Stability of Mannitol to *Lactobacillus plantarum* Degradation in Green Beans Fermented with *Lactobacillus cellobiosus*

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ABSTRACT

Mannitol, in fermented green bean juice, was converted to lactic acid by *Lactobacillus plantarum* when the initial pH was raised to 3.9. However, at pH 3.5, mannitol was stable to anaerobic degradation by a 10^6 CFU/ml inoculum of 19 strains of *L. plantarum* and four isolated homofermentative lactobacilli. Several strains were capable of limited mannitol degradation at an initial pH 3.7. Completely fermented beans were microbiologically stable for at least 6 months under anaerobic conditions at 27°C. It is possible that heterolactic acid-fermented vegetables are microbiologically stable provided fermentable sugars are removed and the pH is lowered below pH 3.7.

INTRODUCTION

VEGETABLES, fermented with the homofermentative bacterium, *Lactobacillus plantarum*, have been shown to be microbiologically stable, provided all detectable sugars are removed, the pH is 3.8 or less and they are stored in anaerobic conditions (Fleming et al., 1983). We have found that *Lactobacillus cellobiosus* can lower the pH to 3.6 and remove all fermentable sugars from green beans with the production of lactic acid, acetic acid, ethanol, and mannitol (Chen et al., 1983a). Lactic acid, acetic acid and ethanol cannot be metabolized in anaerobic conditions, such as occur in hermetically sealed containers. However, to prevent secondary fermentations in green beans, it is also necessary to prevent further metabolism of mannitol.

Mannitol is different from the other fermentation products formed by heterolactic acid bacteria because it can be fermented in an anaerobic environment. Bergey's Manual lists seven species of lactobacilli which are reported to use mannitol, though it is not clear that strict anaerobiosis was maintained in each of those cases. Anaerobic mannitol utilization has been shown for *Aerobacter aerogenes* (Hadjipetrou and Stouthamer, 1965), *Bifidobacterium* (de Vries and Stouthamer, 1968), *Lactobacillus casei* (de Vries et al., 1970), and *Lactobacillus brevis* (Eltz and Vandemark, 1960). Therefore, the microbiological stability of products fermented by heterolactic acid bacteria will be dependent upon establishment of conditions which prevent the growth of organisms which can use mannitol.

Lactobacillus plantarum is one of the *Lactobacillus* species which has been reported to ferment mannitol (Pederson and Albury, 1969). It is also the terminal organism in many natural lactic acid fermentations due to its high acid tolerance. For this reason, *L. plantarum* is the most likely lactic acid species to carry out a secondary fermentation after heterolactic acid fermentation.

The objectives of this study were: (1) to determine the ability of *L. plantarum* to metabolize mannitol anaerobically after *L. cellobiosus* fermentation, (2) to identify conditons which will prevent secondary fermentation by *L. plantarum*, and (3) to evaluate the stability of green beans fermented with *L. cellobiosus*.

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MATERIALS & METHODS

TO INVESTIGATE mannitol stability in bean juice after *L. cellobiosus* fermentation, bean juice was prepared as described previously (Chen et al., 1983a). One liter batches of this juice were fermented by *L. cellobiosus*. After fermentation was completed, the juice was centrifuged at 23,000 x *g* for 10 min to remove cells. Aliquots were adjusted to the desired pH levels with 1N NaOH or HCl and then filter-sterilized with 0.22 μm Millex filters into sterile test tubes. The sterile fermented juice was inoculated with 10^6 CFU/ml *L. plantarum* unless stated otherwise. In all experiments, duplicate tubes were fermented and analyzed. Growth was evaluated by visual inspection and scored as + for definite growth, ± where there appeared to be slight growth and – for no growth. Mannitol analysis was done on samples where growth was observed to determine the extent of fermentation.

Preparation and fermentation of snapped green beans for storage studies were done as described by Chen et al. (1983a).

Analytical procedures for pH, substrate and product analyses using high performance liquid chromatography (HPLC) and plating techniques were the same as described by Chen et al. (1983b).

Isolation of mannitol-utilizing lactic acid bacteria

Unblanched, snapped green beans were covered with an equal weight of brine containing 5.0% NaCl and 0.16% glacial acetic acid to give equilibrated concentrations of 2.5% NaCl and 0.08% acetic acid, as was used in *L. cellobiosus* fermentations, and fermented in a sealed jar at 27°C for 1 mo. The brine was sampled with a sterile syringe through a septum in the jar cap and placed on basal medium (Hansen, 1968) with 2% $CaCO_3$ and mannitol as carbon source. The plates were incubated in an anaerobic jar at 30°C for 2 da. Isolations were made by picking colonies with a transparent clearing zone, indicating acid production from mannitol. The isolates were purified before use by repeating the isolation procedure two more times. The bacteria isolated were rods, which produced little or no CO_2 and almost exclusively lactic acid from glucose and fructose.

