MYCOFLORAL SUCCESSION IN POZOL FROM TABASCO, MEXICO*

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INTRODUCTION

The maize plant, *Zea mays* L., since long ago has been the axis of entire civilizations in the American continent, playing an analogous role to wheat in Europe and rice in Asia. It has been to the present an important human and animal food in Mexico and other Latin American countries. The distribution of maize, cultivation and storage methods, and manners of preparation for human consumption, explain many of the peculiarities of these peoples (Beltrán, 1949).

The *pozol* (from the Aztec *pozolli*, foamy; Robelo, 1918) is a fermented maize dough that, diluted in water, is drunk raw as a basic food by the Indian and mestizo populations in southeastern Mexico; the main states where pozol is eaten are Chiapas, Tabasco, Campeche, and Yucatán, although, on smaller scale, it is also utilized as food in some regions of the states of Veracruz and Oaxaca.

Several authors have published interesting ethnological descriptions in relation to pozol, indicating the alimentary and ceremonial usages of this food by the Maya culture since several centuries ago, even before the Spanish conquest (de Landa, 1560; Tozzer, 1907; Blom, 1944; Duby, 1944; Salinas Ch., 1958; Morley, 1961; Frías, 1964).

In order to determine the nutritive value of pozol, Cravioto *et al.* (1955) conducted a comparative analytical study of this food and the maize kernels used for its preparation. The results showed that pozol has a higher content of protein, niacin, riboflavin, lysine, tryptophan, and some other nutrients than maize; however, the latter contains more thiamine and phosphorus than pozol. Also a better quality of proteins was found in pozol, measured by both

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the esential amino acid composition and growth promoting efficiency in albino rats.

Since most of the biochemical changes are brought about by the microorganisms developing during fermentation in the maize dough to transform it into pozol, a series of microbiological studies was done on the bacteria, yeasts, and molds which comprise the complex microflora of pozol. Among the bacteria studied are *Bacillus cereus* Frank. & Frank. and *Paracolobactrum aerogenoides* Borman *et al.* (Salinas Ch., 1958); *Agrobacterium azotophilum* Ulloa & Herrera and *Achromobacter pozolis* Ulloa & Herrera, two new species recently described, the first of which is a nitrogen fixer that contributes to increasing the nitrogen content in pozol (Ulloa & Herrera, 1972); *Escherichia coli* var. *neapolitana* (Topley & Wilson) Yale and *Pseudomonas mexicana* Fuentes, Herrera & Ulloa (Fuentes, Herrera & Ulloa, 1974).

Among the yeasts recorded are *Candida krusei* (Cast.) Berkhout, *Trichosporon cutaneum* (de Beurm., Gougerot & Vaucher) Ota, and *Hansenula fabiani* Wickerman (Herrera & Ulloa, 1971, 1972); *Hansenula pozolis* Herrera, Ulloa & Fuentes and *Candida parapsilosis* var. *tuxtiensis* Herrera, Ulloa & Fuentes, a new species and a new variety recently reported (Herrera, Ulloa & Fuentes, 1973).

Among the species of molds isolated from pozol are *Aureobasidium pullulans* (de Bary) Arnaud, *Cladosporium herbarum* (Pers.) Link. *Epicoccum* sp., *Fusarium* spp., *Geotrichum candidum* Link, *Monilia sitophila* (Mont.) Sacc., *Mucor racemosus* Fres., *Paeciiomyces fumosoroseus* (Wise) Brown & Smith, *Rhizopus stolonifer* (Ehrenb. ex Fr.) Lind, and *Trichoderma viride* Pers. (Herrera & Ulloa, 1970; Ulloa & Herrera, 1971); *Penicillium claviforme* Bainier, *P. cyclopium* Westling, *P. expansum* Link, *P. italicum* Wehmer, and *P. lanosoviride* Thom (Ulloa & Herrera, 1973 a); and *Phialophora richardsiae* (Melin & Nannf.) Conant (Ulloa & Herrera, 1973b).

All of the species of microorganisms mentioned above have been isolated from pozol of known source but unknown time of fermentation. Most frequently these species have been found growing on samples of pozol that had been shipped from the sites of preparation to the Laboratorio de Micología of the Instituto de Biología of the UNAM. The presence of such species indicates that they are capable of growing on fermented pozol, but they could well be superficial contaminants of the balls of pozol that perhaps would not be present, at least in the same proportion, at the original locations where pozol is prepared and eaten, where the environmental conditions are obviously different.

Among the species isolated from pozol are some known in the literature as pathogenic or potentially pathogenic to man, i. e. *G. candidum, T. cutaneum, C. parapsilosis* and *P. richardsiae* that may cause geotricosis, white piedra, candidiasis, and phaeosporotrichosis in man, respectively, if the conditions are favorable for them to infect a host (Emmons, Binford & Utz, 1970). Other species present in pozol could contribute toward improving its nutritional qualities by fixing nitrogen, as mentioned before, or by synthesizing proteins

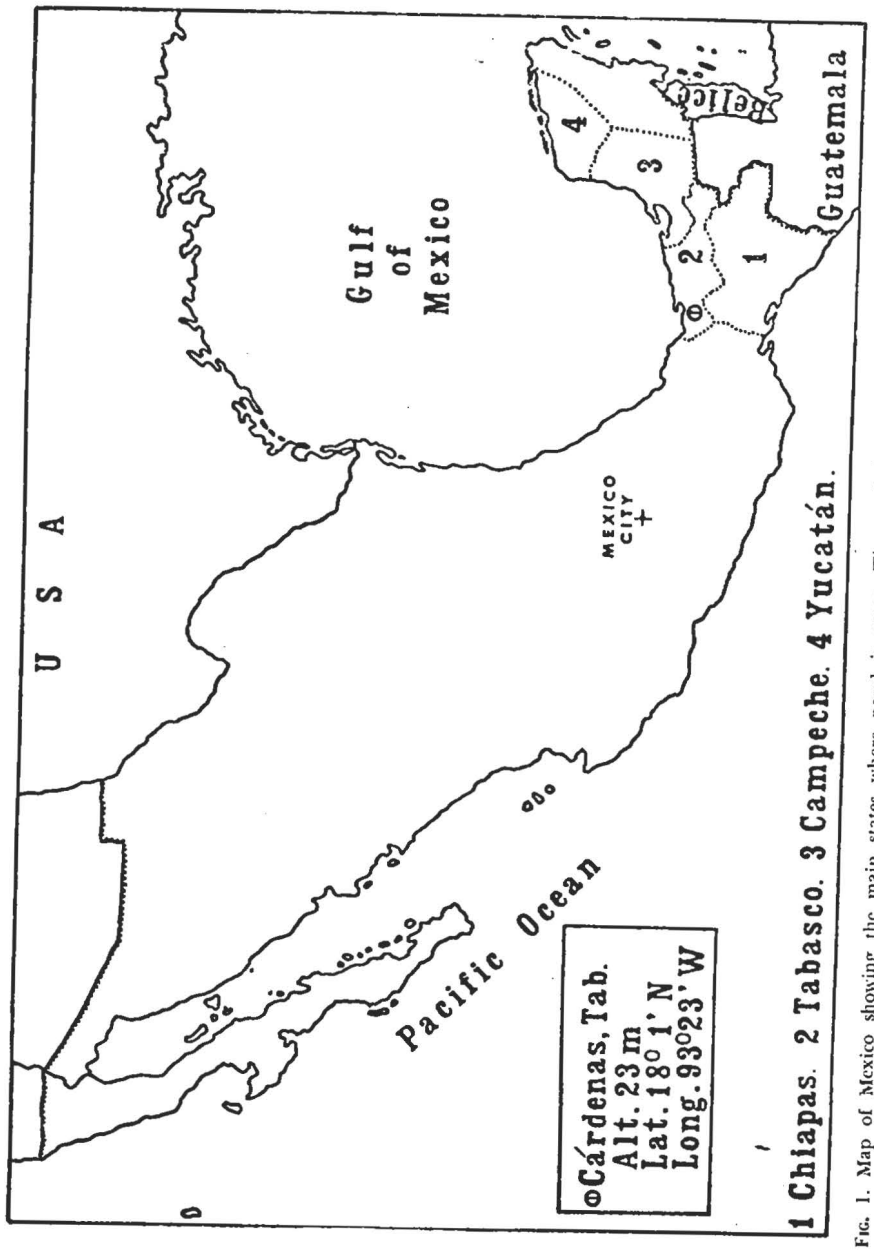

Fig. 1. Map of Mexico showing the main states where pozol is eaten. The studied pozol was prepared at Cárdenas, Tabasco.

and vitamins, as some yeasts can do; but their presence could be variable, unpredictable, and determined by environmental and biotic factors that have not been studied so far.

The present method of making pozol, which does not differ much from that of the Mayan ancestors, is more or less controlled by the people who eat it through the preparation of the balls of maize dough, since this is always obtained by grinding the kernels previously cooked in lime water. However, the fermentation of the maize dough is produced by the bacteria, yeasts, and molds present on the hands of the persons who prepare it, the utensils used over and over again, the air, the water, the banana leaves used to wrap the dough balls, and in general the surroundings of the site of preparation of the food. Even though the inoculation of the dough is not controlled but takes place at random, surprisingly enough, the consumers of pozol seem to obtain a similar product, with respect to appearance and sour taste, every time they prepare it. This would indicate that the fermentation process must be carried on by certain kinds of microorganisms that are favored by the chemical composition of the substrate and the environmental factors involved in such a process. It is important to note that no biochemical studies have been done on the fermentation of pozol, although it can be said that an alcoholic fermentation does not occur, or if any ethanol is ever produced, its concentration must be quite low.

Considering all the aspects mentioned above, it can be seen that it was necessary to study the changes in the mycoflora from the basic material, the maize kernels, through the different stages of preparation and fermentation of pozol under the environmental conditions prevalent at a location where this food is routinely eaten. So, the objectives of this study were to isolate at the manufacturing site the yeasts and molds from maize kernels, kernels cooked in lime water, recently made dough, and dough of known fermentation time, and to record the changes in pH, moisture content, relative humidity, and temperature during the whole process. The site chosen for the study was Cárdenas, Tabasco (Fig. 1), because, firstly, it is a typical region where pozol is prepared daily and eaten as a basic food, mainly by the peasants of low income; secondly, the facilities provided at the Colegio Superior de Agricultura Tropical in the locality; and thirdly, the availability of meteorological data for the area (Table 1). Study of the changes in the mycoflora of pozol at the site of preparation was designed to determine which species are present at the different stages of preparation and fermentation and whether or not species potentially pathogenic or beneficial for the consumers of this food were consistently present.

