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Effect of Lactobacilli Inoculation on Cassava (*Manihot esculenta*) Silage: Fermentation Pattern and Kinetic Analysis*

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ABSTRACT

The major effect of Lactobacillus *inoculation on laboratory cassava* (Manihot esculenta *Crantz*) *silage was a change from a heterofermentative pattern observed in natural silage to a homofermentation. Small amounts of starter culture (1 % v/w) were required to produce a high level of lactic acid (> 28 g kg^{-1} DM) and to reach a pH of at least 4. The Gompertz model was used to evaluate the effect of inoculation level on the lactic formation based on kinetic criteria. Also an empirical pH–lactic acid correlation was proposed to monitor the progress of ensiling, based solely on pH measurements. The simulation model may be used to improve guidelines for silo safety and to evaluate the effect of lactobacilli inoculants.*

Key words: Cassava silage, lactobacilli inoculants, homofermentation, lactic acid, kinetic analysis, empirical pH–lactic acid correlation.

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INTRODUCTION

Silage has an important role in the conservation of organic material. In Africa and South America ensilage has been used to achieve detoxification and conservation of wet cassava (Okigbo 1980). However, the importance of the proper handling of this biotechnological process is often underestimated by farmers (Lindgren *et al* 1988).

In the past few years there has been renewed interest in the inoculation of forage crops (Seale 1986). Lactobacilli inoculation in forage has been extensively studied in different laboratories (eg by Lindgren *et al* 1988) as a way of achieving a rapid decline in pH and of producing a higher level of lactic acid and a lower proportion of volatile fatty acids. Efforts have also been reported indicating that simulation models can be used to improve guidelines for silo safety and to analyse the effects of inoculation (Meiering *et al* 1988).

Ensilage of cassava is a rural practice in the state of Tabasco in South Mexico. Silage inoculation could help to achieve a better product for animal feeding. A systematic study was undertaken to assess the effect of lactobacilli inoculation on cassava silage. In this paper, two experiments are described in which changes in cassava silage have been observed. The first experiment was designed to investigate the effect upon silage of natural fermentation (without inoculation), artificial acidification and lactobacilli addition. The purpose of the second experiment was to observe the effects of increasing amounts of homofermentative bacteria on silage fermentation. A simple mathematical model is presented to evaluate the kinetics of lactic acid formation, and also a correlation between pH and lactic acid is proposed as a guideline to improve silo safety.

EXPERIMENTAL

Cassava roots

In both experiments cassava (*Manihot esculenta* Crantz) with a dry matter (DM) content of 40% and pH 6 was used, and was harvested in Puente de Ixtla, Morelos State, Mexico. The approximate composition was (g kg^{-1} DM): crude protein 18·23, total carbohydrates 750, ash 28·7.

For the first experiment, fresh cassava was chopped in an industrial food processor to give an approximate particle size of 1 to 2 cm, then utilised for the three treatments described below. To obtain a standard starting material for the second experiment, cassava chips were sun dried (92% DM), stored and mixed with water and inoculum for inoculation level studies.

Microorganisms

Three different strains of lactobacilli were selected as silage inoculants. Lactobacilli were isolated from sauerkraut (sour cole) and pozol (sour corn dough). According to the biochemical tests indicated by *Bergey's Manual* (Buchanan and Gibbon 1974) the strains were from the genus *Lactobacillus*. The inocula were grown in Rogosa medium at 35°C for 20 h. The final pH was 3·8, and the optical density at 540 nm

was 4–6 OD units ml^{-1} equivalent to 1–2 g litre of biomass. The pH was adjusted to 6·0 before being used as inoculum.

Treatments

In the first experiment the effects of inoculation were studied by ensiling fresh cassava after the following treatments: untreated, acidified and inoculated. Inoculation was carried out as follows: 18 kg of fresh matter was mixed with 2 litres of fresh starter inoculum (10% v/w). Control natural fermentations (without inoculation) were ensiled without any treatment. Artificial acidification of fresh cassava was made at 0·1% with a mixture of 7:1 v/v of hydrochloric and sulphuric acids. Each of the treated materials was ensiled in triplicate (minimum) silos consisting of sealed polythene bags inside rigid containers. The silages were stored at room temperature for 140 days. Nearly 50 g of sample was taken every month and used for duplicate chemical analysis.

