	Fair	Good
5	*Granulobacter* plus *L. mannitopoeum*	Poor	None	None	None	None
6	*Granulobacter* plus *L. mannitopoeum*	Poor	None	None	None	None

completely dominates the fermentation, then it is impossible to secure a high acid production. The maximum acid production was obtained with a 1 per cent inoculum of *Granulobacter pectinovorum* and a 0.2 per cent of *L. leichmanni*. In other words, to secure a high total acidity of the mash, the combined action of the two organisms is required. These data will be discussed in connection with another experiment.

Although the total acidity was measured after three, seven, fourteen and thirty days no marked gain in acid was found after the third day. The two cultures which received the larger

amounts of *L. leichmanni* did show a decided increase in acid after the thirtieth hour.

Attempts to favor the growth of one of these groups of organisms, and thus overcome the other, by incubating at various temperatures was tested repeatedly. Without exception, the lactic acid organisms soon dominated the fermentation.

The persistence of lactic acid bacteria in corn mash

Laboratory studies have shown that in the usual culture media many of the lactic acid bacteria, especially the high acid formers, exist for only a short time. Whether or not this same condition obtains in such natural substrates as corn mash is not known. It seems probable that these organisms will persist for a much longer time in cereal mashes. To secure an answer to this question a large number of tests with various strains of the lactic acid bacteria, and with various media, have been carried out. Only a summary of the more important points will be presented.

Flasks containing 300 cc. of a 5 per cent corn mash were inoculated with different strains of the lactic acid bacteria, *Lactobacillus leichmanni*, *Lactobacillus intermedium*, and *Lactobacillus mannitopoeum*. Twelve hours later, 30 cc. of the acetone-butyl alcohol organism were added to each flask. *L. leichmanni* is typical of the high acid, non-mannitol-forming, rod-shaped, granulated lactic acid bacteria possessing decided toxic properties; the other two organisms are small rods, representative of the low acid, mannitol-forming bacteria, and are not so toxic to *Granulobacter pectinovorum*. At first all of the cultures showed gas production, especially the flasks inoculated with *L. intermedium* and *L. mannitopoeum*. At the end of twenty-four hours the flasks which received *L. leichmanni* ceased to show gas, and a large deposit of unfermented starch remained. The other cultures gave a better fermentation, and only a small amount of unfermented starch remained.

To test for the presence and the toxicity of these lactic acid bacteria, samples were drawn from the original stock flasks and seeded into tubes of sterilized corn mash, as follows: 1 cc. portions of these mixed cultures were added to duplicate tubes of

20 cc. each of corn mash, and twelve hours later 1 cc. of an active culture of the acetone-butyl alcohol organism was added.

When forty-eight hours old, enrichment cultures were made (that is, transfers to fresh tubes of mash), and an active culture of *Granulobacter pectinovorum* was added. The results of these enrichment cultures, which were carried out simply as confirmatory tests, to eliminate the possibility that the change in reaction due to the inoculum might be the cause of the injury to the butyl alcohol organism, are not recorded in the tables.

The results obtained from the associated growth of the two kinds of organisms in fresh mash are given in table 3. The horizontal columns on the right of this table ("kind of granulobacter fermentation") show the toxic effect of the lactic acid bacteria on the butyl alcohol fermentation. The terms used in this table, for example "none," mean that transfers from the original culture into fresh mash and then seeded with fresh cultures of Granulobacter failed to give any growth. For each test after two, seven, fourteen, twenty-one and forty-two days old, fresh vigorous cultures of the acetone-butyl alcohol organism were added.

The results show definitely that in the mashes of this experiment the toxicity of *L. leichmanni* decreases with an increase in age until, after twenty-one to forty-two days, no injury is noted. The *L. intermedium* culture failed to show any decided change in toxicity after the second day. Apparently this member of the lactic acid family is not very harmful to the growth of *Granulobacter pectinovorum.*

In relation to time, *L. mannitopoeum* behaves in a manner entirely different from the high acid former *L. leichmanni;* the toxicity increased rather than decreased in the older cultures. The formation of neutral substances, alcohol instead of lactic acid, probably accounts for the longevity of this culture in mash.

