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Microbiology of *pozol*, a Mexican fermented maize dough

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Mexican fermented maize dough, *pozol*, including traditional banana leaf-wrapped samples and material in plastic bags, was purchased. All samples were pH 4.7 to 5.7 approx. 12 h after preparation, pH declining to 3.6 to 3.9 after 6 to 9 days storage at ambient temperature. These latter samples had dry matter contents of 31% to 48% (w/w), 0.35% to 0.75% titratable acidity as lactic acid and lactic acid bacteria as predominant microbial flora at about 10^8 c.f.u./ml. The lactic acid bacteria included strains of *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Lactobacillus confusus*, *Lactococcus lactis* and *Lactococcus raffinolactis*. Fungi were not found in the samples stored in plastic bags. The samples wrapped in banana leaf, however, developed a large surface mycoflora within 2 days. This included *Geotrichum candidum*, yeasts and moulds. The majority of the lactic acid bacteria and approx. 50% of yeasts hydrolysed starch to some extent. No *Geotrichum* isolate hydrolysed starch. Lactate was assimilated by all the *Geotrichum* isolates and by 17 of 39 yeast strains.

Key words: *Geotrichum*, lactic acid bacteria, maize fermentation, *pozol*, yeast.

Pozol is a drink made by suspending fermented alkali-treated maize dough in water, with or without other flavouring agents such as salt, sugar, honey, cocoa or chilli (Ulloa *et al.* 1983; Cañas Urbina *et al.* 1993). It is a traditional product made by ethnic groups and *mestizos* in southern Mexico (Ulloa & Herrera 1986).

In the traditional method of preparation (Ulloa 1974; Ulloa *et al.* 1983; Cañas Urbina *et al.* 1993; Wacher *et al.* 1993), maize kernels are boiled in lime water until the kernels are swollen and the husk separates easily. The kernels are dehulled, washed with water, drained and wet ground to make a coarse dough. The dough is shaped into oval balls 5 to 8 cm diam. and 10 to 12 cm long, wrapped in banana or other leaves and stored at ambient temperature for 1 to 5 days before consumption. The dough undergoes an acidic fermentation and surface growth of fungi occurs.

Cañas Urbina *et al.* (1993) recognized two basic types of *pozol* processes: a traditional-type made as above by the indigenous Indians; and a *mestizo*-type, characterized by an additional cooking of the dehulled grains.

During the fermentation, the pH value of the dough falls from an initial value of about 7 to 7.5 to about 5.0 to 5.5 in 12 h and reaches 3.9 to 4.1 in 3 to 4 days (Wacher *et al.* 1993). The microbial flora is dominated by lactic acid bacteria. Wacher *et al.* (1993) reported maximum concentrations (c.f.u./g) of 10^9 lactic acid bacteria, 10^7 aerobic mesophilic bacteria, 10^6 enterobacteriaceae, 10^6 yeasts and 10^4 mould propagules.

The fungi involved in traditional *pozol* fermentations were examined in detail by Ulloa (1974). Amongst a great diversity of species, the most prevalent were yeasts of the genus *Candida*, with populations up to 10^8 c.f.u./g wet dough within 2 days, and filamentous yeasts, such as *Trichosporon cutaneum* and *Geotrichum candidum*, with populations up to 10^7 c.f.u./g in 2 days. Surface mould growth included many different species. Ulloa (1974) noted that some of the surface moulds but none of the yeasts were amylolytic. No liquefaction of the dough occurs, and he suggested that amylolysis was not significant in the fermentation.

The aim of the present work was to enumerate and characterize the microflora present in market samples of *pozol*.

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Materials and Methods

Mestizo-type Pozol *Dough Samples*

The *mestizo*-type dough was not moulded into balls but kept by the vendor as a large mass in a bowl or bucket. Sample A included material with and without cocoa and was purchased in Chiapa de Corzo market, Chiapas. It was described as made that day. Sample B was purchased in the market at Tuxtla Gutiérrez, Chiapas. It was also described as made that day. Sample C, with added cocoa, was purchased in the market at Chiapa de Corzo and was described as 2 days old.

Traditional-type Pozol *Dough Samples*

Sample D, three oval balls wrapped in banana leaf, total weight 900 g, and sample E, three oval balls wrapped in banana leaf, total weight 825 g, were purchased in the market at San Cristóbal de las Casas, Chiapas. Both samples were described as being made that day.