TABLA 1

Climatic characteristics of Cárdenas, Tabasco[a]

	Jan	*Feb*	*Mar*	*Apr*	*May*	*Jun*	*Jul*	*Aug*	*Sep*	*Oct*	*Nov*	*Dec*	*Annual*
Temp. °C[b]	23.0	24.0	26.4	27.9	29.3	29.2	28.8	29.0	28.1	26.7	25.1	23.1	26.7
Precip. mm[c]	141.5	62.0	35.9	60.7	99.8	232.1	208.0	210.8	345.5	452.2	237.3	156.5	2240.3

a Data obtained from the Mexican Meteorological Service.
b Average of 19 years (1941-1960).
c Average of 10 years (1951-1960).

Type of climate according to García (1964): Amw" (i")g, characterized as a hot and humid climate. The mean temperature of the hottest month is over 18°C. with the highest temperature registered before the summer solstice. It has slight oscillation of temperatures during the year; the difference between the coolest month and the hottest one is an average of 6°C. "Canícula" (a dry period between the rainy seasons) is present. Most of the rains occur during the summer; only 10.6% of the total annual rain occurs during the winter.

MATERIALS AND METHODS

Preparation of pozol. The pozol utilized for the present study was prepared on March 21, 1973, by the Hernández family, a kind family of peasants, living in a hut by the Gulf road, near the Colegio Superior de Agricultura Tropical at Cárdenas, Tabasco, where the author carried out the initial phase of the research.

The pozol was made according to the traditional manner as follows: cobs of white maize, *Zea mays* L., were shelled, and about 1½ K of kernels were boiled for one hour or so in about 2 liters of well water to which was added a handfull of lime powder (approximately 10% $Ca(OH)_2$ w/v). When the kernels were swollen and their pericarps peeled off easily, the kernels were cooled, rinsed once with well water, and drained to get what is called "nixtamal". The nixtamal was ground in a manual metal mill to obtain a coarse dough which was then shaped into balls 10-12 cm long and 5-8 cm wide and wrapped in banana leaves (Figs. 2-4). Thirty balls of maize dough, ranging from 70 to 165 g each, were made from the same dough by one person (Mr. Hernández' daughter) at the same time and place. The balls were numbered 1-30; the first 5 were immediately separated to be studied, and the rest were placed in a tray to ferment for increasing periods of time up to 8 days under the same environmental conditions, outdoors, under the shade of a roof made of palm leaves (Fig. 5A).

Measurements of pH, moisture content, relative humidity, and temperature at the different stages of preparation and fermentation of pozol. Five 10 g replicates each of maize and nixtamal kernels were ground in a mortar and suspended in 50 ml of distilled water for pH readings with a Beckman potentiometer. The same procedure was followed for pozol at different fermentation times. The moisture content of maize, nixtamal, and pozol was determined in five 10 g replicates of each material by loss of water after drying in an oven at 90°C for 24 hrs. The temperature and relative humidity around the balls of pozol were recorded during the 8 days that pozol was being fermented

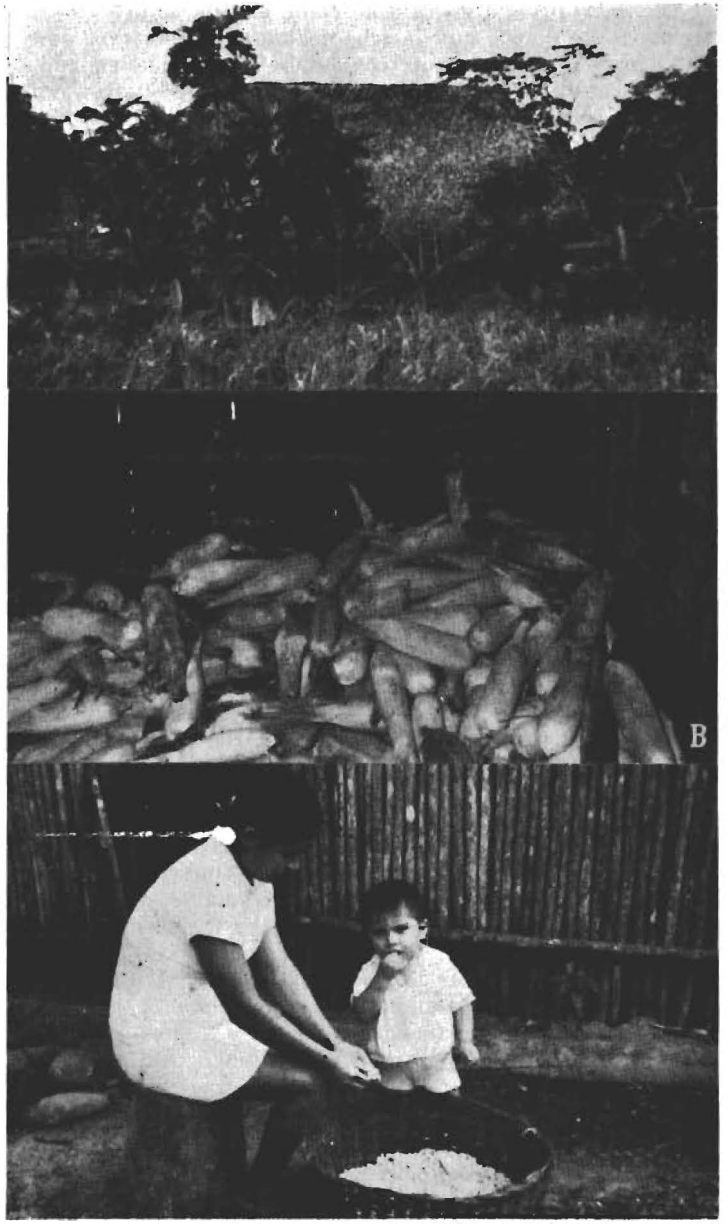

FIG. 2. A, typical hut of the peasants of Tabasco, who eat pozol as a basic food. B, maize stored as ears, and used for making pozol and other foods. C, young girl shelling the cobs.

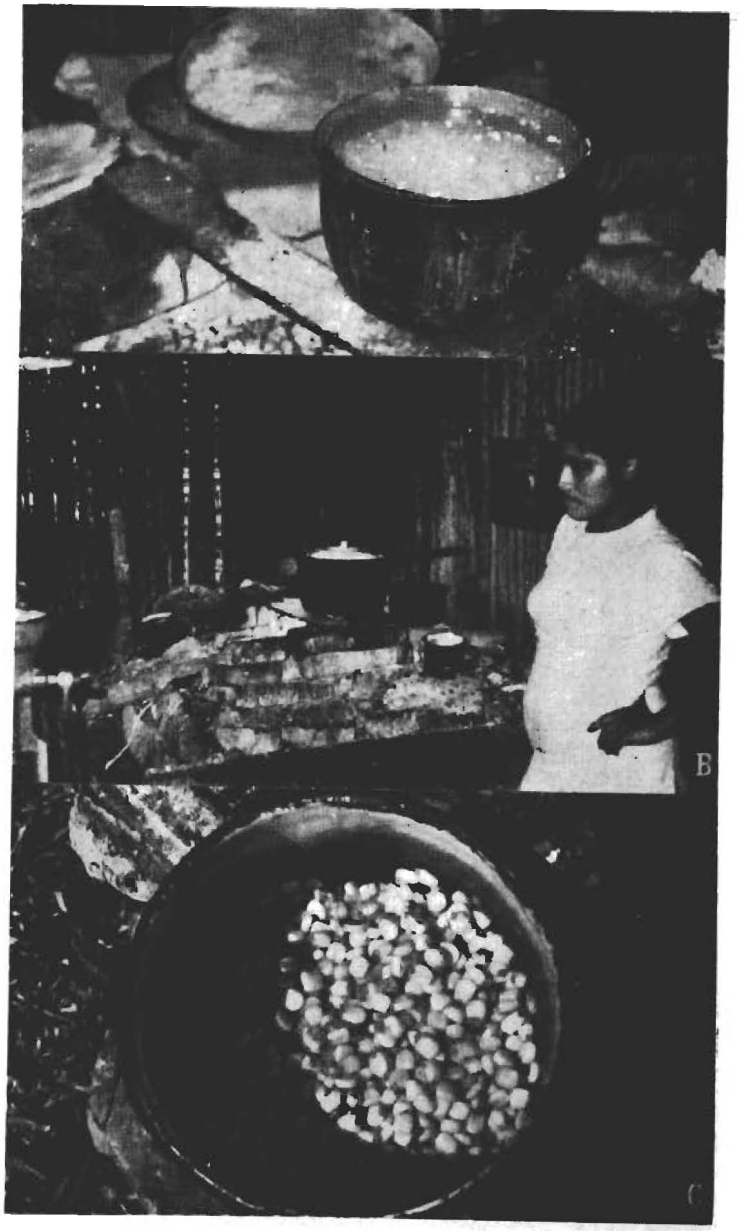

FIG. 3. A and B, maize kernels being cooked in lime water to prepare the nixtamal. C, nixtamal ready to be ground to obtain the dough for pozol.