Additional evidence on the longevity of the lactic acid bacteria is given in table 4. Here the procedure differed from that given in the foregoing experiment. The amount of active culture of *Granulobacter pectinovorum* was reduced from 10 per cent to 2.0 per cent, and also the amount of the lactic acid inoculum was

greatly reduced. Instead of allowing a period of incubation, both cultures were added at the same time. This change in method of inoculation resulted in quite different fermentations. The butyl alcohol organism showed a most active fermentation for more than forty-eight hours, and but little starch was left undigested.

TABLE 4

Relation between age of and different treatments of lactic acid cultures and their inhibiting effect on Granulobacter pectinovorum

In 5 per cent corn mash

NUMBER	KIND AND AMOUNT OF INOCULUM		ORIGINAL FERMENTATION (HEAD)	REACTION AFTER 1 MONTH (0.1 N ACID IN 10 CC. OF CULTURE)		KIND OF GRANULOBACTER FERMENTATION		
						Age in days of the original lactic acid cultures added to corn mash		
	Organism	Per cent		cc.	pH	60	160	365
1	*Granulobacter* alone	2.00	Good	4.5	4.1	Good	Good	Good
2	*L. leichmanni* alone	0.02	None	1.3	4.4	None	None	None
3	*Granulobacter* plus *L. leichmanni*	2.00 0.02	Good	4.4	3.9	None	None	None
4	*L. intermedium* alone	0.02	None	0.8	4.4	Poor	Fair	Fair
5	*Granulobacter* plus *L. intermedium*	2.00 0.02	Good	3.8	4.2	Poor	Fair	Fair
6	*L. mannitopoeum* alone	0.02	None	0.6	4.5	None	Poor	None
7	*Granulobacter* plus *L. mannitopoeum*	2.00 0.02	Good	8.0	3.7	None	Poor	Poor

As shown by Peterson, Fred and Domogalla (1924), the *Granulobacter pectinovorum* fermentation increases the buffer content of the medium. This gain in buffer, which is caused by the increase in soluble nitrogenous compounds, and the production of acids of low dissociation, coupled with the decrease in fermentable sugar, should bring about conditions favorable to the persistence of the lactic acid bacteria. The results shown in Tables

3 and 4 support this statement. The original *Granulobacter pectinovorum* fermentations recorded in table 3 were stopped long before the carbohydrate was consumed, while in table 4 these fermentations continued until almost all of the carbohydrate was destroyed. This difference in the original *Granulobacter pectinovorum* fermentation no doubt accounts for the long persistence of the lactic acid bacteria in the mashes of table 4.

TABLE 5

The production of lactic acid in mixed cultures of Granulobacter pectinovorum and L. leichmanni

Calculated for 1 liter of mash

NUMBER	KIND AND AMOUNT OF INOCULUM		0.1 N ACID IN 10 CC.	SOLVENTS	ZINC LACTATE	
	Organism	Per cent			Weight	Water of crystallization*
			cc.	*grams*	*grams*	*per cent*
1	*Granulobacter* alone	1.0	3.3	12.7	0.0	
2	*Granulobacter* plus *L. leichmanni*	1.0 0.05	4.0	11.2	1.53	16.7
3	*Granulobacter* plus *L. leichmanni*	1.0 0.25	4.6	10.5	2.25	15.1
4	*Granulobacter* plus *L. leichmanni*	1.0 0.5	7.1	0.2	4.84	15.5
5	Pancreatin and *L. leichmanni*	1.0	6.3	0.0	2.97	13.0

* Theory for inactive lactic acid, 18.2 per cent; for the active form, 12.9 per cent.

The presence of the lactic acid bacteria in the old mash was traced by microscopic examinations. When the cultures were two hundred and three hundred sixty-five days old, isolation plates were poured and the organisms secured in this way compared with the original stock lactic acid cultures. No differences in fermentation reactions or toxicity between the old and the new subcultures were noted.