Storage

Samples were kept at ambient temperature (17 to 33°C). *Mestizo*-type doughs were kept in plastic bags whereas the traditional products were stored wrapped in their banana leaves.

Sampling

For samples A, B and C, material for examination was taken from near the centre of the dough mass. For sample A, material with and without cocoa was combined. For the banana leaf-wrapped samples, D and E, surface material was obtained by shaving off slices 1 to 2 mm thick with a sterile spatula and interior material was collected after cutting the ball in half.

Determination of Concentrations of Viable Microbes

Approximately 0.8 g dough was added to 4.5 ml sterile 0.1% peptone/water and further dilutions were prepared in 0.1% peptone/water. Counts of aerobic mesophilic bacteria were made on duplicate spread plates of plate count agar. Colonies 2 mm or more in diam. were counted and were presumed to represent the aerobic microflora, not lactic acid bacteria.

Geotrichum candidum, yeasts and moulds were enumerated on duplicate, surface-inoculated plates of acidified potato/dextrose/agar (PDA; pH adjusted to 3.5 by adding 1.4 ml 10% (w/v) tartaric acid solution to 100 ml molten medium). *Geotrichum candidum* colonies were identified from their characteristic appearance and by microscopical examination (Onions *et al.* 1981).

Lactic acid bacteria were enumerated on duplicate, surface-inoculated plates of MRS agar (Oxoid).

Cultures were placed in plastic bags to prevent drying out and incubated at ambient temperature (17 to 33°C) for 1 to 5 days before colonies were counted.

Isolation of Representative Microorganisms

Representatives of the predominant colony types on MRS and PDA plates were picked and streaked over a plate of the same medium. After incubation at ambient temperature for 1 or 2 days, cultures were examined for uniformity of colony appearance and presumptive lactic acid bacterial cultures were also tested for catalase. In the case of pure cultures, growth from a number of colonies was transferred to duplicate bottles of the appropriate maintenance medium. In the case of mixed cultures, a single colony of the predominant type was picked.

Maintenance and Preservation of Cultures

Lactic acid bacterial cultures were maintained in APT/chalk semi-solid agar medium stored at 5°C. The medium comprised APT broth (Difco) containing 2 g agar/l dispensed in 2-ml amounts in bottles each containing approximately 0.5 g $CaCO_3$. It was sterilized by autoclaving at 121°C for 15 min. All cultures survived 2 months storage in this medium at 5°C but after 4 months two of 46 cultures were non-viable. For long-term storage, cultures were frozen on beads, with 20% glycerol as cryoprotectant (Primrose & Wardlaw 1982).

Yeast and *Geotrichum* cultures were maintained on slopes of glucose/peptone/yeast extract/agar medium (Von Arx & Schipper 1978). The medium contained (g/l): glucose, 40; peptone (Oxoid), 5; yeast extract (Difco), 5; and agar (Difco), 20; at pH 6.2. It was dispensed in 2 ml amounts and sterilized by autoclaving at 121°C for 15 min.

Characterization of Lactic Acid Bacteria

Production of CO_2 from glucose was determined using Gibson's semi-solid tomato juice medium (Harrigan & McCance 1976). *Lactobacillus* and *Leuconostoc* spp. were tested for ammonia production from arginine in MRS broth containing 3 g L-arginine.HCl/l (Harrigan & McCance 1976) whereas *Lactococcus* spp. were tested as described by Abd'el Malek & Gibson (1948). Production of dextran from sucrose was determined by the method of Garvie (1960).

The ability to grow at 10, 15 and 45°C was examined in APT broth. The ability to grow in the presence of 6.5% NaCl was tested in APT broth at 30°C for 5 days.

Hydrolysis of starch was examined on plates of APT agar modified by replacing the 1% glucose in the APT formulation with 0.05% glucose and 1% soluble starch (Lintner's; BDH) (Dunican & Seeley 1962). Plates were prepared as double-layer plates, with a bottom layer of similar medium but without glucose or starch. Cultures were spot inoculated onto the surface of duplicate plates, one of which was incubated in air and the other anaerobically in an anaerobic jar at 30°C for 5 days. Cultures were flooded with Gram's iodine solution to detect hydrolysis of starch.