Studies of the effect of inoculation level on cassava silage fermentation were done using dry cassava meal with a particle diameter between 0·6 and 0·8 mm. Initial conditions were: 45% DM, pH 6, and a packing density of 1·1 kg $litre^{-1}$. The culture of *Lactobacillus* 1 was prepared as above but added in the volume:weight proportions 0, 1, 3, 5 and 10%. Samples of approximately 50 g were packed tightly into small test tubes (22 × 140 mm) sealed with a rubber cap fitted with a Bunsen valve. Duplicate tubes of each treatment were analysed from 0 to 80 h. Incubation temperature was controlled at 30 ± 0·1°C in a thermoregulated water bath. Chemical analysis was carried out in duplicate.

Chemical analysis

pH was measured in 5-g suspensions of fresh sample diluted with 45 ml of distilled water; potentiometric titrations were carried out using 0·1 M NaOH (AOAC 1980) and results were reported as lactic acid (g kg^{-1} DM). Lactic acid determinations were made by the colorimetric method of Barnett (1951) and confirmed by gas chromatography. Dry matter was measured according to the AOAC (1980). Water soluble carbohydrates were measured by the method of Miller (1959), using 3,5-dinitrosalycilic acid. Nucleic acids, mainly RNA, were measured by perchloric acid extraction and ultraviolet absorption at 260 nm (Gomez-Hernandez and Viniegra-Gonzalez 1977). Protein was measured using the Lowry *et al* technique (1951). Fermentation products were analysed by gas chromatography using the procedure described by Gomez-Hernandez and Coronado-Vega (1983). Flieg (Ohyama *et al* 1975) scores were calculated for quality comparison of silages. This 100-point score gives high points for high percentages of lactic acid and lower points according to the appearance of acetic and butyric acid.

THEORETICAL CONSIDERATIONS

Simulation of lactic acid fermentation using a Luedeking and Piret (1959) type model requires accurate biomass, substrate and product measurements (Aborhey and Williamson 1977). By analogy with chemical kinetics, alternative models have

been presented in order to simulate fermentation kinetics (Bovee *et al* 1984) without biomass measurements, but these kinds of model are not easy to apply in very heterogeneous systems. Modelling of acidification rate in silage fermentation is very important and can be used as a guideline to improve silo safety. Here, an alternative is presented to simulate the kinetics of lactic acid production in cassava silage by monitoring the product formation alone. This can be achieved by using an S-shaped equation like the Gompertz model, that is, a logistic-like curve (Ratkowsky 1983):

$$dP/dt = kP \ln(P_{max}/P) \quad (1)$$

where P is the lactic acid concentration, t is time, k is the acidification rate constant with the significance of a specific parameter, and P_{max} is the highest product concentration. The integrated form of the Gompertz model allows a time–product algebraic relation (Draper and Smith 1981):

$$P = P_{max} \exp\{-b \exp(-kt)\} \quad (2)$$

This model has been used in the simulation of kinetic acidification using a heterogeneous mixed inoculum (Saucedo-Castañeda and Gomez 1989). The limiting conditions for these equations are: if $t \rightarrow 0$, then $P \rightarrow P_0 = P_{max} \exp(-b)$ and, if b is a positive defined number, then P_0 has a small numerical value not necessarily zero (eqn **2**); if either $P = 0$ or $P = P_{max}$, then $dP = 0$ (eqn **1**). The log function P_{max}/P does not give unreal values when P approaches zero, since the product formation rate is also a function of product concentration (eqn **1**). The Gompertz model (eqn **1**) is a logistic function without symmetry in the inflexion point: its flexibility allows the acidification kinetics to be described accurately within defined limits. Parameters can be estimated by using the method of Marquardt (1963), which minimises the sum of squares in a nonlinear model.