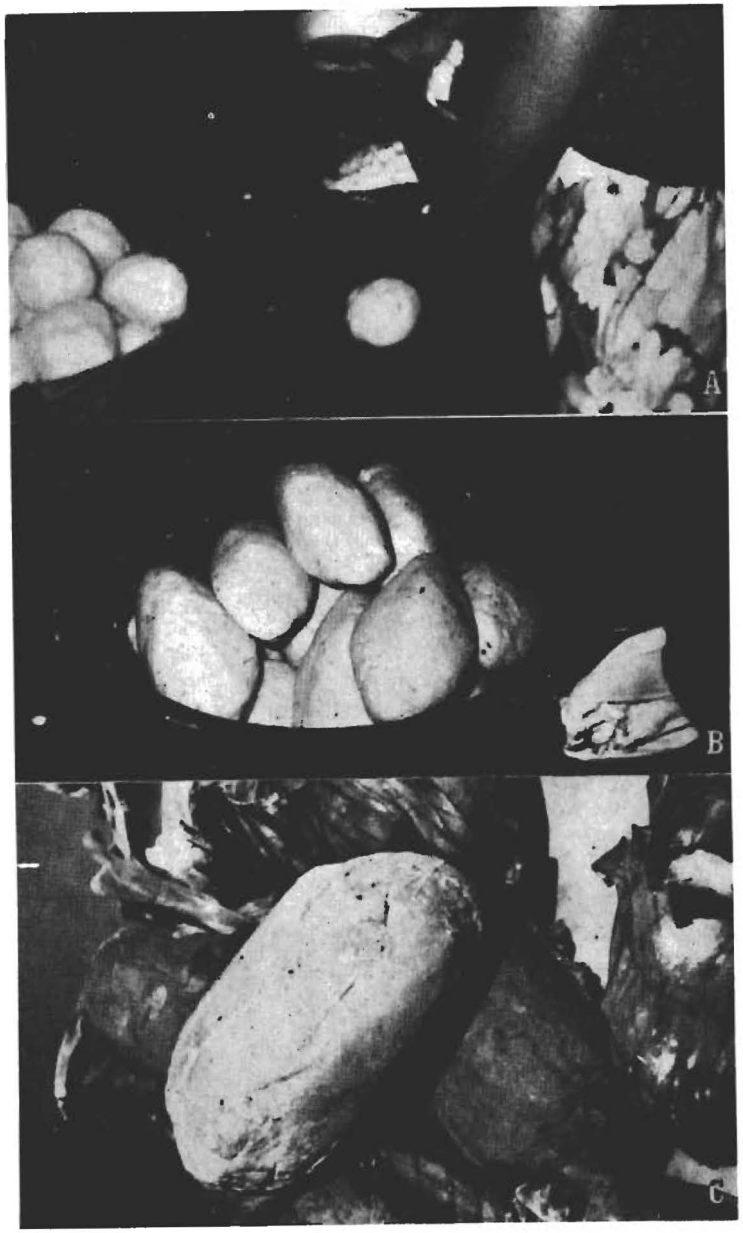

FIG. 4. A and B, the nixtamal is ground to make the dough balls, which are wrapped in banana leaves and allowed to ferment. C, moldy ball of pozol after 8 days of fermentation.

by means of a hygro-thermograph (Bacharach Instrument Co.), a hygrometer (Lambrecht), and an indoor-outdoor thermometer (407 Airguide); the outdoor bulb was permanently placed inside one ball of pozol in order to register its changes in temperature (Figs. 5A and 5B).

Study of the mycoflora at the different stages of preparation and fermentation of pozol. From randomly taken samples of maize and nixtamal kernels, there were drawn 100 kernels of each kind; the maize kernels were surface disinfected with a 1.0% solution of sodium hypochlorite for one minute and plated on petri dishes containing malt extract agar (MEA), placing 10 kernels/plate. The same was done with the nixtamal kernels, except that they were not immersed in the disinfectant solution in order to see whether or not the treatment with heat and lime water would have disinfected the kernels. The plates were incubated for 5 days at room temperature (28-30°C) and the colonies of molds and bacteria that developed from the kernels were counted and isolated in MEA tubes for further study. No yeasts were isolated from maize and nixtamal kernels.

At each fermentation stage of pozol (8 hrs, 1, 2, 4, 8 days) 5 balls were separated from the pile. From each ball serial dilutions were made in sterilized water, increasing the dilutions from $1:10^6$ for recently made pozol up to $1:10^{10}$ for pozol fermented 8 days. From each dilution 0.5 ml samples were inoculated onto petri dishes containing the following solidified culture media: malt extract agar, Brewer's anaerobic agar (BAA), tryptone glucose extract agar (TGEA), medium 77 for *Azotobacter* (Allen, 1951) modified with the addition of 2% sucrose instead of glucose and 2% purified agar (77SA), brilliant green bile agar (BGBA), and selective medium for lactobacilli (SL). The last medium is prepared by Merck, and the rest, except 77SA, by Difco. All of the inoculated plates were incubated at room temperature (28-30°C) for 2-3 days; the yeast and mold colonies that developed were counted and transfers from the representative kinds were made into tubes containing the same media as the plates on which they were growing. A plate of TGEA inoculated with a $1:10^6$ dilution of a recently made ball of pozol is shown in Figure 5C, and it gives an idea of the numbers and kinds of colonies that grew.

The molds isolated from maize and nixtamal kernels, and from pozol, were grown at 26°C on plates of MEA for 2 weeks to study their macroscopic characteristics, and in slide cultures of the same medium for 3-5 days to study their microscopic structures.

The yeast isolates were cultured in the media that are mentioned in the results section when *Trichosporon cutaneum* is described. All media used were Difco, except the pieces of carrot, cucumber, and chalk, as well as Fowell's (sodium acetate 0.5 g, agar 2.0 g, distilled water 100 ml), Gorodkowa's (glucose 0.1 g, peptone 1.0 g, sodium chloride 0.5 g, agar 2.0 g, distilled water 100 ml), and chalk agar (glucose 5.0 g, calcium carbonate 0.5 g, agar 2.0 g, distilled water 100 ml). All media mentioned above were utilized to see whether the isolated yeasts could produce sexual spores.

The tests of fermentation of carbohydrates by the yeast isolates were done

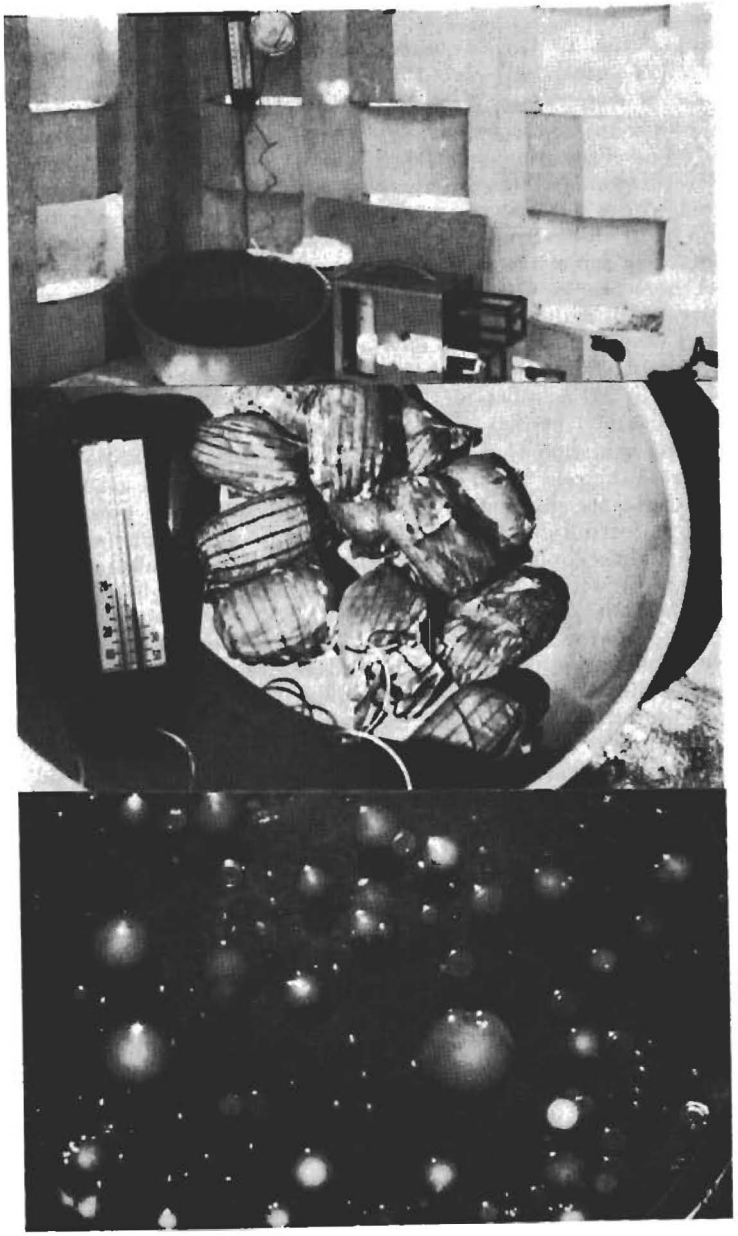

FIG. 5. A and B, experimental conditions during the fermentation of pozol. C, colonies of bacteria and yeasts developed on a plate of tryptone glucose extract agar, after inoculation with a $1{:}10^9$ dilution of recently made pozol.

according to Wickerham's method (*in* Lodder, 1970), and the tests of assimilation of carbon and nitrogen compounds were done with auxanograms (Lodder & Kreger-van Rij, 1952). All cultures were incubated at 26°C except when indicated otherwise.

All mold and yeast colonies illustrated in this paper are 2-3 weeks old on MEA; the microscopic structures shown were formed in the same medium at 3-5 days.

Considering that several yeasts isolated from the pozol studied could possibly be new species of *Candida*, they are being carefully studied and will be described in a separate paper when a definit identity is obtained; however, in this paper such Candidas are included in the counts as *Candida* spp. Nos. 1, 2, 3, and 4 (Tables 4-6).

RESULTS AND DISCUSSION

The changes in pH and moisture content that occurred during the process of preparation and fermentation of pozol are shown in Figure 6. The acid pH of the maize kernels was raised to 7.5 due to the treatment with lime water, which itself had a pH of 11.75. The maize dough obtained from the nixtamal kernels had a slightly lower pH (6.8), probably due to a more homogeneous mixing of the kernles in grinding. As fermentation proceeded the pH gradually decreased reaching 3.93 on the eighth day. The low moisture content of the maize kernels (6.24%) increased to 22.7% after cooking in lime water, and to 30.5% in the dough, since more water was added to the dough to make the shaping of the balls easier. During the following 8 days, the moisture content of pozol remained quite stable, around 30%, because the banana leaves used to wrap the balls retarded the evaporation of water, although the surface of the balls dried somewhat as the days passed. It is important to note that moisture content of pozol balls was determined by oven drying slices comprising portions of the surface and the interior of the balls; hence, the figures reported represent the overall moisture content of pozol balls. The graphed data of Figure 6 resulted from an average of five determinations at each stage of the process.