An experiment similar to the foregoing was carried out with the

stock cultures kept at 37°C. instead of room temperature, 20 to 22°C. Since it was assumed that the organisms would probably die much quicker at the higher temperature, samples were drawn every five days and tested for the presence of harmful organisms. The lactic acid bacteria were alive regardless of the medium in which the cultures had been kept, whether mash fermented with Granulobacter or unfermented mash. These tests were discontinued after thirty days.

Solvent and lactic acid production in mixed cultures of Granulobacter pectinovorum and L. leichmanni

In a mixed culture the rôle played by each microörganism can be followed chemically by determining the product most characteristic of each: for Granulobacter, the solvents, acetone, and butyl alcohol; for *L. leichmanni*, lactic acid. A fermentation in which Granulobacter predominates will be high in solvents and low in lactic acid, and the reverse will be true where *L. leichmanni* is the dominating factor. It must be borne in mind, however, that the lactic acid organism can not grow to any extent upon the unmodified corn mash, and a suitable medium for its development depends upon the amylolytic and proteolytic action of Granulobacter. By varying the amount of inoculum in a series of flasks, fermentations grading from an essentially Granulobacter to that of a chiefly *L. leichmanni* type may be secured. The data from such a series are given in table 5. In order to make certain that lactic acid was being measured, the zinc salt was prepared and its water of crystallization was determined. The data show that solvents and lactic acid are inversely proportional to one another. As the quantity of *L. leichmanni* used in the inoculum was increased, solvent production decreased and the amount of zinc lactate increased.

An interesting result of the associated action of these bacteria is the effect on the relative quantity of d and l lactic acid produced. As shown in a previous publication *L. leichmanni* produces in pure culture almost entirely levo-lactic acid. In the case of no. 5, which was a pure culture of *L. leichmanni*, the water of crystallization is 13.0 per cent and is very close to that for the

levo enantiomorph. In nos. 2, 3, and 4, which were mixed cultures of the two bacteria, the water of crystallization shows the production of a larger quantity of dextro-acid by *L. leichmanni* than in no. 5. The effect of association on the forms of lactic acid and other products will be dealt with more fully in a later publication.

The nature of the inhibiting agent

The product of the lactic acid-forming organism, responsible for the injurious effect on the solvent-forming bacteria, has not been definitely identified, although the subject has been studied and much discussed. In view of our knowledge of the harmful influence exerted by acid-producing organisms on their associates, it would seem that we should look first to acidity as the cause of this antibiotic relation.

Speakman (1920) showed that during a normal fermentation of corn mash by *Granulobacter pectinovorum* a typical curve of acidity may be traced, which consisted of three phases. There is observed a rapid rise in the production of acid to a maximum. This is followed by a decided fall to a minimum and finally a slow rise from the minimum. Reilley and others (1920) have shown that the acids accumulated during the first phase of the curve are chiefly acetic and butyric with evidence of a third unknown kind. Schmidt, Peterson and Fred (1924) have since proved leucic acid to be present. Thaysen (1921) pointed out that a "serious infection" greatly modifies the normal acid curve as to time and extent of rise and fall. Such modifications are accompanied by irregularities in the entire fermentation, showing usually a rise of acidity above the maximum. Reilley and his associates state in their publication, that such erratic curves of acidity are associated with large amounts of lactic acid. The bacteria most commonly found to be responsible for the changes here noted belong to the lactic-acid-producing group. In all the earlier discussions at hand concerning this interference with the work of *Granulobacter pectinovorum* wherever reference is made to the influence of contamination, it seems to have been taken for granted that the results were due to the acidity produced by the

foreign organisms. More recently, however, doubt has been expressed as to the validity of this belief. On the basis of work recently done, Speakman and Phillips (1924) contend that the effect can not be due to the acid produced by the lactic acid bacterium, and they adopt the tentative hypothesis that the inhibitory agent is a product of the nitrogen metabolism of the contaminating organism. Scarcity of experimental evidence on this point and lack of agreement as to the part played by the acid produced by the lactic acid bacteria, appeared to warrant a more intensive laboratory study of this problem.

