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Evidence for a Lactic Streptococcal Role in Nigerian Acidic Cassava (*Manihot esculenta* Crantz) Fermentations[1]

M. O. ABE[2] and R. C. LINDSAY*

Department of Food Science
University of Wisconsin, Madison, Wisconsin 53706

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ABSTRACT

The predominant acid-producing organism isolated from Nigerian cassava mixed fermentation cultures was *Streptococcus faecium*. *Corynebacterium manihot* was also abundant in the mixed cultures, but contrary to earlier reports, this organism grew slowly and lacked significant acid-producing capabilities. Cultural characteristics of *S. faecium* indicated that it was the primary fermentation organism in acidic cassava fermentations rather than the earlier indicated *C. manihot*. Diacetyl production in both milk cultures and fermented cassava was demonstrated for *S. faecium*. Detection of diacetyl in fermented cassava products indicated that *S. faecium* may also play an additional role in the flavor development of these products.

Cassava or manioc (*Manihot esculenta* Crantz) is a widely-grown tropical root crop that is consumed in a variety of forms as a food staple in many cultures. This popular carbohydrate source contains the cyanogenic glycoside, linamarin, which degrades to hydrocyanic acid (HCN) and glucose under the influence of the enzyme, linase, and/or low pH conditions (*2*). As a part of the traditional processing or preparation of popular Nigerian food items, such as gari, cassava roots are macerated and allowed to undergo a natural, acidic fermentation process which results in the detoxification of the cassava (*1,3*). This has been reported to occur through the liberation of HCN at low pH (ca 3.9) through the combined activities of *Corynebacterium manihot* and *Geotrichum candidum* (*3*), and substantial amounts of lactic acid that have been found in fermented cassava (*1*). Acid production has been ascribed to *C. manihot* because it was the predominant organism that was isolated in the initial 48 h of fermentation while flavor production was attributed to *G. candidum* which grew abundantly in the latter stages of the 4-day fermentation (*3*). In some instances fermentation occurs as a result of natural microflora associated with cassava roots and utensils, while in more centralized processing situations the juice from previous batches is used as an inoculum for fresh cassava pulp (*1*).

Corynebacterium spp. do not consistently ferment sugars, but when they do high acidities are seldom produced. Many species within the genus oxidize glucose completely to carbon dioxide and water (*7*). The fact that high acidity is seldom produced by members of the genus *Corynebacterium* strongly suggests that the organism responsible for lowering the pH of the fermenting cassava pulp is not *C. manihot*. The consistent isolation of this organism as the dominant species in the first stage of the fermentation (*3*) may have resulted from inappropriate selection of the isolation medium. *C. manihot* easily grows on nutrient agar, but lactic-acid bacteria are nutritionally fastidious and were probably selectively excluded from observation. This paper reports the results of a re-investigation of the microbiology of Nigerian cassava fermentation using isolation media suitable for detection of lactic lacid bacteria.

MATERIALS AND METHODS

Isolation and characterization of streptococci

Mixed cultures from cassava mash fermented locally in Nigeria (Adeladan Amodo, a village on the Ondo Road) were streaked on Plate Count agar (Difco Laboratories, Detroit, Michigan) and Nutrient agar (Difco) to determine microorganisms capable of growing on simple media, and APT agar (Difco) was employed to enhance growth of lactic acid bacteria (*8*). Sodium azide (0.04%) added to APT agar and KF Streptococcus agar (Difco) were used for selective streptococcal growth and presumptive indication of streptococci species (*11*).

Isolated colonies were picked from each of the agar plates, and were transferred into Nutrient, APT and Brain Heart Infusion (BHI, Difco) broths. Cultures were maintained by transferring into appropriate broth every 48 h, and separate cultures were carried at 30 and 37 C. Selected cultures were gram-stained, and checked for morphology.

Isolated colonies from the mixed cultures were inoculated into sterile skimmilk and sterile litmus milk to determine acid production. Suspected streptococci from KF Streptococcus agar were streaked on Low Glucose Agar (LGA, Difco), and were tested for catalase activity by flooding the incubated plates with 3% hydrogen peroxide. The colonies were also tested for presence of iron-porphyrin compounds by flooding incubated LGA plates with benzidine reagent before adding 3% hydrogen peroxide (*5*). Finally, the suspected streptococcus colonies were transferred into pyruvate and sorbitol media (*6*).