Cultures

Fourteen different strains of *L. plantarum* with culture numbers 82, 340, 341, 343, 352, 354, 363, 963, 965, 1193, 1194, 1752, 1939, and 1988 were obtained from the National Institute for Research in Dairying (Reading, England). *Lactobacillus plantarum* YIT 0068 was obtained from Yakult Institute for Microbiological Research (Tokyo, Japan). *Lactobacillus plantarum* WSO, *L. plantarum* 15, *L. plantarum* 16, and *Leuconostoc mesenteroides* 43 were obtained from the culture collection of this laboratory. *Lactobacillus cellobiosus* ATCC 11739 was provided by the Northern Regional Research Laboratory of USDA-ARS.

Storage stability studies

Green beans fermented by *L. cellobiosus* were washed with running tap water, repacked into 8-oz jars with 120g of beans and an equal weight of brine which was clarified by centrifugation at 23,000 x *g* for 20 min. The sealed jars were stored at 27°C. Three jars stored at 27°C were analyzed at 1, 2, 3, and 6 mo after packing. Brine pH was determined at each sampling period. Turbidity was estimated by visual inspection. An equal weight of beans and brine was blended in a Waring blender and filtered through a 0.22 μm Millex filter for analysis. The analysis of fermentation end products was done as described in the previous paper (Chen et al., 1983b).

RESULTS & DISCUSSION

LACTOBACILLUS PLANTARUM WSO has been selected for use in controlled cucumber fermentations because of low CO_2 production and tolerance to low pH (Etchells et al., 1973). It was used as the primary test organism in this investigation due to its high acid tolerance. Its ability to ferment mannitol anaerobically was demonstrated by the fact that it grew in bean juice with 2.5% NaCl, previously fermented with *L. cellobiosus*, which had been adjusted to pH 3.9 prior to filter sterilization. Fig. 1 shows the time course for changes in mannitol, lactic acid, acetic acid, and ethanol during this secondary fermentation. The most important point was that very close to 2 moles of lactate were produced per mole of mannitol metabolized. This contrasts with *L. casei* (de Vries et al., 1970) in which acetate, formate, and lactate were formed from mannitol. *Lactobacillus plantarum* apparently used an external electron acceptor to convert the third mole of NADH produced by mannitol fermentation back to NAD. Acetic acid appears to be one accpetor, since the concentration decreased 5 mM, while a 5 mM increase in ethanol occurred. However, this only accounts for 36% of the excess electron pairs required for the degradation of mannitol, so other unidentified acceptors must be used by *L. plantarum*. The same pattern of mannitol fermentation, acetic acid decline and ethanol increase was observed in juice which did not contain NaCl and in juice in which the primary fermentation was done with *L. mesenteroides*.

Since this *L. plantarum* strain can rapidly use mannitol, assurance of microbiological stability for *L. cellobiosus*-fermented beans will be dependent upon exclusion of mannitol-fermenting organisms or setting conditions which will prevent the growth of such organisms. If vegetables are to be fermented in bulk tanks, they will have to be removed from the tanks and repackaged prior to sale. It was considered impractical to prevent some contamination by *L. plantarum* during required post-fermentation handling procedures. As a result, the effect of pH on the ability of *L. plantarum* to grow in fermented juice which did not contain measurable amounts of glucose, fructose, or sucrose was investigated. Data in Table 1 show that after 2 wk incubation at 30°C, *L. plantarum* did not grow at an initial pH of 3.5 with an inoculum of 10^6 CFU/ml. At pH 3.7, 10^6 CFU/ml grew, but 10^5 did not. The growth at pH 3.7 was quite limited since only 5 mM mannitol of the 32 mM initially present disappeared. Since the recommended level of *L. plantarum* WSO inoculum for controlled culture cucumber fermentations is 10^6 CFU/ml (Etchells et al., 1973), it is unlikely that an unintentional contamination in the range of 10^6 CFU/ml would occur. Therefore, beans completely fermented with *L. cellobiosus* would be expected to be stable to a secondary *L. plantarum* WSO fermentation provided the final pH was less than 3.7.