The species of molds isolated from maize and nixtamal kernels, as well as those from pozol, were identified from the relevant monographs (Carmichael, 1957; Raper & Fennell, 1965; Barron, 1968; Zycha & Siepmann, 1969; Ellis, 1971). The percentages of the species isolated from maize and nixtamal kernels are shown in Table 2. Despite the low moisture content of maize kernels, *Fusarium moniliforme* Sheldon was quite abundant (77%), although *Cladosporium cladosporioides* (Fresen.) de Vries was isolated from only a few kernels (3%). Apparently these two molds were killed by the treatment with lime and heat since they were not found in nixtamal, neither in the kernels nor in the lime water. Other species, such as *Aspergillus carbonarius* (Bainier) Thom (1%), *A. parasiticus* Speare (2%), *Monilia sitophila* (10%), and *Trichoderma viride* (18%), as well as many bacteria (70%), were isolated from

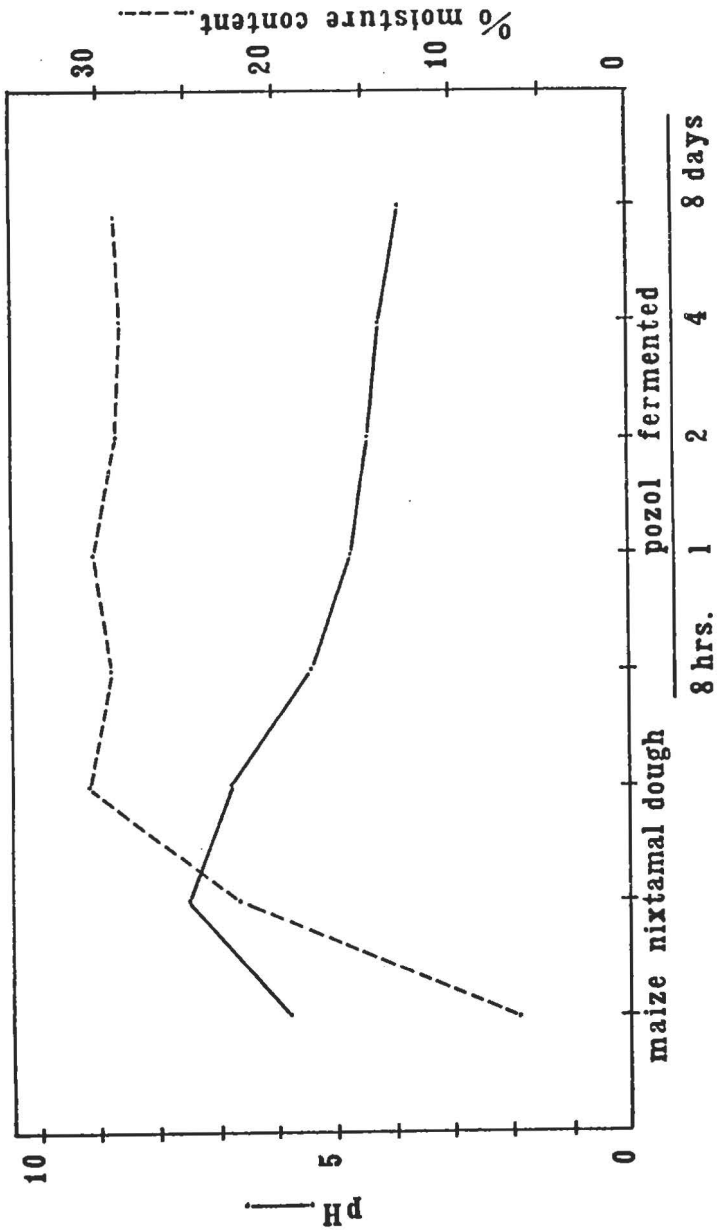

FIG. 6. Changes in pH and moisture content during the different stages of preparation and fermentation of pozol. The plotted figures are the average of 5 readings at every stage.

TABLA 2

Percentages of molds and bacteria isolated from maize and nixtamal kernels on MEA medium[a]

	Maize	*Nixtamal*[b]
Aspergillus carbonarius	0.0	1.0
A. parasiticus	0.0	2.0
Cladosporium cladosporioides	3.0	0.0
Fusarium moniliforme	77.0	0.0
Monilia sitophila	0.0	10.0
Trichodrema viride	0.0	18.0
Bacteria	0.0	70.0
Kernels free of molds and bacteria	20.0	5.0

[a] Results from 100 kernels of each kind. Ten kernels/plate.
[b] The figures do not add to 100 because some kernels had more than one kind of mold.

nixtamal. Due to the fact that these molds were absent in the maize kernels and the lime water it is very probable that they were added with the well water used to rinse the nixtamal and eliminate most of the lime before grinding it to make the dough, by the air, or with the utensils and hands during the manipulation of nixtamal.

The species of molds isolated from maize and nixtamal kernels are illustrated in Figures 7-8. *Monilia sitophila* was found in nixtamal and in pozol after 2 days of fermentation; whereas some, i. e. *Aspergillus carbonarius, A. parasiticus,* and *Trichoderma viride,* which were present in nixtamal, were not found in pozol.

In Figure 9 is shown the course of environmental and pozol temperatures, and relative humidity during the fourth day of pozol fermentation (March 26); the data for the other days were very similar (Table 3). Figure 9 shows

TABLA 3

Cardinal temperatures (°C) of environment and % relative humidity during the period of pozol fermentation

		Temperature			*Relative humidity*	
March	*Day of fermentation*	*minimum*	*maximum*	*mean*	*minimum*	*maximum*
22	0	22.0	33.3	25.0	55	84
23	1	22.0	34.0	26.0	50	94
24	2	20.0	37.8	26.0	32	65
25	3	20.0	38.0	27.0	50	88
26	4	19.0	32.5	24.0	50	96
27	5	21.0	33.3	25.0	47	94
28	6	21.0	41.1	31.0	21	72
29	7	22.5	40.0	31.2	26	70
30	8	23.5	39.5	27.0	25	65

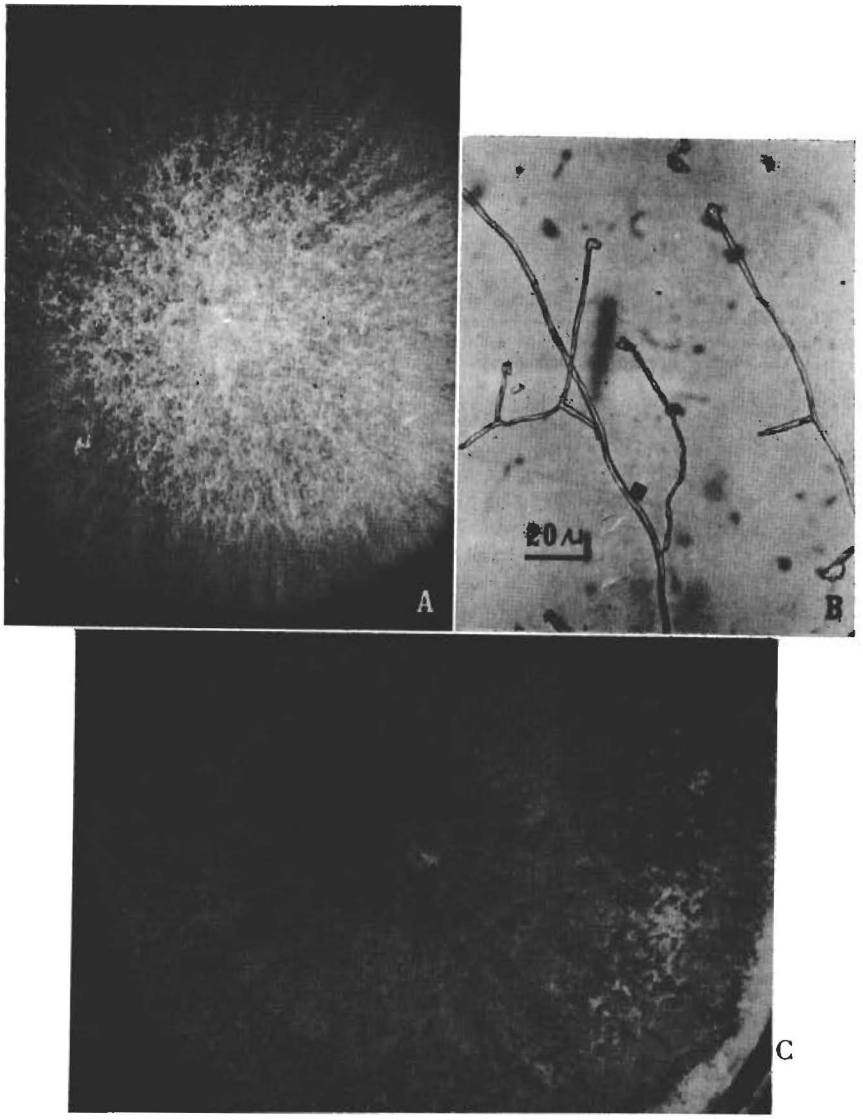

FIG. 7. *Fusarium moniliforme* and *Trichoderma viride*.—A, colony of *Fusarium moniliforme*, isolated from maize kernels. B, phialides of *F. moniliforme* with long chains or with mucilaginous heads of microconidia X 500. C, colony of *Trichoderma viride*, isolated from nixtamal kernels.