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[2]*On leave from: Department of Food Science and Technology, University of Ife, Ile-Ife, Nigeria.*

Characteristics of cassava culture organisms

Pure cultures of *C. manihot* and *G. candidum* obtained from the Federal Institute of Industrial Research, Oshodi, Nigeria, *G. candidum* ATTC #12784 (American Type Culture Collection, Rockville, MD), and *S. faecium* isolated from naturally fermenting cassava mash were grown at 37 C for 18 h in steamed (1 h at ca 100 C) skimmilk and sterile litmus milk to determine acid-producing capabilities and aroma development in these media.

A limited number of model-system cassava fermentations were carried out using greenhouse-cultured cassava roots (Variety 53101, J. Omeumu, Dept. of Pathology, University of Wisconsin). Cassava roots (ca 200 g each) were washed, peeled, and grated to yield a mash. Fifty-gram lots of mash were inoculated with 1 ml of appropriate seed culture, placed in cheese cloth bags, and placed in 250-ml foil-covered beakers for incubation at 35 C for 1 week. Cultures employed in these trials included natural mixed cultures from fermenting cassava which had been transferred to Nutrient broth before use, and mixtures of pure cultures of *C. manihot* and *G. candidum*.

Laboratory-fermented and naturally-fermented cassava mash, traditionally processed gari, and the previously described milk cultures were analyzed by the gas chromatographic headspace analysis procedure developed by Morgan and Day (*13*). Corresponding unfermented control samples were also analyzed where appropriate to verify production of volatile compounds by fermentation organisms. Analysis conditions included nitrogen-purging (10 ml/min) of volatile compounds from NaCl-saturated aqueous sample systems, and collection of volatile compounds at the head of either a Porapak Q column (12 ft × 1/8 inch stainless steel; Waters Associates, Framingham, Mass.) or a 3% 1,2,3-tris-(2-cyanoethoxy)-propane (Tris) on Chromosorb G column. In each instance 10 g of sample was combined with 5 ml of saturated NaCl solution and 1 g of NaCl to provide a solution for analysis that would contain the same proportions of volatile compounds as the original sample in each instance.

The Porapak Q column was operated isothermally at 155 C, and the Tris column was held initially at 50 C for 5 min, then was programmed at 4 C/min to 175 C and held. Injector and detector temperatures on the Varian Model 1740 gas chromatograph were maintained at 250 C, and the carrier gas was nitrogen at a flow rate of 25 ml/min. Tentative identifications of votalile compounds were assigned on the basis of coincidence of relative retention times of unknowns with those of authentic compounds, and where appropriate occurrence of distinctive aromas were detected through a column-effluent splitter assembly. Peak areas were determined by the triangulation procedure.

RESULTS AND DISCUSSION

After incubation at 37 C for 48 h, large yellow colonies were observed on Nutrient agar plates streaked with mixed cassava cultures. On similarly incubated plates of Plate Count Agar which were streaked with the same mixed cultures, two different types of colonies were observed. There were many pinpoint colonies which were characteristic of lactic acid bacteria, and an abundance of large yellow colonies which overgrew the pinpoint colonies. The pinpoint colonies probably were not observed on the Nutrient agar plates because Nutrient agar is a less nutritive medium than Plate Count Agar.

The yellow colonies on both the Nutrient agar and Plate Count Agar were picked into tubes of Nutrient broth which were incubated at either 30 or 37 C. No growth was observed in cultures incubated at 30 C, but cultures incubated at 37 C showed heavy growth after 96 h of incubation. Examination of the culture organisms by the usual gram-staining techniques showed that the yellow colonies were composed of gram-positive, club-shaped rods with granules that stained gram-positive. These characteristics agreed closely with those described for *C. manihot* previously isolated and reported in earlier investigations of cassava fermentations (*3*).