Despite the fact that *L. plantarum* WSO has been used in cucumber fermentations in part because of its high acid tolerance, it is possible that other *L. plantarum* strains might be able to initiate growth at a lower pH. Therefore, 18 additional *L. plantarum* strains were tested for their ability to ferment mannitol. Each of these strains grew anaerobically on basal medium (Hansen, 1968) with mannitol as the carbon source. Data in Table 2 show that with a 10^6 CFU/ml inoculum, only one of these organisms showed

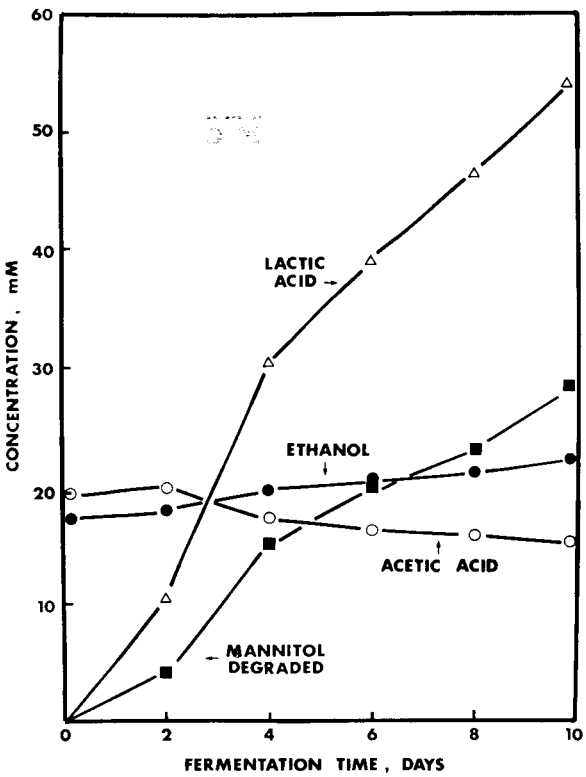

Fig. 1—Anaerobic degradation of mannitol by L. plantarum in fermented green bean juice. The green bean juice contained 31.9 mM of mannitol, 44.5 mM of lactic acid, 19.9 mM of acetic acid, an 17.3 mM of ethanol. The pH was raised to pH 3.9. The coefficients of variation were mannitol, 8.7%; lactic acid, 7.2%; acetic acid, 1.3%; and ethanol, 2.6%.

Table 1—Effect of initial pH upon the ability of L. plantarum WSO to grow anaerobically in L. cellobiosus-fermented bean juice[a]

Inoculum (no./ml)	pH value			
	3.5	3.7	3.9	4.1
10^6	–[b]	+	+	+
10^5	–	–	+	+
10^4	–	–	+	+
10^3	–	–	±	+
10^2	–	–	±	+
10^1	–	–	–	+

[a] The juice contained 22.6 mM mannitol, 56.0 mM lactic acid, 32.3 mM acetic acid, and 18.4 mM ethanol.
[b] –, No growth; ±, slight growth; +, growth.

Table 2—Effect of pH on the ability of different strains of L. plantarum to grow in L. cellobiosus-fermented juice[a]

L. plantarum strain no.	Initial pH value	
	3.5	3.7
WSO	–[b]	+
15	–	+
16	–	±
442	–	±
YIT 0068	–	±
82	–	±
341	–	±
343	–	±
352	–	±
1194	–	±
1988	–	±
1939	–	±
1193	–	±
340	–	–
363	–	–
965	–	–
1752	–	–
963	–	–
354	–	–

[a] The juice contained 27.8 mM mannitol, 42.9 mM lactic acid, 35.0 mM acetic acid, and 19.0 mM ethanol.
[b] –, No growth; ±, slight growth, <2.5 mM mannitol fermented; +, growth, >2.5 and <6.0 mM mannitol fermented.

definite growth at pH 3.7, while 11 showed only slight growth. None of the organisms grew at pH 3.5.

We also isolated homofermentative, rod-shaped organisms after 30 days of a natural fermentation of fresh, unblanched, snapped green beans. With a 10^6 CFU/ml inoculum, each of these isolates grew at pH 3.7, but not at pH 3.5 (Table 3). Analysis of the remaining mannitol after 2 wk incubation showed that 6 mM or less mannitol was used just as occurred with *L. plantarum* WSO.

The results of this survey indicated that none of the organisms tested have a greater ability to use mannitol at low pH than *L. plantarum* WSO. Provided post-fermentation contamination levels are controlled to be less than 10^5 CFU/ml, *L. cellobiosus*-fermented beans would be expected to be stable to a secondary *L. plantarum* fermentation when the final pH was less than 3.7.

All completed *L. cellobiosus* fermentations of green beans that we have done have had a final pH of 3.5–3.6, so they would be expected not to be susceptible to a secondary *L. plantarum* fermentation. This was tested by inoculating four jars of fermented beans with 10^6 CFU/ml of *L. plantarum* WSO. After 2 wk of incubation, the mannitol level was 39.2 ± 2.7 mM compared to an initial concentration of 40.2 ± 1.0 (Table 4). This indicated that the pH after *L. cellobiosus* fermentations was low enough to prevent growth of *L. plantarum* on mannitol.