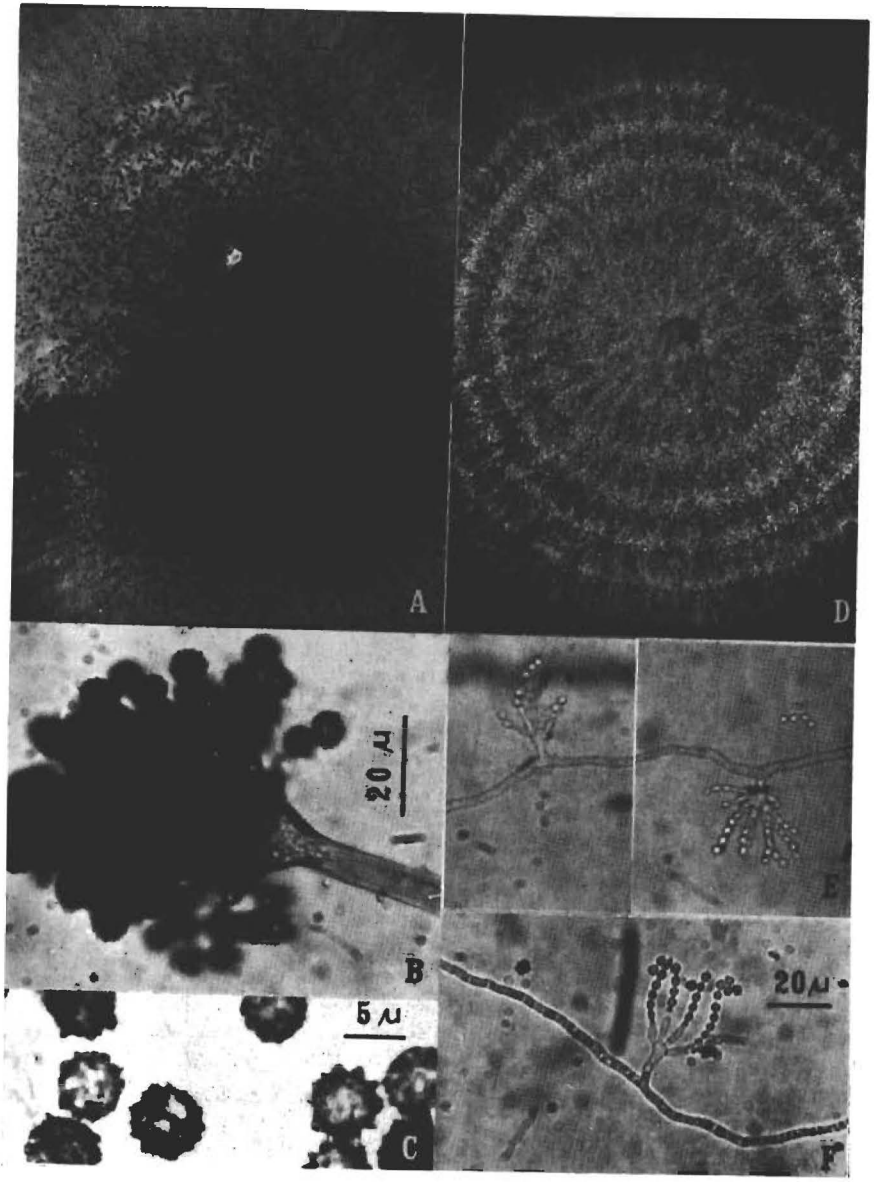

FIG. 8. *Aspergillus carbonarius* and *A. parasiticus*.—A, colonies of *A. carbonarius*. B, conidiophore head of *A. carbonarius* with phialospore chains, X 800. C, echinulate phialospores of *A. carbonarius*, X 2000. D, colony of *A. parasiticus*. E-F, conidiophore heads of *A. parasiticus* with phialospore chains, X 500. Both species were isolated from nixtamal kernels.

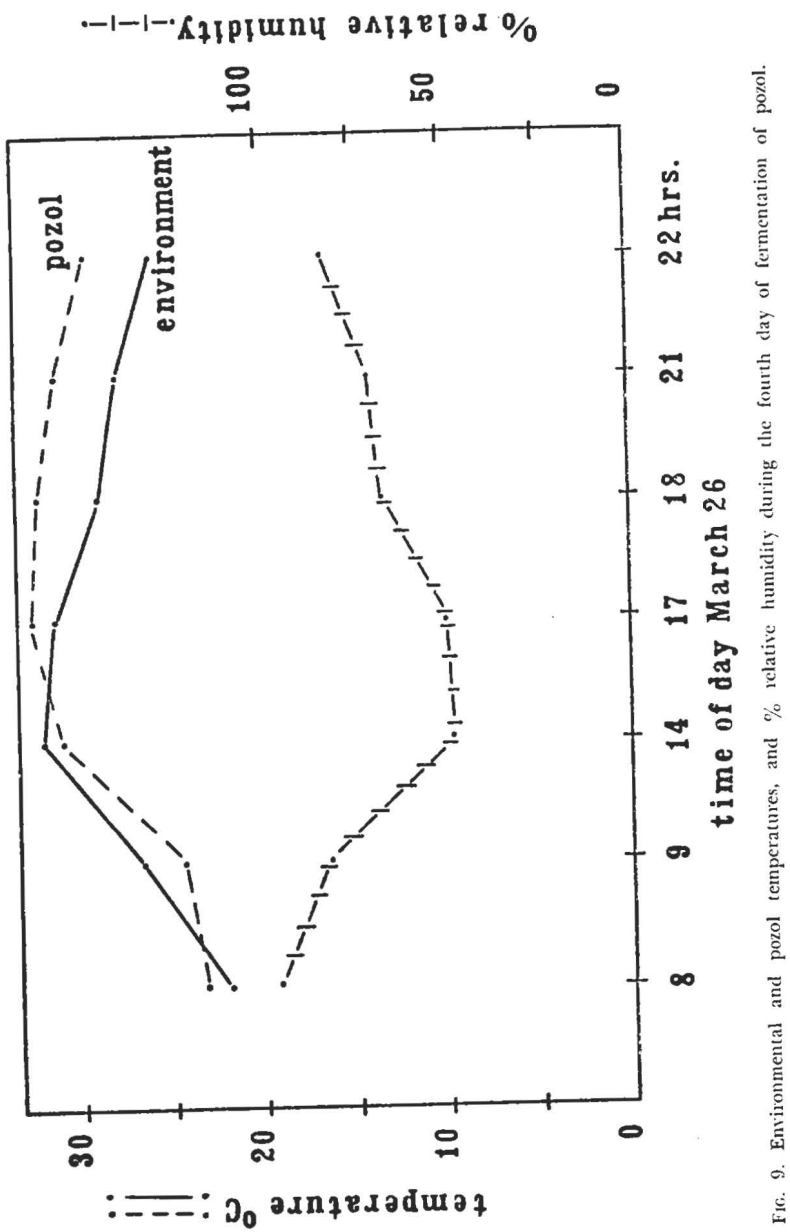

FIG. 9. Environmental and pozol temperatures, and % relative humidity during the fourth day of fermentation of pozol.

that relative humidity decreased as environmental temperature increased and viceversa. With respect to pozol temperature, it was found that pozol balls warmed and cooled slower than environment. This could be due to the insulating properties of the banana leaves that covered the balls. It is also probable that the microorganisms metabolizing during fermentation liberated some heat.

In Tables 4-6 are shown the numbers of yeast and mold colonies developed on six culture media inoculated with serial dilutions of pozol of different fermentation times. Six media were used, ranging from rich semisynthetic ones, such as MEA, BAA, TGEA, BGBA, and SL, to a poor synthetic one, 77SA, in order to isolate a wider spectrum of microorganisms. MEA was utilized because it is a good medium for molds and yeasts; BAA because most of the yeasts and bacteria of pozol previously studied are facultative with respect to oxygen requirements and can grow in microaerophilic conditions (Herrera & Ulloa, 1970). TGEA is adequate for bacteria, although yeasts and molds can also grow on it; BGBA is selective for coliform bacteria, and SL for lactobacilli; 77SA was utilized to see if nitrogen fixers could be isolated.

It is important to mention that great amounts of bacteria were present on

TABLA 4

Counts of yeast and mold colonies[a] isolated from pozol at different fermentation stages on MEA and BAA.

	Candida spp. Nos. 1+2+3+4[b]	*T. cutaneum* + *G. candidum*[b]	*C. cladosporioides*	*M. sitophila*	*M. rouxianus*
MEA					
just made	30×10^{7}	—	—	—	—
8 hrs	55×10^{8}	2×10^{9}	—	—	—
1 day	4×10^{9}	2×10^{9}	1×10^{9}	—	—
2 days	12×10^{10}	10×10^{9}	1×10^{9}	—	1×10^{10}
4 days	20×10^{10}	10×10^{9}	1×10^{9}	1×10^{10}	2×10^{10}
8 days	25×10^{10}	20×10^{9}	1×10^{10}	2×10^{10}	—
BAA					
just made	50×10^{7}	—	—	—	—
8 hrs	133×10^{8}	—	—	—	—
1 day	6×10^{9}	2×10^{9}	1×10^{9}	—	—
2 days	128×10^{9}	20×10^{9}	1×10^{9}	—	—
4 days	307×10^{9}	10×10^{9}	—	—	—
8 days	114×10^{9}	20×10^{9}	—	1×10^{10}	—

[a] Average of the numbers of colonies obtained from 5 balls of pozol for every fermentation stage.

[b] The figures for the colonies of these species were added because they could not be differentiated morphologically at the time of counting.

— Colonies absent.

TABLA 5

Counts of yeast and mold colonies[a] isolated from pozol at different fermentation stages on TGEA and 77SA.

	Candida spp. Nos. 1+2+3+4[b]	*T. cutaneum* + *G. candidum*[b]	*C. cladosporioides*	*M. sitophila*	*M. rouxianus*
TGEA					
just made	32×10^7	—	—	—	—
8 hrs	64×10^8	—	—	—	—
1 day	5×10^9	—	1×10^9	—	—
2 days	70×10^9	2×10^{10}	1×10^{10}	—	—
4 days	59×10^{10}	—	1×10^9	—	—
8 days	90×10^9	—	—	1×10^{10}	—
77SA					
just made	—	—	—	—	—
8 hrs	—	—	3×10^9	—	—
1 day	—	—	1×10^9	—	—
2 days	7×10^9	—	—	—	—
4 days	6×10^9	—	—	—	—
8 days	—	—	—	—	—

[a] Average of the numbers of colonies obtained from 5 balls of pozol for every fermentation stage.
[b] The figures for the colonies o these species were added because they could not be differentiated morphologically at the time of counting.
— Colonies absent.

the plated media inoculated with pozol dilutions, with numbers similar or surpassing the numbers of yeasts and molds, mainly at the beginning of fermentation. Among the bacteria seen were bacilli of various kinds and sizes, cocobacilli, micrococci, streptococci, and staphylococci, but they were not identified because this was beyond the scope of the present study; this; however, does not mean that bacteria could be any less important than yeasts in the process of pozol fermentation. Even though the bacteria were not studied, the media used to isolate them are included in this work because yeasts and molds were also isolated on such media. It would be interesting to study the bacteria of pozol.

Due to the fact that yeast colonies are very similar macroscopically and that the isolated yeasts could be differentiated from each other only after fermentation and assimilation tests, it was necessary to report the total numbers of colonies developed on the media utilized; however, such numbers really correspond to the four species of *Candida* mentioned above. A similar situation occurred with the white, filamentous, very similar, colonies of *T. cutaneum* and *Geotrichum candidum,* which were differentiated from each other only when the microscopical study was done and the fermentation and assimila-

TABLA 6

Counts of yeast and mold colonies[a] isolated from pozol at different fermentation stages on BGBA and SL.