RESULTS AND DISCUSSION

Effect of inoculation of fresh cassava roots

Inoculation of fresh cassava chips had some effect on the pH (Table 1) and lactic acid production, but more noticeable were the changes in fermentation pattern in the various treatments (Table 1). Natural fermentation indicated a good level of lactic acid (19·1 g kg^{-1} DM) but with significant amounts of ethanol (5·2 g kg^{-1} DM) and volatile fatty acids (total VFA = 10·4 g kg^{-1} DM). Addition of 10% v/w inoculum produced levels of lactic acid varying from 14·6 up to 26·5 g kg^{-1} DM, but with significantly lower levels of ethanol and VFA as compared with natural fermentation. On the other hand, artificial acidification produced a lower pH (3·16), but with practically no lactic acid nor VFA and with significant higher levels of ethanol (6·5 g kg^{-1} DM).

The suitability of the experimental model was tested with an 11-tonne rural silo. A lactic acid concentration of 22·8 g kg^{-1} DM and a pH of 4·05 were found in the rural silo. These results are in good agreement with those found in the current study (Table 1). Lactic acid fermentation is inhibited at low pH (Seale 1986). Our evidence with inorganic acids agrees with this conclusion.

TABLE 1
Comparison of fermentation products in fresh cassava silage: natural fermentation, lactobacilli inoculated and acid addition[a]

Treatments	*Source*	*Lactic acid* ($g\ kg^{-1}$ *DM*)	*Ethanol* ($g\ kg^{-1}$ *DM*)	*Total VFA* ($g\ kg^{-1}$ *DM*)	*pH*
Natural flora	Roots	19·1±1	5·2±0·2	10·4	4·11±0·14
Lactobacillus 1	Sauerkraut	26·5±1	1·7±0·8	0·7	3·82±0·08
Lactobacillus 2	Sauerkraut	14·6±1	1·0±0·5	<0·1	4·07±0·09
Lactobacillus 3	Pozol	24·8±3	<0·1	<0·1	3·98±0·06
Acid addition		<0·1	6·5±1·9	<1·6	3·16±0·15

[a] Mean values from 30 to 140 days.
Total VFA: sum of acetic, propionic and butyric acid.
Error expressed as standard error of mean.

TABLE 2
Comparison of fermentation products in fresh cassava silage: natural fermentation, *Lactobacillus* 1 inoculated and acid addition[a]

Parameters	*Treatments*		
	Natural flora	Lactobacillus *1*	*Acid addition*
Ethanol[b]	5·2	1·7	6·5
Acetic acid[b]	9·4	0·7	1·2
Propionic acid[b]	1·0	<0·1	<0·1
Butyric acid[b]	<0·1	<0·1	<0·1
Lactic acid[b]	19·1	26·5	<0·1
Lactic acid %[c]	54·9	91·1	<1·3
Flieg index	77·0	100·0	<1·0

[a] Mean values between 30 and 140 days.
[b] $g\ kg^{-1}$ DM.
[c] Percentage of fermentation products.

By using the same data as in Table 1, a more detailed comparison of the effect of the addition of 10% v/w inoculum of *Lactobacillus* 1, natural fermentation and inorganic acidification is shown in Table 2, where a homolactic fermentation (>90% of all products) was associated with lactobacilli inoculation, as compared with only 54·9% of lactic acid in the silages which had undergone a natural fermentation. An alcoholic fermentation was observed in the acid treatment. The Flieg index comparison shows a high value for inoculated silos as compared with natural fermentation; a very low Flieg index was observed in the acid-treated silos. Since the Flieg index has been correlated to the intake of silages by animals, this gives further support to the use of starter cultures in cassava silage.