Cultures were tested for fermentation of carbohydrates in API 50CH galleries (API bioMérieux) using API CHL medium for *Lactobacillus* and *Leuconostoc* spp. and API CHS medium for *Lactococcus* spp., according to the manufacturer's instructions. Cupules were sealed with sterile liquid paraffin and cultures were incubated at 30°C for 7 days. Cultures were identified using APILAB Plus V.3.2.2 and the strip 50 CHL V4.0 database.

Characterization of Yeasts and Geotrichum

Yeast and *Geotrichum candidum* cultures were only examined for their ability to metabolize lactate and starch. Inoculum suspensions were prepared by suspending growth from malt extract/agar plates in sterile distilled water to give just visibly turbid suspensions.

Growth on lactate was examined in yeast/nitrogen-base medium (Difco) containing 5 g lactic acid/l. The medium was sterilized by membrane filtration. Tubes were each inoculated with a loopful of test organism suspension, incubated at 25°C for 7 days and examined for turbidity.

Growth on starch was examined in yeast/nitrogen-base medium containing 4.5 g soluble starch/l (Lintner's). Starch solution, 5 g/l, was sterilized by autoclaving at 121°C for 15 min and filter-sterilized yeast/nitrogen-base was aseptically added to it. Tubes were each inoculated with a loopful of test organism suspension, incubated at 25°C for 7 days and examined for turbidity.

Starch hydrolysis was examined on plates having a lower layer of MEA medium (Oxoid) and an upper layer of MEA plus 1% soluble starch (Lintner's). A loopful of culture suspension was spot inoculated on the surface of a plate and cultures were incubated at 25°C for 5 days. Cultures were flooded with Gram's iodine solution to detect starch hydrolysis.

Chemical Analysis

Approximately 1.9 g *pozol* dough was suspended in 2 ml distilled

Table 1. Concentrations of viable microorganisms and pH values in *pozol* doughs stored at ambient temperature.*

Type of dough	Sample	Incubation period (days)	pH	Concentration of microorganism (log_{10} c.f.u./ml)			
				Lactic acid bacteria	Aerobic, catalase +ve bacteria	Yeasts	Geotricum candidum
Mestizo	A	0.5	4.7	7.7	4.8	3.9	1.7
		2	4.0 to 4.4	nd†	nd	nd	nd
		7	3.6	7.5	4.5	3.8	< 2.8
Mestizo	B	0.5	5.7	7.8	3.5	3.2	nd
		2	4.0 to 4.7	nd	nd	nd	nd
		7	3.8	7.9	5.0	3.2	< 2.8
Mestizo	C	0.5	nd	nd	nd	nd	nd
		2	4.0 to 4.4	7.9	1.5	4.1	0.8
		9	3.6	7.8	4.6	4.8	< 2.8
Traditional	D, surface	0.5	4.7	nd	nd	nd	nd
		2	4.0 to 4.4	nd	nd	nd	nd
		6	4.2	8.3	nd	7.2	6.3
	D, interior	0.5	4.7	nd	nd	nd	nd
		2	4.0 to 4.4	nd	nd	nd	nd
		6	3.9	7.9	4.1	< 3.8	< 3.8
Traditional	E, surface	0.5	4.7	nd	nd	nd	nd
		2	4.0 to 4.4	nd	nd	nd	nd
		6	4.0 to 4.4	8.5	nd	7.6	6.8
	E, interior	0.5	4.7	nd	nd	nd	nd
		2	4.0 to 4.4	nd	nd	nd	nd
		6	3.7	8.0	3.1	< 2.8	< 2.8

*Doughs were purchased in markets and stored at ambient temperature (17 to 33°C).
†For details see Materials and Methods.
nd—Not determined.

water and the pH measured with paper indicator strips (samples incubated for 0.5 to 4 d) or with a pH meter (samples incubated 7 or 9 days). Suspensions were tested for glucose with Diastix glucose test papers (Laboratorio Miles de México; sensitivity 1 g glucose/l).

Water contents of *pozol* doughs were measured by determining loss in weight on drying under an i.r. lamp.

To determine titratable acidity, 5 g (wet weight) *pozol* dough was suspended in 25 ml distilled water. Duplicate 10-ml volumes were titrated with 0.1 M NaOH and phenolphthalein as pH indicator.