	Candida spp. Nos. 1+2+3+4[b]	*T. cutaneum* + *G. candidum*[b]	*C. cladosporioides*	*M. sitophila*	*M. rouxianus*
BGBA					
just made	—	—	—	—	—
8 hrs	—	—	—	—	—
1 day	—	6×10^9	—	—	—
2 days	—	2×10^{10}	—	—	1×10^{10}
4 days	4×10^9	2×10^{10}	—	—	—
8 days	—	—	—	—	—
SL			—	—	—
just made	5×10^9	—	—	—	—
8 hrs	5×10^9	—	—	—	—
1 day	3×10^9	—	—	—	—
2 days	6×10^9	6×10^9	—	—	—
4 days	25×10^9	—	—	—	—
8 days	8×10^9	—	—	—	—

[a] Average of the numbers of colonies obtained from 5 balls of pozol for every fermentation stage.
[b] The figures for the colonies of these species were added because they could not be differentiated morphologically at the time of counting.
— Colonies absent.

tion tests were carried out. These last two species were found to be non fermenters.

The figures reported in Tables 4-6 show that the numbers of yeasts and molds increased as pozol fermentation proceeded and that more species and greater numbers were isolated on MEA, BAA, and TGEA. The fewest microorganisms were isolated on 77SA, and this could mean that few nitrogen fixers were present in pozol. However, several species of bacteria which were isolated on MEA and TGEA (media that are not selective for growing nitrogen fixers because they contain nitrogen sources) when cultured in a 15% suspension of maize dough, or in Burk's medium, were able to reduce acetylene to ethylene; this reatcion indicates the capacity to fix nitrogen (Dilworth, 1966) and has been utilized for *Agrobacterium azotophilum,* which was isolated from pozol of Chiapas (Taboada, Herrera & Ulloa, 1971; Ulloa & Herrera, 1972).

It is very significant that *Geotrichum candidum, Trichosporon cutaneum,* and *Monilia sitophila,* species that had been reported from pozol from several localities in Chiapas, were again found, now in pozol from Tabasco; also, other species of *Cladosporium* and *Mucor, C. herbarum* and *M. racemosus,*

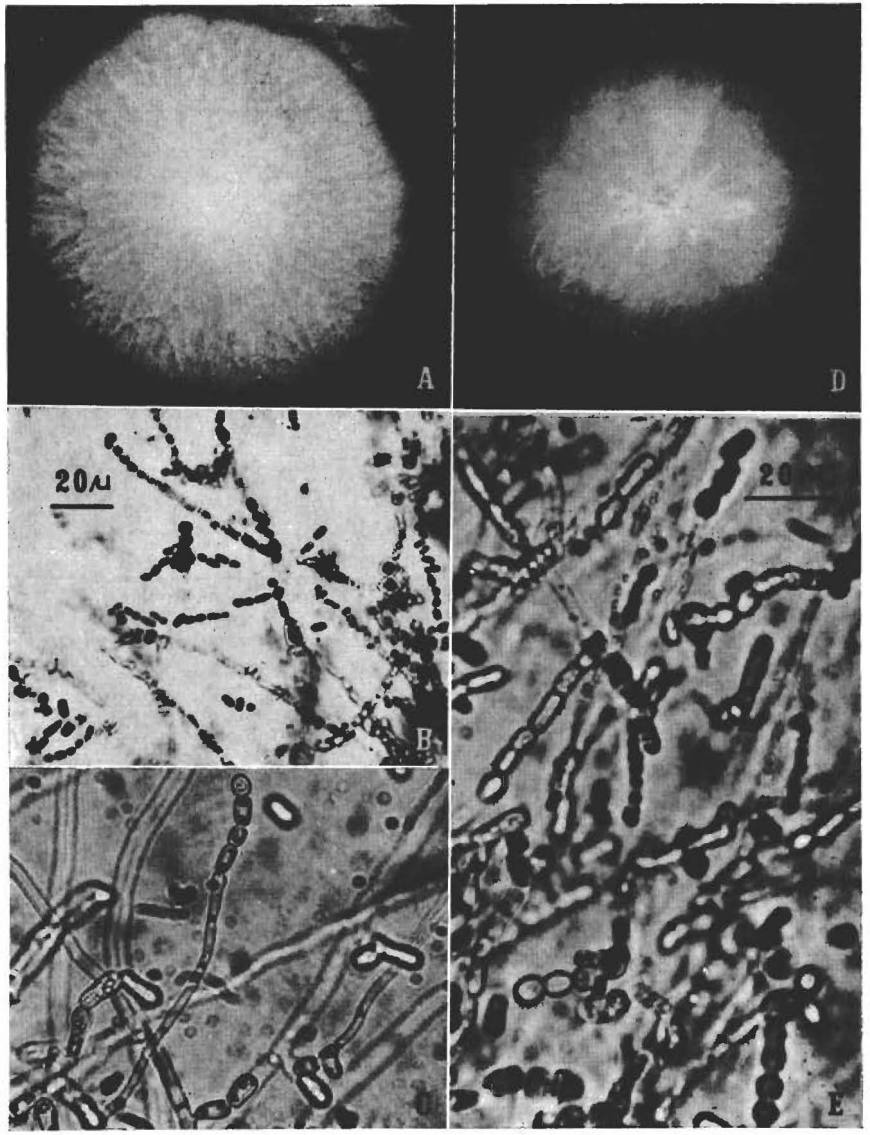

FIG. 10. *Geotrichum candidum* and *Trichosporon cutaneum.*—A, colony of *G. candidum,* B-C, arthrospore chains of *G. candidum,* X 500. D, colony of *T. cutaneum,* E, arthrospore and blastospore chains of *T. cutaneum,* X 800. Both species were isolated from pozol.

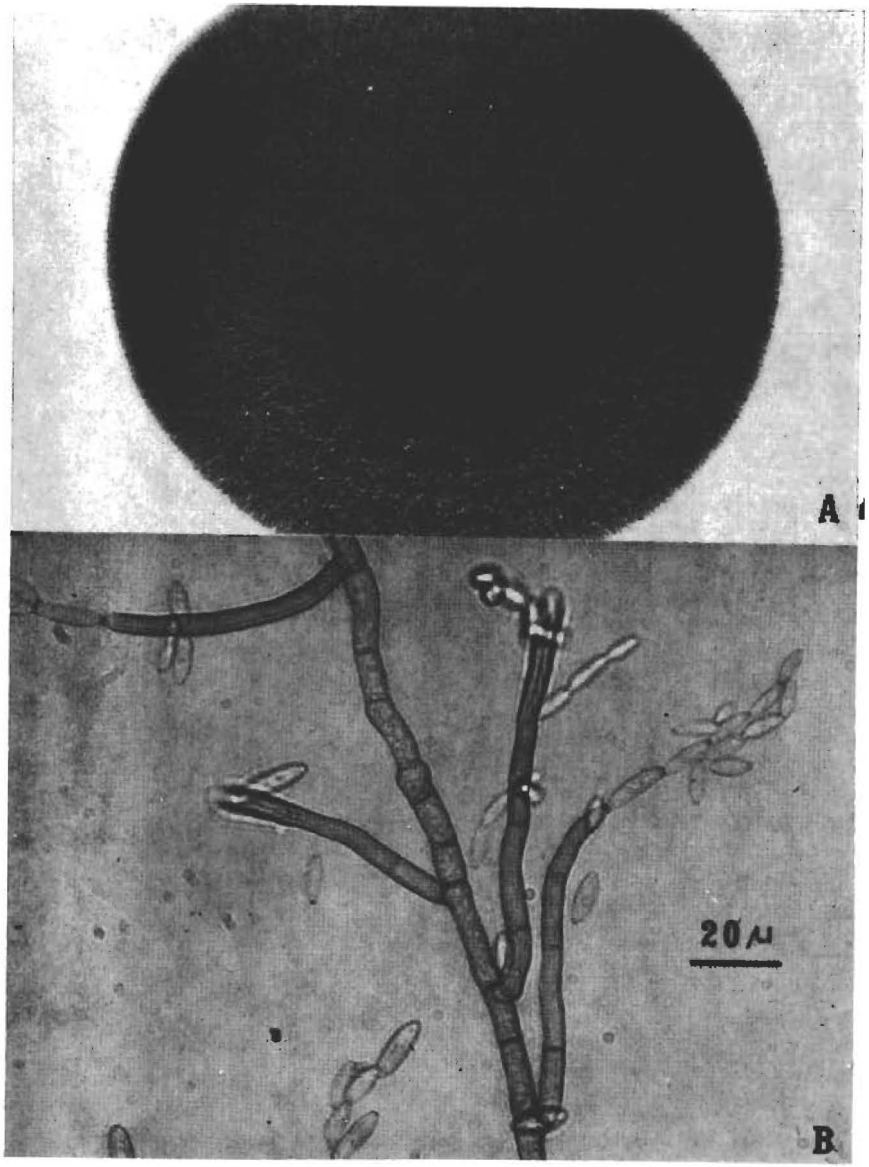

FIG. 11. *Cladosporium cladosporioides* isolated from maize kernels and pozol. A. colony. B, conidiophore with blastospore chains, X 625.