Some factors that influence acid production in corn mash inoculated with the lactic acid bacteria alone

As reported by Speakman and Phillips (1924) all attempts to secure high acid production in corn mash alone inoculated with these contaminating forms of lactic acid bacteria failed. When glucose was used and Sorensen's phosphate added to the medium as a buffer substance the result was entirely different. Under the above condition the lactic acid bacteria form large amounts of titratable acid as shown in table 6. In the absence of *Granulobacter pectinovorum*, but in the presence of a buffer and of glucose, *L. leichmanni* produces a fairly high concentration of acid. The results show that the presence of a fermentable carbohydrate, like glucose, is not sufficient to bring about acid production in mash. It is essential that a buffer be present to take care of the free acidity. A similar result can be accomplished by pre-digesting the corn mash with pancreatin. A sufficient quantity of available carbohydrates and protein is thus produced and the buffer capacity of the medium is increased. In such a medium *L. leichmanni* produced a considerable quantity of lactic acid. From 500 cc.of culture, 1.47 gm. of zinc lactate with 13.04 per cent water of crystallization was obtained. Corn mash media are unsuitable for the growth of *L. leichmanni* in several respects: lack of fermentable sugar, low buffer capacity and possibly insufficient soluble protein and phosphates. If these deficiencies are corrected, no difficulty is experienced in bringing about growth of the organism and acid production.

TABLE 6

The formation of acid in corn mash inoculated with Lactobacillus leichmanni alone

4 per cent corn mash

NUMBER	TREATMENT	TITRATABLE ACID AFTER						
		At beginning	1 day	2 days	3 days	5 days	10 days	15 days
		cc.†	cc.	cc.	cc.	cc.	cc.	cc.
1	Mash alone	0.25	0.50	1.0	1.2	1.2	1.2	1.4
2	Mash plus 0.2 per cent phosphate*	0.70					3.0	3.2
3	Mash plus 2.0 per cent glucose	0.30	1.4	1.7	2.1	2.2	2.2	2.2
4	Mash plus 2.0 per cent glucose 0.2 per cent phosphate	0.75	3.6	5.2	6.2	6.6	6.8	7.4
5	Mash plus 2.0 per cent glucose 0.5 per cent phosphate	1.65	3.6	5.0	6.0	6.6	7.1	7.7
6	Mash plus 2.0 per cent glucose 1.0 per cent phosphate	2.35	3.2	4.8	6.0	7.4	7.5	8.2
7	Mash predigested with Takadiastase	0.40			1.0	1.1	1.7	
8	Mash predigested with pancreatin	0.50			5.9	6.3		

* Sorenson's phosphate $Na_2HPO_4 + 2\ H_2O$ was used.

† 0.1 N acid in 10 cc. of medium.

TABLE 7

The influence of the products produced by L. leichmanni in corn mash on the growth of the butyl-alcohol organism

5 per cent corn mash plus 1 per cent of glucose

NUMBER	AGE OF LACTIC ACID CULTURE	TREATMENT	GROWTH OF GRANULOBACTER IN MASH PREVIOUSLY INOCULATED WITH THE LACTIC ACID BACTERIA			REACTION
			Growth	Gas	Head	
	hours					pH
1	24	Sterilized	Good	Profuse	None	5.8
2	24	Neutralized and sterilized	Good	Profuse	Good	
3	48	Sterilized	Fair	Slight	None	4.7
4	48	Neutralized and sterilized	Good	Profuse	Good	
5	72	Sterilized	None	None	None	4.5
6	72	Neutralized and sterilized	Good	Profuse	Good	

The effect of heat and neutralization of acids on the fermentation of corn mash by Granulobacter pectinovorum

The following experiments were arranged with reference only to the rôle played by acids in the antagonistic effect on Granulobacter. A direct comparison was made of the true acidity produced by the lactic-acid bacteria and of the corresponding acidity produced by additions of pure acids to the culture medium. After sterilization, the lactic acid bacteria were inoculated into the flasks and allowed to grow for various lengths of time. At the end of the period specified, the cultures were heated sufficiently to kill the lactic acid organism. Five flasks of each group were then inoculated with a vigorous culture of *Granulobacter pectinovorum,* and development recorded as shown in the accompanying tables. A sixth flask in each group was reserved for the determination of titratable acidity, and the pH value produced by the bacteria.