Results and Discussion

Macroscopic Observations

All the doughs had a coarse texture, with small, gas-filled holes. The surface and the interior of *pozol* doughs A, B and C, stored in plastic bags, remained similar in appearance over the 7 days of incubation. In contrast, the surfaces of *pozol* doughs D and E, which were wrapped in banana leaf, showed substantial white fungal growth by 7 days. The fungal growth was greatest at joins in the banana leaf wrappings, presumably due to the ingress of air, and was not evident in regions where the banana leaf was tightly appressed to the surface of the dough ball.

Chemical Characteristics

The approx. 0.5-day-old-samples had pH values between 4.0 and 4.7. During 6 to 9 day's storage, the pH values declined to 3.6 to 3.9 in all samples, except for the surfaces of doughs D and E, which remained at pH 4.0 to 4.4 (Table 1).

Titratable acidity, glucose and moisture content were assayed only in *pozols* that had been incubated for 7 or 9 days. The concentrations of titratable acidity (as lactic acid, weight/wet wt) were relatively low at 0.45%, and 0.35% in the two traditional *pozols*, D and E, respectively, but rather higher at 0.6% to 0.75% in the *mestizo pozols*. Free glucose was < 2 g/l in all samples. The moisture contents of pozols A, B, C, D and E were, respectively, 69%, 59%, 71%, 52% and 58%.

Microbial Floras

The freshly purchased *mestizo pozol* dough samples, A and B, (approx. 0.5-day-old) already contained high numbers of lactic acid bacteria (Table 1). The numbers of aerobic, catalase-positive bacteria, yeasts and *Geotrichum candidum* propagules were relatively low (Table 1). It is clear that the predominant initial microflora to develop in *pozol* was a lactic acid bacterial one and it is likely that the high

Table 2. Characteristics of 46 strains of lactic acid bacteria isolated from *pozol* doughs.

Group	No. of strains	Characteristic*							Identification
		Cell shape	CO_2 from glucose	Dextran from sucrose	NH_3 from arginine	Growth†		Amylolysis	
						45°C	6.5% NaCl	Aerobic	Anaerobic
I	21	Cocci, short rods	+	+	−	−	+(−)	−	+	*Leuconostoc* sp.
II	2	Cocci	+	−	−	−	+	−	+	*Leuconostoc* sp.
III	4	Rods	+	+	+	±	+	−	d	Heterofermentative *Lactobacillus* sp.
IV	10	Rods	−	−	−	−	+(−)	−	+	Homofermentative *Lactobacillus* sp.
V	6	Cocci	−	−	+	−	−	±	+	*Lactococcus* sp.
VI	2	Cocci	−	+	−	−	−	±	+	*Lactococcus* sp.
VII	1	Cocci	−	−	−	−	−	±	+	*Lactococcus* sp.

*+, Positive reaction or growth; −, negative reaction or growth; ±, weak reaction; d, different for different strains; +(−), most strains positive but some strains negative.
†All strains grew at 10°C.

concentrations present were a consequence of contamination during grinding of the grains (Wacher *et al.* 1993).

The numbers of viable lactic acid bacteria increased only slightly after 6 or 7 days' incubation, compared with the numbers present at 0.5 day, and reached around 10^8 c.f.u./ml in all samples. Likewise, the number of aerobic, catalase-positive bacteria and yeasts did not increase during incubation of *pozols* A, B and C, and remained relatively low. *Geotrichum candidum* was not isolated from *mestizo pozol* doughs. In contrast, the mycoflora on the surface of *pozol* doughs D and E included up to 4×10^7 c.f.u. viable yeasts and 6×10^6 c.f.u. *Geotrichum candidum*/ml (Table 1).

These observations clearly show that fungi, including yeast, *Geotrichum candidum* and other moulds, were not involved to a significant extent in the fermentation of the *mestizo pozol* doughs stored in plastic bags nor in the interior of traditional, banana-leaf-wrapped *pozols*. However, both traditional *pozols* had large yeast, *Geotrichum* and mould floras on their surfaces.