Effect of inoculation level on cassava silage fermentation

Under the conditions of this experiment, *Lactobacillus* 1 has demonstrated the highest acidification rate at a high dry matter content (40% DM), a

TABLE 3
Comparison of fermentation products after 72 h in laboratory cassava silage made using different levels of inoculation

Parameters	*Inoculation level* (% v/w)				
	0	*1*	*3*	*5*	*10*
Ethanol[a]	4·5	3·7	2·6	2·9	ND
Acetic acid[a]	5·8	1·0	0·6	1·8	ND
Propionic acid[a]	<0·1	<0·1	<0·1	<0·1	ND
Butyric acid[a]	3·9	0·1	0·1	0·3	ND
Lactic acid[a]	17·1	28·3	29·2	30·1	32·4
Lactic acid %[b]	54·5	85·2	89·6	85·5	91·1[c]
pH	4·4	4·0	3·9	3·9	3·8
Flieg index	42·0	100·0	100·0	100·0	100·0

[a] g kg^{-1} DM.
[b] Percentage of fermentation products.
[c] From Table 2.
ND, Not determined.

homofermentative pattern and a capability of producing a pH of at least 4. For these reasons and following the criteria defined earlier (Seale 1986). *Lactobacillus* strain 1 was selected for the following studies. The effect of inoculation level on fermentation pattern, biomass variation, pH evolution and lactic acid formation on laboratory cassava silage was evaluated.

Ethanol and acetic and butyric acid production were diminished at a higher inoculation level (Table 3). With inoculation levels between 1 and 10%, at least 85% of fermentation products (organic acids and ethanol) were identified as lactic acid (Table 3). By comparison with natural silos, the homofermentative pattern in the inoculated silos had given a higher percentage of final lactic acid and a higher Flieg index.

A comparison of means of final pH and final lactic acid concentration, at the different inoculation levels, was carried out by using Tukey's test (Steel and Torrie 1980). For both lactic acid and pH, no differences ($P<0·05$) were found between inoculation levels of 1 and 5%. The product yield coefficient $Y_{p/s}$ (g lactic acid g^{-1} substrate consumed) has shown a constant higher level of 0·73 in treatments where lactobacilli was added (Table 5), indicating that this strain was more efficient at producing lactic acid than the natural microbial flora.

A comparison of these results (Table 3) with those of fresh cassava silage (Table 2) indicated that there was an increase of 22% in the final lactic acid concentration when using the 10% level of inoculation. This was probably because dried cassava meal (used in the second experiment) could be expected to have a lower level of competitive bacteria. Analyses of actual numbers of lactobacilli were not carried out, but nucleic acid and protein analyses indicated very small changes throughout the fermentation processes. In Table 4 are shown only the results of nucleic acids. The insignificant changes of protein and nucleic acids during the process suggested

TABLE 4
Final and maximum increment of nucleic acids in cassava silage at different inoculation levels

Inoculum (% v/w)	*RNA* ($g\ kg^{-1}$ *DM*)	
	Maximum	*Final*
0	0·20±0·02	0·10±0·03
1	0·25±0·01	0·25±0·01
3	0·48±0·01	0·38±0·01
5	0·52±0·05	0·39±0·02
10	0·63±0·01	0·62±0·02

Error expressed as standard error of mean.

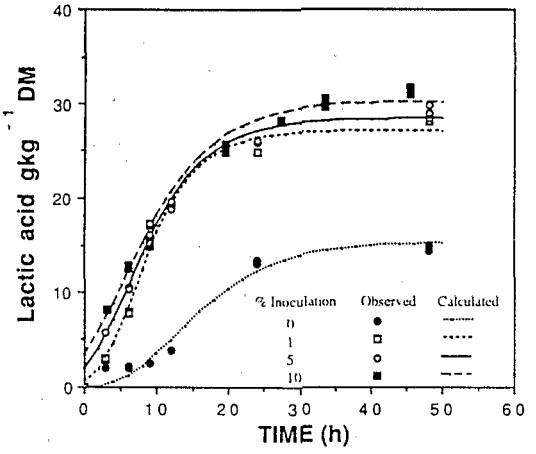

Fig 1. Influence of inoculation level (% v/w) of *Lactobacillus* 1 on lactic acid formation in cassava silage.