The outline of this experiment and the results obtained are shown in table 7. While the lactic acid bacteria alone in corn mash plus glucose do not produce as high a titratable acid as is found in mixed cultures containing *Granulobacter pectinovorum,* the hydrogen-ion concentration is not greatly different. It is well-known that a pH value of 4.8 to 4.6 is unfavorable to *Granulobacter pectinovorum;* and that pure cultures of the lactic acid bacteria will, in glucose mash, produce a value even lower than these figures.

The effect of the acid produced by these lactic acid bacteria in preventing the growth of the *Granulobacter pectinovorum* is clearly shown from the figures of this table. If old mash cultures of the lactic acid bacteria are inoculated with Granulobacter, there is no sign of growth. However, when these same cultures are neutralized, the butyl-alcohol organism develops. This fact has been noted where *Granulobacter pectinovorum* had been dormant for as long as eight days. The mash culture was merely neutralized, and no new inoculation of Granulobacter made.

Additional data concerning the nature of the toxic agent were obtained from cultures which had been seeded with *L. leichmanni*

and *Granulobacter pectinovorum*. Within three days after inoculation these cultures reached a maximum acidity of 11.5 cc. of 0.1 N acid in 10 cc. These cultures were kept for two weeks, but did not show any gain in total acidity beyond that found on the third day. As found by transfers to fresh media, the lactic acid bacteria under the conditions of this test were entirely destroyed before the end of one week. The complete destruction of the lactic acid bacteria in these cultures offered an opportunity to measure the *Granulobacter pectinovorum* fermentation in highly toxic cultures in which the acidity may be neutralized but the cultures not subjected to the heat of sterilization. To carry out this test large flasks of the mash were neutralized, inoculated with a fresh culture of Granulobacter, and a representative sample removed for solvent analysis. The fermentation in this neutralized but unheated mash was not as vigorous as commonly noted in fresh mash. However, by the second day there was a strong gas production and well formed head. The analysis for solvents of this 6 per cent mash is shown below:

	Total solvents in 1000 cc. of culture
	gm.
At the end of second fermentation neutralized and reinoculated....	8.88
At beginning of second fermentation.....	2.11
Gain.....	6.77

According to the conditions of this test, neutralization alone without heat removes the toxic agent from corn mash cultures. The evidence points strongly to acidity as the prime cause in the injury to the butyl-alcohol fermentation.

That the lactic acid radical is not only non-toxic to Granulobacter, but is actually destroyed by the organism, was proved by adding calcium lactate (0.12 to 0.24 per cent) to the corn mash before inoculation, and analyzing the culture at the end of the fermentation. Such cultures, if anything, gave a better head and gas production than the controls. A careful chemical analysis failed to show any lactic acid in the flasks containing the smaller amount of calcium lactate and only a trace in those to which the larger quantities had been added.

Effect of varying amounts of organic and inorganic acids.

In tables 8 and 9 are given the results of this study. The relation between hydrogen-ion concentration and growth of *Granulobacter pectinovorum* is the important point brought out by these

TABLE 8

The influence of varying amounts of organic acids on the growth of Granulobacter pectinovorum