Storage studies were carried out on two lots of fermented green beans. All detectable sugars were removed from one lot of beans (Table 5), while another lot had about 12 mM glucose remaining (Table 6). With all sugar removed, turbidity did not develop and the pH did not change. HPLC analysis showed that the amount of fermentation products, including mannitol, was stable for 6 mo (Table 5). When 12 mM glucose remained, a slight turbidity developed, but the pH did not change. Data in Table 6 show that the glucose disappeared after 1 mo of storage and a 20 mM increase in ethanol was observed. The mannitol had not changed after 6 months. Yeast cells were observed by microscopic examination of the brine. These changes showed that remaining glucose could be metabolized by yeasts, but that the yeasts present in the product did not degrade the mannitol.

The results of these experiments on the stability of *L. cellobiosus*-fermented beans and bean juice indicated that the usual terminal pH of the fermentation was low enough to prevent a secondary fermentation by *L. plantarum*. Since simply increasing the pH of fermented bean juice allowed growth of *L. plantarum*, this indicated that low pH was the factor responsible for growth inhibition. Since *L. plantarum* is the most acid-tolerant organism normally found in vegetable fermentations, these results suggest that heterolactic-fermented beans are likely to be stable to a secondary bacterial fermentation provided sugars are removed.

It is well known that acid-tolerant yeasts can grow in fermented cucumbers when sugars remain after the lactic acid fermentation is completed (Etchells et al., 1975). One experiment in this investigation showed that yeasts could ferment residual glucose, but they did not metabolize mannitol. Since yeasts are the only group of organisms, other than lactic acid bacteria, which grow in the acid and salt conditions associated with fermented and acidified vegetables, an important question is whether there are any yeasts which use mannitol anaerobically. Barnett (1976) and Phaff et al. (1978) have stated that yeasts can not use sugar alcohols anaerobically, but we have been unable to find any systematic investigations on this point.

Further studies are needed to test the stability of heterolactic-fermented vegetables to spoilage by yeasts or lactic acid bacteria under a variety of storage conditions. However, these initial results indicate that *L. cellobiosus*-fermented beans can be made microbiologically stable for extended periods of time without use of pasteurization, refrigeration or other preservation techniques.

Table 3—Effect of pH on the ability of isolated homofermentative lactobacilli to grow in L. cellobiosus-fermented juice[a]

Strain no.	pH value	
	3.5	3.7
2	–[b]	+
3	–	+
10	–	+
12	–	+

[a] The juice contained 26.2 mM mannitol, 40.3 mM lactic acid, 33.2 mM acetic acid, and 17.1 mM ethanol.
[b] –, No growth, +, growth, but <6 mM mannitol fermented.

Table 4—Mannitol stability in green beans to secondary fermentation by L. plantarum WSO

Jar No.	Mannitol (mM)[a]	
	Before inoculation	After inoculation
1	41.3	42.5
2	40.9	41.4
3	39.2	38.4
4	39.5	36.7
Mean	40.2 ± 1.0	39.2 ± 2.7

[a] Mannitol concentrations before and after inoculation with L. plantarum WSO were not significantly different.

Table 5—End product stability in green beans during storage at 27°C after all sugars were removed by L. cellobiosus fermentation[a]

Storage time (months)	End products			
	Mannitol	Lactic acid	Acetic acid	Ethanol
0	44.2	49.8	28.2	21.2
1	45.0	50.2	27.4	23.3
2	44.3	49.4	27.7	23.7
3	44.7	50.9	28.9	27.5
6	44.6	51.4	30.1	24.8

[a] The initial substrate concentrations in the brined beans were 6.8 mM sucrose, 34.5 mM glucose, 40.6 mM fructose, and 4.9 mM malic acid. The pH after L. cellobiosus fermentation was 3.48.

Table 6—End product stability of L. cellobiosus-fermented beans containing residual glucose during storage at 27°C[a]

Storage time (months)	End products (mM)				
	Glucose	Mannitol	Lactic acid	Acetic acid	Ethanol
0	12.1	55.9	46.9	44.9	10.6
1	–	55.2	51.0	44.5	30.4
2	–	55.7	52.8	45.6	33.3
3	–	54.5	53.3	46.8	33.3
6	–	55.7	55.7	47.4	32.4

[a] The initial substrate concentrations in the brined beans were 6.2 mM sucrose, 44.8 mM glucose, 52.5 mM fructose, and 5.6 mM malic acid. The pH after L. cellobiosus fermentation was 3.59.

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