Growth of the mixed culture on the APT agar medium was very rapid and nonpigmented colonies were observed after 24 h of incubation at 30 C. Incorporation of manganese ions (Mn^{++}) and citrate into the basal nutrient medium results in a unique medium for supporting growth of lactics which do not grow on Nutrient agar or grow poorly on Plate Count Agar (*8*). Colonies from APT agar medium were transferred into APT broth and incubated at 30 C; this resulted in a heavy turbid growth in the tubes after 48 h. These cultures were again streaked on APT agar to which 0.04% sodium azide (NaN_3) had been added. Pinpoint colonies appeared on this medium after 48 h at 30 C, and provided strong evidence for the presence of lactic acid bacteria in cassava fermentation mixed cultures.

Pinpoint colonies picked from the APT-sodium azide plates were transferred daily into APT and BHI broths. Heavy growth was observed in all the cultures and each culture was gram-stained and examined under the microscope for gram reaction and morphology. All of the organisms observed were gram-positive cocci in chains which was indicative of *Streptococcus* spp. A further indication that these organisms were *Streptococcus* sp. was their growth as pinkish colonies on KF agar.

Results of the morphological and physiological examinations of primary streptococcal isolate from cassava fermentation mixed cultures are summarized in Table 1. The fact that the organism under investigation gave negative catalase and benzidine tests indicated very strongly that it was a lactic acid bacterium. The morphological characteristics and reaction on the KF Streptococcus agar clearly placed the organism in question in the *Streptococcus* group. Additional tests showed that the organism grew well in skimmilk giving rapid coagulation, and readily changed blue litmus to colorless. The organism also tolerated 6.5% sodium chloride, and showed growth at 45 C. Since the streptococcal isolate from the mixed cassava culture was

TABLE 1. *Physiological and morphological characteristics of primary acid-producing isolate from Nigerian cassava fermentation mixed cultures.*

Tests	Results
Gram reaction	Positive
Morphology	Cocci in chains
Catalase test	Negative
Benzidine test	Negative
Growth on APT+sodium azide	Positive
Growth on KF Streptococcus agar	Pinkish colonies
Growth in pyruvate medium	Negative
Growth in sorbitol medium	Negative
Growth in a medium containing 6.5% NaCl	Positive
Growth at 45 C	Positive
Reaction in steamed skimmilk	Coagulation
Reaction in litmus milk	Colorless in 12 h and coagulation

able to grow at 45 C and tolerated 6.5% sodium chloride, it fell in Sherman's enterococcus division that includes *Streptococcus faecalis* and *Streptococcus faecium* (*4*).

Pyruvate is used as an energy source only by *S. faecalis* (*6*) and use of pyruvate as an energy source is employed as an aid in differentiating between *S. faecalis* and *S. faecium*. The inability of the isolate from the cassava fermentation mixed culture to grow in a pyruvate medium confirmed that the organsim was *S. faecium* and not *S. faecalis*. Further, the organism was unable to grow in sorbitol medium. *S. faecalis* can grow in both pyruvate and sorbitol media while *S. faecium* does not grow in these media (*6*).

Inoculation of steamed skimmilk and litmus milk with both single-strain and mixed-strain cultures gave results which supported the hypothesis that *S. faecium* was the principal acid-producing organism in mixed-strain natural cassava cultures (Table 2). Acid production in milk was not observed for pure cultures *C. manihot* or *G. candidum* or for a mixture of these two organisms. These organisms produced only weak fatty acid-like aromas while *S. faecium* gave a pronounced diacetyl aroma similar to that observed for the natural mixed-strain cassava cultures.

TABLE 2. *Behavior of organisms from cassava cultures grown for 18 h at 37 C in steamed skimmilk and litmus milk.*

Culture	Coagulation of steamed milk	Aroma	Reaction in Litmus milk
Natural mixed cassava culture	+	Strong diacetyl	Coagulation and colorless
S. faceium (Isolate)	+	Strong diacetyl	Coagulation and colorless
G. candidum (Isolate[1] & ATTC #12784	—	Weak, fatty acid-like	No reaction
C. manihot (Isolate[1])	—	Indistinct	No reaction
Mixed culture of *G. candidum*[1] and *C. manihot*[1]	—	Weak, fatty acid-like	No reaction

[1]Obtained as pure culture from the Federal Institute of Industrial Research, Oshodi, Nigeria.