In 5 per cent corn mash

NUMBER	KIND OF ACID	1 N ACID	REACTION	KIND OF GRANULOBACTER FERMENTATION		
				Growth	Gas	Head
		per cent	*pH*			
1	Lactic	0.2	5.7	Good	Profuse	Good
2	Lactic	0.4	5.6	Good	Profuse	Good
3	Lactic	0.6	5.6	Good	Profuse	Good
4	Lactic	0.8	5.1	Good	Profuse	Good
5	Lactic	1.0	4.8	Good	Medium	Fair
6	Lactic	1.2	4.7	Fair	Slight	None
7	Lactic	1.4	4.6	None	None	None
8	Lactic	1.6	4.3	None	None	None
9	Acetic	0.2	5.9	Good	Profuse	Good
10	Acetic	0.6	5.8	Good	Profuse	Good
11	Acetic	1.0	5.4	Good	Profuse	Good
12	Acetic	1.6	5.0	Good	Profuse	Good
13	Acetic	2.2	4.8	Fair	Medium	Good
14	Acetic	2.6	4.7	Fair	Slight	None
15	Acetic	3.0	4.6	Poor	None	None
16	Butyric	0.2	6.0	Good	Profuse	Good
17	Butyric	0.6	5.7	Good	Profuse	Good
18	Butyric	1.0	5.1	Good	Profuse	Good
19	Butyric	1.6	4.9	Good	Profuse	Good
20	Butyric	2.2	4.8	Fair	Medium	Fair
21	Butyric	2.6	4.7	Poor	Slight	None
22	Butyric	3.0	4.6	Poor	Slight	None
23	Butyric	3.2	4.5	None	None	None

tests. It is not the percentage of the acid but the pH value which determines its inhibiting effect on the butyl-alcohol fermentation. The mineral acids are even more effective in preventing growth of *Granulobacter pectinovorum* than the organic acids. This injurious effect varies slightly among the acids

tested. As seen in table 9 *Granulobacter pectinovorum* will grow in slightly lower pH with hydrochloric than with either sulfuric or phosphoric acids, but the difference is not great. Since *Granulobacter pectinovorum* has the power to destroy these organic acids, and thus reduce the hydrogen-ion concentration, it is to be expected that the organism will start at a slightly higher

TABLE 9

The influence of varying amounts of inorganic acids on the growth of Granulobacter pectinovorum

In 5 per cent corn mash

NUMBER	KIND OF ACID	1 N ACID	REACTION	KIND OF GRANULOBACTER FERMENTATION		
				Growth	Gas	Head
		per cent	*pH*			
1	Sulphuric	0.1	6.0	Good	Profuse	Good
2	Sulphuric	0.2	5.8	Good	Profuse	Good
3	Sulphuric	0.4	5.6	Fair	Medium	None
4	Sulphuric	0.6	5.2	None	None	None
5	Sulphuric	0.8	5.0	None	None	None
6	Hydrochloric	0.1	5.8	Good	Profuse	Good
7	Hydrochloric	0.2	5.6	Good	Profuse	Good
8	Hydrochloric	0.4	5.4	Good	Medium	None
9	Hydrochloric	0.6	5.2	Fair	Medium	None
10	Hydrochloric	0.8	5.0	Poor	None	None
11	Hydrochloric	1.0	4.8	None	None	None
12	Phosphoric	0.1	6.0	Good	Profuse	Good
13	Phosphoric	0.2	5.9	Good	Profuse	Good
14	Phosphoric	0.4	5.8	Good	Profuse	Good
15	Phosphoric	0.6	5.7	Good	Medium	Fair
16	Phosphoric	0.8	5.6	Fair	Slight	None
17	Phosphoric	1.0	5.4	Fair	Slight	None

acidity when this is due to organic acids, than when it is caused by unfermentable compounds.

The results here reported have reference only to the pH value necessary to prevent initiation of growth of the butyl-alcohol organism. Nothing has been ascertained as to the pH value which would check growth when once under way. It would no doubt be lower than that required to prevent the beginning of development, as Granulobacter is known to produce a very con-

siderable buffer effect when active in a medium. In mixed cultures, the development appears always to be in favor of the lactic acid-forming organism. In fact some hydrolysis of starch in the corn mash is necessary before the lactic acid organism has a suitable supply of carbohydrate upon which to act. During this initial growth, the Granulobacter would probably form the buffers mentioned.