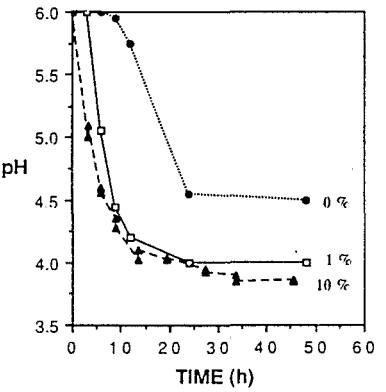

Fig 2. Influence of inoculation level (% v/w) of *Lactobacillus* 1 on the pH evolution of cassava silage.

that, under those conditions, lactic acid production could be associated mainly with maintenance metabolism rather than with changes in cell biomass.

Kinetic evaluation

The effects of inoculation level on the kinetics of lactic acid formation and pH changes are shown in Figs 1 and 2, respectively. In Fig 1 the lactic acid profile for 3% was very similar and is not shown. In the same way, in Fig 2 the pH profiles for 3 and 5% are not shown.

Product–time data were fitted to the integrated form of the Gompertz model (eqn 2) by the Marquardt algorithm. In Table 5 are shown the simulation results, major kinetic characteristics and the goodness of fit of the nonlinear regressions. The estimations of P_{max} and k are shown in Fig 3. The product mean deviation (PMD), $\{\sum (Pi_{obs} - Pi_{cal})\}^{1/2}/n$, was calculated as a practical measurement of the deviation

TABLE 5
Simulation results in Gompertz model and major kinetic characteristics of cassava silage at different inoculation levels with *Lactobacillus* 1

Inoculum (% *v/w*)	*b*	*Correlation coefficient*	*PMD* (*g kg*$^{-1}$ *DM*)	$-dpH/dt^a$	$Y_{p/s}$
0	5·57	0·97	0·275	0·10	0·40
1	4·11	0·99	0·231	0·20	0·73
3	2·83	0·99	0·235	0·19	0·74
5	2·72	0·99	0·275	0·18	0·74
10	2·20	0·98	0·311	0·17	ND

[a] Initial rate of pH decrease, pH units per hour.
b = Constant related to initial conditions of Gompertz model (eqn **2**).
PMD, product mean deviation. $\{\sum (Pi_{obs} - Pi_{cal})\}^{1/2}/n$.
$Y_{p/s}$: Product yield, in g lactic acid g^{-1} glucose consumed.
ND, Not determined.

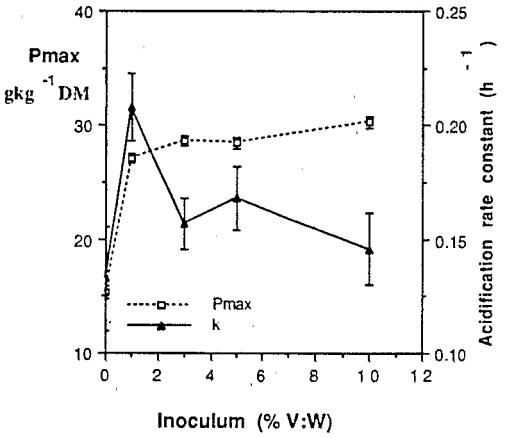

Fig 3. Influence of inoculation level of *Lactobacillus* 1 on the maximum lactic acid accumulation (P_{max}) and the acidification rate constant (k) of cassava silage fermentation.

between observed and calculated lactic acid (Table 5). The mean overall was 0·26 g kg^{-1} DM, giving a very good agreement between experimental data and predicted curves (Fig 1).

The effect of inoculum size on cassava silage acidification kinetics was evaluated through the estimated P_{max} and k (eqn **1**) and by the corresponding final pH and pH decline rate. There were only small changes in final pH (Table 3) and final lactic acid production using inoculation levels between 1 and 10% compared with a greater change between 0 and 1% (Fig 3). This apparent saturation phenomenon can be explained as the inhibition of fermentation by the undissociated molecule of lactic acid at low pH, since optimal lactic fermentation occurs at pH values from 5 to 6, where lactic acid is dissociated to a large extent (Seale 1986). The maximal acidification rate constant (Fig 3) and pH decline rate (Table 5) were found at 1%: more rapid production of lactic acid initially can be explained by the end product inhibition and the increment of initial product at a higher inoculation level (Fig 1).