Characteristics of the Lactic Acid Bacteria

Overall, 46 lactic acid bacterial strains were isolated: 20 from approx. 0.5-day-old *pozol* doughs and 26 from doughs after incubation for 2 to 9 days. The strains could be assigned to seven main groups (Table 2). The predominant types of lactic acid bacteria isolated were *Leuconostoc* spp. (23 of the 46 strains), homofermentative lactobacilli (10/46), lactococci (9/46) and heterofermentative, dextran-producing lactobacilli (4/46). *Leuconostoc* spp. and homofermentative lactobacilli were isolated from all five *pozol* doughs. The relatively small number of strains isolated means that it is not possible to say whether or not there were other differences in the bacterial floras of the different *pozols* or if the composition of the floras changed with time.

Sugar fermentation patterns were determined for 14 strains. Of five Group I strains examined, one was identified as *Leuconostoc mesenteroides* (in very good identification according to the APILAB Plus database) but the others, along with one Group II strain, did not yield acceptable identifications. The two Group III strains examined were identified as *Lactobacillus confusus* (very probable identification although doubtful profiles). Three Group IV strains were identified as *Lactobacillus plantarum* (very good identifications). Two Group V strains were *Lactococcus lactis* (very good identification) and a Group VI strain was identified as *Lactococcus raffinolactis* (excellent identification).

Characteristics of Yeast and Geotrichum *Strains*

The yeast and *Geotrichum candidum* isolates were only examined for their ability to grow on lactate or starch as sole sources of C and energy and for their ability to hydrolyse starch. Slightly over half of the yeast strains hydrolysed starch to some extent, only producing a clearing beneath the colony, but only three of the 39 strains grew with starch in yeast/nitrogen-base medium (Table 3). None of the *Geotrichum* strains either hydrolysed or grew on starch.

All the *Geotrichum* strains and 17 of the 39 yeast strains grew on lactate as sole C and energy source (Table 3). The proportion of yeast isolates able to grow on lactate was higher among those isolated later in the fermentation than among those isolated initially. *Geotrichum* is commonly observed in the later stages of lactic fermented foods (Chapman & Sharpe 1981; Steinkraus *et al.* 1983) and it is possible that the use of lactate is an important factor in its ecology (Collard & Levi 1959). It is also possible that the utilization of lactate by the fungi on the surface of the traditional *pozol* balls accounts for the higher pH of the surface dough than that of the interior dough (Table 1).

Table 3. Ability of yeast and *Geotrichum candidum* strains isolated from fermenting *pozol* doughs to grow on lactate or starch as sole sources of C and energy or to hydrolyse starch.

Source of strain			Proportion of strains exhibiting property			
Type of dough	Sample*	Incubation period (days)	Yeast strain			*Geotrichum*†
			Hydrolysis of starch‡	Growth on:		Growth on lactate
				Starch	Lactate	
Mestizo	A + B	0.5	5/7	0/7	1/7	0/0
	A + B + C	2 to 9	7/15	1/15	7/15	0/0
Traditional	D + E	0.5	3/4	1/4	3/4	1/1
	interior	2 to 7	1/2	0/2	2/2	2/2
Traditional	D + E	0.5	2/4	0/4	0/4	0/0
	surface	2 to 7	3/7	1/7	4/7	5/5
Totals		0.5	10/15	1/15	4/15	1/1
		2 to 9	10/24	2/24	13/24	7/7

*For details see Materials and Methods.
†No *Geotrichum candidum* strain hydrolysed or grew on starch.
‡Starch-hydrolysing strains only produced clearing on starch agar medium under the colony.

Microbiology and Biochemistry of the Fermentation

It is evident that the *pozol* fermentation is initially a lactic acid bacterial fermentation. In the absence of access to air, as with the *mestizo*-type products, other microbes are not present at concentrations sufficient to cause significant biochemical changes in the substrate. Where access to air is allowed, as with the traditional banana-leaf-wrapped *pozol* balls, extensive growth of fungi occurs on the surface. It is possible that this surface mycoflora contributes to flavour and, hence, the traditional-style *pozol* dough might be viewed as a fungal-ripened, lactic-fermented product.

None of the lactic acid bacteria hydrolysed starch under aerobic conditions but about two thirds of them exhibited some hydrolysis under anaerobic conditions. Similarly, almost half of the yeast strains isolated from *pozol* samples exhibited some amylolytic activity. It is not known what role, if any, amylolysis plays in the fermentation. Certainly, no very active amylolysis occurs since there is no tendency for fermenting *pozol* dough to liquefy. This does not exclude the possibility, however, that a low level of amylolytic activity is necessary for maximum microbial growth and acidification of the dough.

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