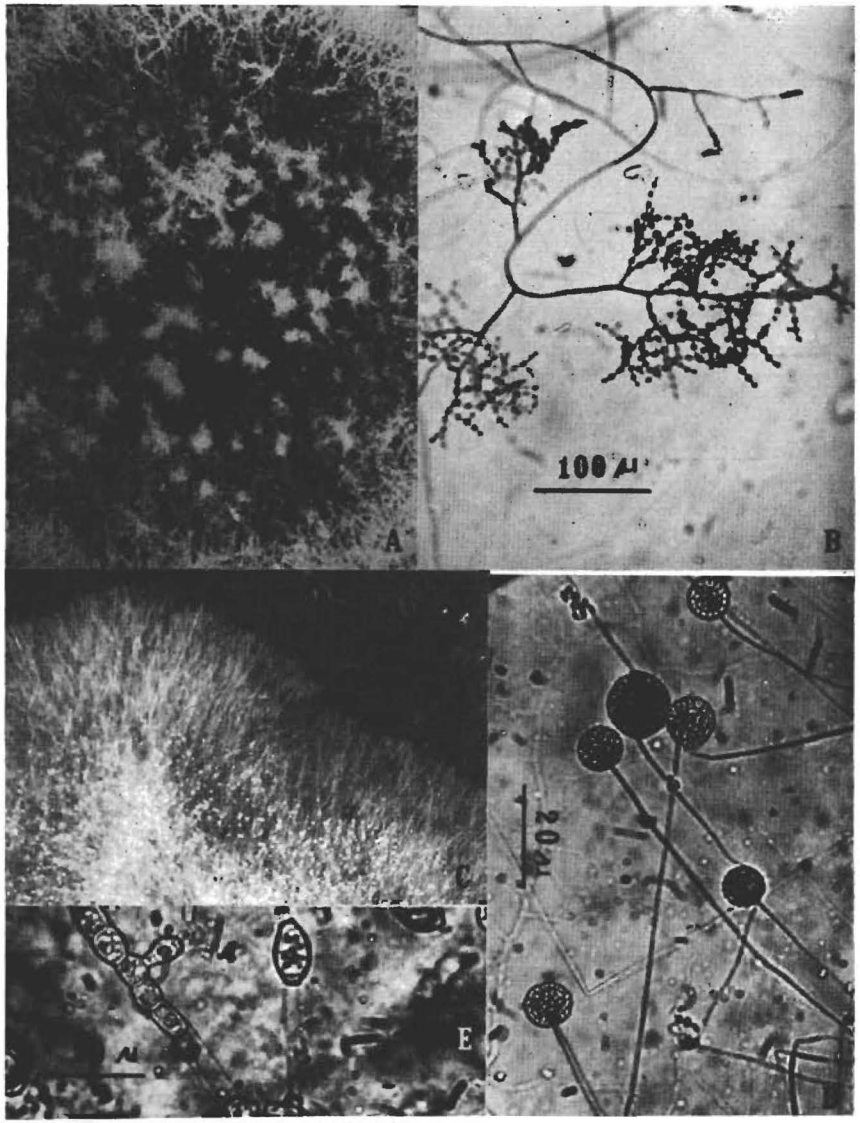

FIG. 12. *Monilia sitophila* and *Mucor rouxianus*.—A,B. *M. sitophila*, isolated from nixtamal kernels and pozol. A. colony. B. conidiophore with blastospore chains. X 200. C,D,E. *Mucor rouxianus*, isolated from pozol. C. colony. D. sporangia. X 800. E. chlamydospores, X 800.

had been isolated from pozol from Chiapas, and now *Cladosporium cladosporioides*, and *Mucor rouxianus*, which is in the same section as *M. racemosus*, were isolated form pozol from Tabasco. Yeasts of the genus *Candida*, such as *C. krusei* and *C. parapsilosis*, or of the perfect state *Hansenula*, such as *H. fabianii* and *H. pozolis*, have been found in fermented pozol from different places in Chiapas (Herrera & Ulloa, 1971, 1972; Herrera, Ulloa & Fuentes, 1973). Now, several yeast isolates obtained from pozol from Tabasco have been found to belong to the genus *Candida*. All these findings would indicate that despite the expected variation in pozol microflora, due to the uncontrolled manner of preparation, the microflora exhibits a certain constancy in occurrence of the same or closely related species of molds and yeasts.

From the collateral observations done on the bacteria, the findings of previous investigations, and the yeast and mold counts done at the different fermentation times of pozol, it can be postulated that bacteria, yeasts of the genus *Candida* and *T. cutaneum*, and *G. candidum* (Fig. 10) are present from the first hours of fermentation and continue growing for several days, and that as the surface of the pozol balls progressively dries and their pH is lowered, molds such as *Cladosporium cladosporioides*, *Monilia sitophila* and *Mucor rouxianus* also invade to substrate to constitute a complex mixture of bacteria, yeasts, and molds. The species of molds isolated from pozol are illustrated in Figures 10-12.

The yeast isolates obtained from pozol were morphologically and physiologically studied. Of such isolates, one corresponded to *Candida* sp. No. 1; seven to *Candida* sp. No. 2; three to *Candida* sp. N. 3; two to *Candida* sp. No. 4; and four to *Trichosporon cutaneum*. Only the latter yeast is described below.

Trichosporon cutaneum (de Beurm., Gougerot & Vaucher) Ota (Fig. 10 D-E)

Growth on MEA: After 5 days the colony shows good growth and is white to cream-colored, opaque, and with fringed border. True mycelium and arthrospores, are formed. The size of the arthrospores is extremely variable, usually (3.5-7) × (3.5-14) µ. Some blastospores are formed. The mycelial cells reach 28 µ in length.

Growth in liquid malt extrat: After 3 days a pellicle is formed; it is dull, white or cream-colored, smooth, wrinkled, or folded, thick, and often creeping. The morphology of the cells is similar to that developed on MEA.

Fermentation:

Glucose —
Galactose —
Sucrose —
Maltose —
Lactose —
Cellobiose —
Trehalose —
Melibiose —
Raffinose —
Melezitose —
Inulin —

Assimilation:

Glucose +	Melibiose −
Galactose +	Raffinose +
L-Sorbose −	Melezitose +
Sucrosa +	Inulin −
Maltose +	Soluble starch −
Cellobiose −	D-Xylose +
Trehalose +	D-Arabinose −
Lactose +	L-Arabinose +
D-Ribose +	
L-Rhamnose +	D-Glucitol −
Ethanol +	α-Mehyl-D-Glucoside +
Glycerol +	Salicin −
Erythritol +	DL-Lactic acid +
Ribitol −	Succinic acid +
Galactitol +	Citric acid −
D-Mannitol −	Inositol +

Assimilation of potassium nitrate: Negative.
Assimilation of potassium nitrite: Positive.
Growth in vitamin-free medium: Negative.
Vitamins stimulating growth: Thiamine and boitin.
Growth at 37°C: Positive.

It is important to note that all yeasts isolated from pozol, in fact all the microorganisms so far reported from this food, are not capable of hydrolyzing starch, which is the main substance of maize dough. This would explain why pozol is not liquefied, at least during the time it is normally allowed to ferment for human consumption, a time that depends upon the taste of the consumers, and which can vary from 1 to 5 days up to 2 or more weeks. Of course, molds such as *Monilia sitophila* and *Mucor rouxianus* can hydrolyze starch, but they are rarely found in the interior of pozol balls, and their growth is not so abundant.

The study of the microbial flora of pozol has been interesting. New species of bacteria have been found. A new bacterium, *Agrobacterium azotophilum,* has interesting properties, such as the capacity to fix nitrogen while growing in some by-products and wastes of the sugar industry (Taboada, Ulloa & Herrera, 1973); the same bacterium has been found to produce *in vitro* potent antibiotics against several species of *Aspergillus* and *Penicillium, Monilia sitophila, Rhizopus stolonifer,* and *Trichoderma viride* (unpublished data). The presence of antifungal substances produced by *A. azotophilum* while growing in pozol might be related to the fact that molds do not invade the balls of pozol during the first few days. However, this situation is variable and depends on whether or not the bacterium is present, the pH, moisture content of pozol, and temperature.

As was mentioned before, pozol is consumed raw, and consequently great numbers of living bacteria, yeasts, and molds are ingested. So far, there is no evidence that routine ingestion of pozol could be associated with diseases of the consumers, but considering the fact that pozol has been prepared and eaten for centuries with no apparent harm to the people who normally eat it, the possibilities seem to be low. Perhaps some immunity has been acquired by such people, but there is no scientific knowledge about this.

Other fermented maize products formerly domestically prepared by certain ethnic groups, because of the importance of such products in the diet of large populations have been studied for large scale preparation under controlled conditions of quality and wholesomeness. For instance, the "mahewu", a traditional beverage of the Bantu people in South Africa, is now prepared by lactic fermentation, mainly carried on by *Lactobacillus delbrueckii* (Leichmann) Beijerinck, of a gruel obtained from sprouted maize kernels (Schweigart & Wit, 1960; Schweigart & Fellingham, 1963). The same Bantu consume an alcoholic beverage, named "kaffir beer", which is prepared on an industrial scale from malted sorghum or maize kernels (Schwartz, 1956); in the preparation process there is a lactic fermentation, produced by *Lactobacillus brevis* (Orla-Jensen) Bergey *et al.,* followed by an alcoholic fermentation, produced by *Saccharomyces cerevisiae* (Hansen), *Candida krusei* (Cast.) Berkhout, and *Kloeckera apiculata* (Reess emend. Klöcker) Janke (van der Walt, 1956). In the case of mahewu, the protein content is 7%-9% and of poor quality, and the B vitamin content remains similar to that of maize, although different supplements can be added without affecting acceptability (Schweigart & Wit, 1960). Kaffir beer was found to be deficient in lysine but the content of tryptophan is higher than that of maize (Horn & Schwartz, 1961). In neither mahewu nor kaffir beer is an increase in total nitrogen content reported.

Taking into account the fact that during the fermentation of the maize dough to get pozol there is an increase in the total nitrogen content, some essential amino acids, such as lysine and tryptophan, and some vitamins, such as riboflavin and niacin, due to the presence of nitrogen fixing microorganisms (Cravioto *et al.,* 1955; Ulloa, Herrera & de la Lanza, 1971) and of various yeasts and molds, it would be desirable if pozol production were also industrialized for human or/and animal consumption.

From the results obtained in this study and previous investigations on the microbial flora of pozol, it can be seen that certain microorganisms are consistently present in the fermentation process of this food. However, in order to know which species should be utilized to prepare pozol under controlled conditions of quality and wholesomeness, it would be necessary to know which microorganisms produce what chemical change in the substrate. Biochemical studies on the fermentation process of pozol are lacking.

The microbiological studies on pozol, mainly the finding that nitrogen fixation occurs in this food, have stimulated an interest in microbiological and biochemical studies of other fermented foods native to Mexico, such as "tesgüino" and "pulque". Tesgüino is a fermented beverage which is drunk

mainly by the Tarahumar and Tepehuan Indians of northwestern Mexico, who prepare it by alcoholic fermentation of malted maize kernels that have been ground and cooked; *Saccharomyces cerevisiae* was found to be important in the fermentation process (Herrera & Ulloa, 1973). Pulque is an alcoholic beverage which is drunk mainly by the Indian population of the central part of Mexico and is produced by fermentation of the sugary secretion of "maguey" (*Agave atrovirens* Karw. and other species of *Agave*); the fermentation of pulque involves *Saccharomyces carbajali* Ruiz-Oronoz and other yeasts species (Sánchez-Marroquín, 1962). By utilizing the acetylene reduction technique, nitrogen fixing microorganisms have been found in tesgüino and pulque, but the isolated microorganisms have not been identified (Herrera, Taboada & Ulloa, 1972; Taboada, 1973).