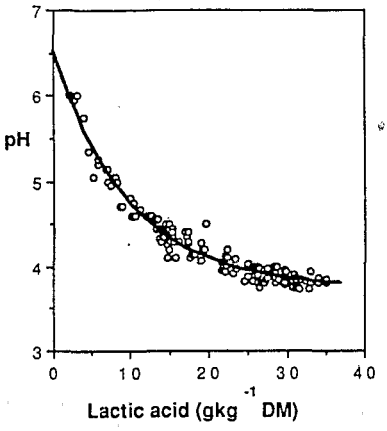

Fig 4. Correlation of pH and lactic acid concentration in inoculated cassava silage (○, experimental data; ——, mechanistic model).

Further, at rates of 3 % of inoculation or above, the final pH (Table 3) was very close to the pK value of lactic acid (3·84), probably due to the formation of a buffer between the lactic acid and its salt. This diminishes the risk of secondary fermentation.

Under these experimental conditions more than 90 % of lactic acid was formed in 24 h (Fig 1). This high acidogenic activity could be due to a better distribution of active liquid inocula in silos.

Simulation of the kinetics of lactic acid formation by the Gompertz model can be used to realise a quantitative selection of lactobacilli inoculants based on kinetic criteria. It can also be used in microcomputers as a comprehensive formula for predicting the effects of inoculation. Further experimental work is desirable to evaluate other factors in silage fermentation.

Regardless of inoculation level, changes in acid production were similar. There must be a relationship between pH and the level of lactic acid. An empirical correlation between pH and lactic acid was found using 200 pairs of data from inoculated silages (Fig 4). A mechanistic model (Draper and Smith 1981) was used to predict the experimental data:

$$-\mathrm{dpH}/\mathrm{d}P = K(A - \mathrm{pH}) \qquad (3)$$

where P is lactic acid, the pH decline is proportional to pH at certain product concentration, A is the minimum pH value and K is the acidification constant. Data were fitted to the integrated form of eqn (3) (Draper and Smith 1981):

$$\mathrm{pH} = A\{1 + B\exp(-KP)\} \qquad (4)$$

The parameters were estimated using the method of Marquardt and were as follows:

$$A = 3{\cdot}74 \pm 0{\cdot}01 \qquad B = 0{\cdot}75 \pm 0{\cdot}01 \qquad K = 0{\cdot}102 \pm 0{\cdot}003$$

The correlation coefficient was 0·97. This predictive equation is only valid between the limits of the observed data used to estimate it (pH between 6 and 3·8 and lactic acid between 0 and 35 g kg^{-1} DM). The expected value for A could be 3·84 (pK of the lactic acid) and 0·60 for B (by using pH 6 at $P = 0$ in eqn 4). Predictions using

estimated and expected A and B values were analysed by using the estimated K value. Final pH at 35 g kg^{-1} DM remains fairly constant with both calculations; a more important difference was noted in the initial pH value. These results agree with the asymptotic characteristic of the model (eqn **4**) and can explain the poor predicted fit at low lactic acid concentration (Fig 4). Even when the prediction is limited, under controlled conditions this simple expression (eqn **4**) can be used as a useful tool to monitor acidification in cassava silage by the sole pH measurement.

CONCLUSION

Evidence was presented in support of a favourable effect of inoculation of cassava silage by lactobacilli. The major effect was a change from a heterofermentative pattern observed in natural silage to a homofermentative pattern with inoculation. Best results were obtained with a strain of *Lactobacillus* isolated from sauerkraut. A detailed analysis of this induced fermentation indicated a relatively low requirement of inoculation (1 % v/w) for obtaining a fermentation with high levels of lactic acid production. The Gompertz model was used to evaluate the effect of lactobacilli inoculation level on the lactic acid formation based on kinetic criteria. Further the empirical pH–lactic acid correlation proposed in this work could lead to the development of improved guidelines for silo safety. It is concluded that inoculation of cassava silage with small volumes of starter culture could have a significant effect in reducing heterofermentation of available carbohydrates and could lead to a better quality product for animal production.

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