The non-toxic effect of filtrates of cultures of L. leichmanni in the Granulobacter fermentation

The question has been asked, Is the antagonistic effect of the lactic acid bacteria due to non-acid substances which pass through a filter? To find an answer to the question, a great number of filtration experiments were carried out. Cultures of the lactic acid bacteria of various ages were passed through Berkefeld and Pasteur Chamberland filters of varying porosity. The bacteria were removed in this way and the neutralized filtrate added to corn mash plus *Granulobacter pectinovorum*. All attempts, however, to suppress the growth of the acetone-butyl-alcohol-forming organism with these filtrates failed.

Similar results were secured from experiments with collodion sacks. Here the different cultures were grown in the same medium separated only by the thin membrane. If it is assumed that the bacteria produce inhibiting substances which will diffuse through a membrane, then the harmful effect of the lactic acid cultures should be readily detected by the use of the special culture tube for collodion sacks (Mulvania, 1924). Here again, no injury other than that common to an acid reaction was ever noted.

SUMMARY

In the mash inoculated with *Granulobacter pectinovorum*, the common types of cereal lactic acid bacteria grow rapidly producing large amounts of lactic acid.

The formation of the lactic acid in the mashes is dependent upon the associated action of the two groups—the lactic acid and the butyl-alcohol organisms. The presence of the lactic acid

bacteria alone is not sufficient. The growth of *Granulobacter pectinovorum* favors the development of the lactic acid bacteria in several ways; by hydrolysis of the starch to fermentable carbohydrates, by proteolysis of the nitrogen compounds to amino acids—production of buffer substances. Although this association is beneficial to the lactic acid bacteria, it is extremely harmful to the solvent-forming organism.

These harmful lactic acid bacteria may be divided into various groups. The high acid-forming organisms described under the name *Lactobacillus leichmanni* are the most injurious to the butyl-alcohol fermentation. Next in order of their inhibiting effect, belong the organisms described as mannitol-formers, *Lactobacillus mannitopoeum*, *Lactobacillus gracile*, and *Lactobacillus intermedium*.

The persistence of the lactic acid bacteria in mash for long periods of time depends on the kind of organism and also on the degree of the associated growth with Granulobacter. The high acid-producing bacteria such as *L. leichmanni* persist for only a short time if they have had an abundance of fermentable sugar and available nitrogen. Quite the contrary is true of the mannitol-forming group of lactic acid bacteria; these usually produce much smaller amounts of acid and survive for a much longer period. In the mash alone, without *Granulobacter pectinovorum*, the lactic acid bacteria persist for at least one year, perhaps for a longer time. The presence of a protective colloid, starch, and the presence of only a small amount of fermentable sugar no doubt favor the longevity of the lactic acid bacteria. Strange to say, somewhat similar results in relation to longevity of lactic acid bacteria are obtained when the *Granulobacter pectinovorum* produces a very vigorous fermentation. Here also the conditions favorable to longevity of the lactic acid bacteria are present; namely, large amounts of buffer substances and relatively small amounts of acid. The life of the lactic acid bacteria is comparatively short, provided the growth of *Granulobacter pectinovorum* is sufficient only to hydrolyze much of the starch, but not to carry it through to the formation of neutral substances, and thus large amounts of acid are formed and only small amounts of buffer substances.

There is no evidence to indicate that lactic acid is more harmful to the growth of *Granulobacter pectinovorum* than the other organic or inorganic acids. When additions of pure acetic, lactic, butyric, sulphuric, hydrochloric and nitric acids are added to mash in amounts sufficient to give pH values of about 4.7 to 4.8, there is a well-defined inhibiting effect on the growth of the acetone-butyl alcohol organism. If this acidity is neutralized, the injurious property is removed. Similar results are obtained with mashes in which the acidity is produced by the lactic acid bacteria, provided the mashes are kept until all of the living lactic acid bacteria have died off. The evidence, taken as a whole, points to acidity as the chief factor injurious to the growth of *Granulobacter pectinovorum.*

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