All the findings on pozol that have been discussed and the comments made on other fermented beverages of indigenous origin indicate that it would be interesting to undertake similar studies on other fermented foods, particularly those made from maize, of which there are about ten native to Mexico and a similar number in other Latin American countries that have not been explored at all (Cruz-Ulloa & Ulloa, 1973); it is possible that, as in the case of pozol, new species of bacteria, yeasts, or molds could be discovered.

Hesseltine (1965) in his paper on various fermented foods, mainly from China, Indochina, Japan, Indonesia, and other oriental countries, considers that fermenting foods with fungi has certain disadvantages. Any additional processing results in additional time, equipment, and labor cost. Also, some loss will occur in washing away of nutrients in preparation for fermentation. The following question is then raised by Hesseltine: "why the fermentation of foods is used on such a wide scale in various parts of the world where food often is in short supply?". Hesseltine makes some comments in trying to answer this question, considering that the fungi involved synthesize one or more desirable enzymes, whose action on the substrate presumably enhances the digestibility of the foods consumed. Also, fermentation almost invariably adds to the flavor of the food and changes the texture, color, or physical state. Millions of people live in areas where the climate is warm or tropical accompanied by high humidity; the fermentation of foods is done to prevent the growth of spoilage or food poisoning microorganisms. In some instances, the fermentation may increase the protein and vitamin content of foods; thus, riboflavin and niacin increase about threefold during the fermentation of soybeans to produce tempeh.

In the case of pozol fermentation, the disadvantage is that there is no control in its preparation, with the consequence that undesirable microorganisms can grow in it. Ulloa & Herrera (1970) have shown that if the maize kernels used to make pozol are contaminated with the aflatoxins produced by *Aspergillus flavus* Link ex Fr., most of the aflatoxins are destroyed during the treatment with lime water and heat but the remaining toxins persist during the fermentation of the dough. On the other hand, the advantages of fermenting the dough are the preservation of pozol without refrigeration under the tropical

conditions of the regions where it is routinely eaten, probably due to its acidity, but mainly the improvement of the nutritional qualities of this maize product due to the development of certain bacteria, yeasts, and molds.

The great increase in the human population of the world demands not only a greater production of carbohydrates but, surely in a more urgent manner, a greater production of proteins. An interesting approach to this problem has been made by Gray (1964, 1966, 1970) who suggests that certain of the fungi imperfecti can make large contributions to the world protein pool by converting carbohydrates and inorganic nitrogen to protein.

The bacteria, yeasts, and molds that develop during the natural process of pozol fermentation convert part of the carbohydrates and inorganic nitrogen into proteins of their cells and are responsible for the improvement in the nutritional qualities of this maize product that is a dietetical discovery of the ancient civilizations of Mexico.

CONCLUSIONS

Most of the microorganisms present in the maize kernels used to make pozol are destroyed during the treatment with lime water and heat to produce the nixtamal. It is during the processing of nixtamal that inoculation of the maize dough takes place; since no sanitary measures are taken by the people who prepare pozol, their hands, the water, the utensils used, the air, and in general the surroundings of the place of preparation of the food, are all probable sources of microorganisms. Nevertheless, despite the excepted variation in the microflora of pozol due to the uncontrolled manner of preparation, there are several species of yeasts and molds which have always been found in pozol from different places and prepared at different times.

It is important to note that in the tropical region where pozol is prepared and consumed, the fluctuations of weather are not as great as in the temperate or more northern areas of the country. So, the consistent occurrence of certain species of microorganisms in pozol could be related to the type of substrate and the environmental conditions prevalent at the areas where pozol is prepared.

It is very significant that *Geotrichum candidum, Trichosporon cutaneum,* and various species of *Candida* have always been associated with the natural process of pozol fermentation. Molds such as *Cladosporium cladosporioides* or *C. herbarum, Monilia sitophila* and *Mucor rouxianus* or *M. racemosus* (which is in the same section as *M. rouxianus*) are also common in pozol.

Bacteria of various kinds are also very abundant in pozol, particularly at the beginning of fermentation; and among them are some able to fix nitrogen.

If pozol is ever to be prepared under controlled conditions of quality and wholesomeness, it will be necessary to study the chemical changes that each of the commoner species found in pozol produces in this food, as well as to perform pathogenicity tests with the species potentially pathogenic to man or to animals. Only after doing this it would be possible to select for the desirable microorganisms in order to prepare a food product of constant qualities.

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SUMMARY

This paper presents the results of a mycological study of pozol, a food product of Mayan origin that is prepared by fermentation of maize dough; the fermented dough is diluted in water and drunk as a basic food by the Indian and mestizo populations of southeastern Mexico.

The pozol studied was prepared at Cárdenas, Tabasco, México, a tropical location where pozol is routinely consumed, mainly by the peasants.

The objectives of the study were to isolate at the site of manufacture the mycoflora present at different stages of preparation of pozol: maize kernels, kernels cooked in lime water, recently made dough, and dough fermented for 8 hours, and 1, 2, 4, and 8 days; and to record pH, moisture content, relative humidity, and temperature during the whole process.

The acid pH of maize kernels (5.75) was raised to 7.5 due to the treatment with lime water, which had itself a pH of 11.75. The pH of the dough (6.8) gradually decreased as fermentation proceeded, reaching 3.93 on the eighth day. The low moisture content of maize kernels (6.24%) increased to 22.7% after cooking them in lime water, and to 30.5% in the dough. During the 8 days that pozol was being fermented, the moisture content remained around 30%.

From the maize kernels were isolated *Fusarium moniliforme* (77%) and *Cladosporium cladosporioides* (3%). From the lime treated kernels were isolated *Aspergillus carbonarius* (1%), *A. parasiticus* (2%), *Monilia sitophila* (10%), and *Trichoderma viride* (18%). Of these molds, only *C. cladosporioides* and *M. sitophila* were isolated from pozol after 2 days of fermentation.

During each of the 8 days of pozol fermentation, the relative humidity of the ambient air decreased as the temperature increased and vice versa. Pozol temperature varied less than environmental temperature; the balls o pozol warmed and cooled slower than the environment.

Six culture media were inoculated with serial dilutions of pozol, ranging from $1:10^6$, for recently made pozol, up to $1:10^{10}$, for pozol fermented 8 days. Although many bacteria were found in pozol, they were not studied in detail but some of them were found to be able to reduce acetylene to ethylene, a reaction that indicates their capacity to fix nitrogen.

The numbers of yeasts and molds increased as fermentation of pozol proceeded. Yeasts of the genus *Candida* as well as *Trichosporon cutaneum* and *Geotrichum candidum* were found from the first hours of fermentation and continued to be present for several days. As the surface of pozol balls progressively dried, and their pH became more acid, molds such as *Cladosporium cladosporioides, Monilia sitophila* and *Mucor rouxianus* also invaded pozol to constitute a complex mixture of bacteria, yeasts, and molds.

The presence in the pozol from Tabasco of some yeast and mold species previously found in pozol samples from other locations indicates a certain consistency in the pozol microflora since the same or closely related species occur in the natural proces of pozol fermentation.

RESUMEN

Este trabajo presenta los resultados de un estudio micológico del pozol, un alimento de origen maya que es preparado por fermentación de masa de maíz; la masa fermentada es desleída en agua y bebida como alimento básico por las poblaciones indígena y mestiza del sureste de México.

El pozol estudiado fue preparado en Cárdenas, Tabasco, México, una localidad tropical en donde el pozol es rutinariamente consumido, principalmente por los campesinos.

Los objetivos del estudio fueron los de aislar, en el sitio de elaboración, la micoflora presente en las diferentes etapas de preparación del pozol: granos de maíz, granos de maíz cocidos en agua de cal, masa recién hecha, y masa fermentada durante 8 horas, y 1, 2, 4 y 8 días; y registrar pH, contenido de humedad, humedad relativa y temperatura durante todo el proceso de preparación del pozol.

El pH ácido de los granos de maíz (5.75) fue elevado a 7.5 debido al tratamiento con agua de cal, la cual tenía un pH de 11.75. El pH de la masa (6.8) disminuyó gradualmente durante su fermentación, alcanzando 3.93 en el octavo día. El bajo contenido de humedad de los granos de maíz (6.24%) aumentó a 22.7% después de ser cocidos en el agua de cal, y a 30.5% en la masa. Durante los 8 días que el pozol estuvo fermentando el contenido de humedad permaneció alrededor de 30%.

De los granos de maíz fueron aislados *Fusarium moniliforme* (77%) y *Cladosporium cladosporioides* (3%). De los granos tratados con agua de cal fueron aislados *Aspergillus carbonarius* (1%), *A. parasiticus* (2%), *Monilia sitophila* (10%) y *Trichoderma viride* (18%). De estos mohos, sólo *C. cladosporioides* y *M. sitophila* fueron aislados del pozol después de 2 días de fermentación.

Durante cada uno de los 8 días que el pozol estuvo fermentando, la humedad relativa del aire disminuyó a medida que la temperatura aumentó y viceversa. La temperatura del pozol varió menos que la temperatura ambiental; las bolas de pozol se calentaron y enfriaron más lentamente que el medio ambiente en que se encontraban.

Seis medios de cultivo fueron inoculados con diluciones en serie de pozol, las cuales variaron de $1:10^6$, para el pozol recién hecho, hasta $1:10^{10}$, para el pozol fermentado 8 días. Aunque muchas bacterias fueron aisladas del pozol no fueron estudiadas en detalle pero algunas de ellas pudieron reducir acetileno a etileno, reacción que indica la capacidad de fijar nitrógeno.

Los números de levaduras y mohos aumentaron a medida que aumentó la fermentación del pozol. Levaduras del género *Candida,* así como *Trichosporon cutaneum* y *Geotrichum candidum,* fueron aisladas desde las primeras horas de la fermentación y continuaron estando presentes durante varios días. A medida que la superficie de las bolas de pozol se fue secando, y su pH se hizo

más ácido, mohos tales como *Cladosporium cladosporioides, Monilia sitophila* y *Mucor rouxianus* también invadieron el pozol para constituir una compleja mezcla de bacterias, levaduras y mohos.

La presencia en el pozol de Tabasco de algunas especies de levaduras y mohos previamente encontradas en muestras de pozol de otras localidades indica una cierta consistencia en la microflora del pozol, ya que las mismas especies, o especies estrechamente relacionadas, están involucradas en el proceso natural de la fermentación del pozol.