Analysis of milk cultures for volatile compounds showed that both the *S. faecium* culture and the natural mixed-strain culture gave substantial diacetyl peaks although the actual levels of diacetyl were not quantified (Table 3). Analyses of laboratory-fermented and naturally-fermented cassava mash (Table 3) also showed the presence of diacetyl in these products. The role of diacetyl in cultured dairy product flavors is well documented (*12*). Data for volatile compounds in traditional gari and sun-dried fermented cassava (Table 4) show that diacetyl is carried through to the finished product, and the presence of diacetyl in these products indicates that *S. faecium* may also play an additional role in development of flavors in fermented cassava products. While the other volatile compounds tentatively identified in the samples are commonly found in lactic fermentations (*9,10*), only acetaldehyde has a sufficiently low flavor threshold to have a possible direct influence on flavors of cassava products. However, ethanol could react with fatty acids to yield esters that could contribute to flavors.

Both laboratory-grated cassava mash and steamed skimmilk samples inoculated with either *C. manihot* or *G. candidum* or combinations thereof showed very little indication of fermentation activity. *S. faecium* apparently was not present in the natural microflora of laboratory-grated cassava samples. As a result acid production did not take place in any of these samples, and molds quickly overgrew the samples.

SUMMARY AND CONCLUSIONS

S. faecium was isolated as the acid-producing organism in Nigerian cassava fermentation mixed cultures. Earlier investigations of the microbial succession in cassava fermentation (*3*) indicated that the acid-producing organism was *C. manihot*. *C. manihot* was abundant in the fermentation cultures, but since this organism grew slowly, and is oxidative rather than

TABLE 3. *Relative abundance of volatile compounds found in gas chromatographic headspace analysis of fermented cassava and skimmilk cultures.*

Peak no.	Tentative identity	Relative retention time[1] Observed	Relative retention time[1] Authentic	Absolute areas of peaks (cm^2) Lab-fermented cassava with mixed culture	Natural Nigerian fermented cassava mash	*S. faecium* in steamed skimmilk	Natural mixed cassava culture in steamed skimmilk
1	Acetaldehyde	0.17	0.18	33	12	172	22
2	Ethanol	0.29	0.34	96	976	512	249
3	Acetone	0.42	0.48	1	16	10	3
4	Diacetyl	1.00	100	4	22	10	11

[1]Relative retention time based on diacetyl equal to 1.00; Packed column: 10 ft × 1/8 inch O.D. stainless steel, Porapak Q.

TABLE 4. *Relative abundance of volatile compounds found in gas chromatographic analysis of headspace volatiles of gari, fermented casava, and* S. faecium *in skimmilk.*

Peak no.	Tentative identity	Relative retention Time[1] Observed	Relative retention Time[1] Authentic	Absolute areas under peaks (cm^2) Traditionally processed gari from Nigeria	Sun-dried fermented cassava from Nigeria	Lab fermented cassava with mixed culture	*S. faecium* in steamed skimmilk
1	Acetaldehyde	0.08	0.07	5	9	12	96
2	Ethanol	0.21	0.19	—[2]	15	7731	164
3	Acetone	0.48	0.42	3	1923	—	—
4	Diacetyl	1.00	1.00	15	256	11	128

[1]Relative retention time based on diacetyl equal to 1.00; Packed column, 12 ft × 1/8 inch O.D. stainless steel, 3% 1,2,3-tris-(cyanoethoxy) propane on Chromosorb G.
[2]Not detected in the product.

fermentative, it is very unlikely that *C. manihot* is responsible for any acid production in cassava fermentations. Based on growth and acid-producing characteristics observed, it is proposed that *S. faecium* is the most important acid-producing organism present in Nigerian cassava mixed-strain fermentations. Diacetyl production by *S. faecium* isolated from cassava mash cultures was also demonstrated, and indicates that this organism may contribute flavors in addition to acidity to fermented cassava products. The role of *C. manihot* and *G. candidum* in the overall fermentation of cassava by natural mixed-strain cultures has not been determined as yet.

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