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Spherical Lactic Acid-producing Bacteria of Southern-grown Raw and Processed Vegetables

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The frequency and levels of population of the spherical lactic acid-producing bacteria were determined on raw and processed yellow summer and zucchini squash, a variety of greens, green beans, okra, southern peas, and butter and lima beans, and on fresh cucumbers and corn flowers. Six taxa occurred consistently: *Leuconostoc mesenteroides*, yellow-pigmented streptococci, *Streptococcus faecium*, *Aerococcus viridans*, and *S. faecalis* and *S. faecalis* var. *liquefaciens*. The same taxa occurred with the same order of frequency on processed, frozen vegetables, but with a marked decrease in the occurrence of *S. faecalis* var. *liquefaciens*. *S. lactis*, *S. cremoris*, *S. equinus*, *S. bovis*, and pediococci were isolated infrequently. No other member of the viridans group of the streptococci and no member of the pyogenic group was isolated. Approximately 88% of the cultures were identified. Total counts of the lactic-acid-producing bacteria rarely exceeded 10^5 per gram of sample, and there was a reduction by 90% during the second year of study, probably because of drought. Only one bacterial species was found on 40% of the raw and 34% of the processed vegetable samples. Two or more species or taxa were present on the remainder of 153 raw and 56 processed vegetable samples. *A. viridans* was present on squash, greens, okra, and southern peas, and its frequency of occurrence on vegetables suggests that plants are its natural habitat.

Each of the few studies conducted to determine the frequency and numbers of the spherical, lactic acid-producing (LAP) bacteria in nature has been directed toward a bacterium or a restricted group of specific interest. Stark and Sherman (20) confined their study to the distribution of *Streptococcus lactis* on fresh vegetables. The surveys made by Mundt (14, 15) and associates (17) were limited to the enterococci. That by Geldreich, Kenner, and Kabler (7) was restricted to *S. faecalis* and its variant *liquefaciens*. Langston and Bouma (10) recovered enterococci and pediococci from freshly cut orchard grass and alfalfa.

The present studies were designed to determine the frequency of occurrence and the levels of population of the members of the *Streptococceae* on plants and raw vegetables destined for freezing-processing. The data are related to the same information obtained with the frozen vegetable. It is the intent of the study to place the presence of the enterococci into a perspective in relation to the other taxa of the tribe, in order that a more complete understanding of distribution and significance may be gained.

MATERIALS AND METHODS

Acquisition of samples. Yellow summer and zucchini squash, mustard, spinach, turnip and dandelion greens, and green beans were sampled both in the fields and at the time of delivery to the processing plants. Southern peas, also known as purple-hull, clay, and field peas, and butter and lima beans were sampled only after harvesting and shelling. Cucumbers and corn flowers, while not processing vegetables, were included to extend the range of observations. All products were weighed for analysis as received, except the squash and the cucumber. Parings approximately 0.6 cm thick were taken from these for analysis. All samples of processed vegetables were taken either at the ends of processing lines and frozen for transportation to the laboratory, or they were acquired as commercially frozen samples at the processing plants.

Plating and selection of cultures. Osterized aqueous suspensions of all samples were surface-plated serially on a modification of the culture medium of De Man, Rogosa, and Sharpe (3). The modifications of the medium included the addition of 0.02% NaN_3, substitution of di- for tri-ammonium citrate, the inclusion of 1.5% agar, the replacement of 40% of the required dextrose with mannitol, and the addition of 1 ml of 1% 2,3,5-triphenyl tetrazolium chloride (TTC) per

100 ml of medium after sterilization. Colonies were enumerated after incubation at 32 C for 3 days. All colonies on sets of plates, or within sectors of plates, for a total of 20 to 35 colonies per sample, were transferred to azide dextrose-0.1% yeast extract (ADYE) broth containing bromothymol blue.

Screening, purification, and identification. Preliminary screening for prompt recognition of the slime-producing *Leuconostoc mesenteroides* and the yellow-pigmented streptococci was accomplished on tryptic agar or Trypticase Soy (TS) Agar containing 4% sucrose and 0.1% yeast extract. Selections were made from the remaining streaks to ADYE broth for the maintenance and study of the cultures.

The pattern of properties given in *Bergey's Manual*, supplemented with other criteria, was used for the grouping and identification of cultures. Phenol red broth base was used in determining ability of the bacteria to grow at 10 C and at 45 C, in the presence of 6.5% NaCl, and at *p*H 9.6. Azide dextrose-agar containing 1:2,500 potassium tellurite, Barnes' medium (1) containing TTC, TS-5% bile salt agar, and TS-5% human citrated blood were employed to determine the characteristics exhibited by the bacteria on these media. The final *p*H was, contrary to Fischer's observations (5), sometimes influenced by alteration in the ratio of volume to surface area; therefore, this value was determined on 15-ml quantities of TS-2% glucose broth in test tubes with an outer diameter of 15 mm after incubation for 7 days at 32 C. Aerogenesis was determined in TS-2% glucose broth which concontained 10% gelatin and was sealed as suggested by Gibson and Abdel-Malek (8). Peroxidase and heme-requiring catalase formation were determined on the heated blood medium of Whittenbury (22). and the mode of dissimilation of malate also was determined according to Whittenbury's procedure (23).

RESULTS

The same six identifiable species or groups of the LAP bacteria recurred on the vegetables throughout the season. These are *S. faecalis*, *S. faecalis* var. *liquefaciens*, *S. faecium*, yellow streptococci, *L. mesenteroides*, and *Aerococcus viridans*, in the order of presentation in the tabulated data. Other members of the LAP bacteria occurred too inconsistently or too infrequently to warrant listing in the tabulated data, and these are included with the unidentified bacteria as miscellaneous. Of 4,665 cultures, 88% were identified.

Frequency in occurrence. The data on the frequency of occurrence of the LAP bacteria on each type of raw and frozen vegetable are recorded in Tables 1 and 2. None of these bacteria was recovered from 23 (14.7%) of 156 samples of raw vegetables, nor from 11 (13.7%) of 80 samples of processed vegetables. Most of the failures to isolate the bacteria occurred during the second year of sampling, when, in contrast with the preceding year, conditions of marked drought prevailed. Rainfall was abundant during the early summer of the first full year of the study, resulting in the recovery of the LAP bacteria from all of 18 samples of squash, with an average total LAP population of 9.7×10^4 per g of peel. During the following year, the LAP bacteria were obtained from only 12 of 18 samples of squash, with an average population for the 12 samples of 1.1×10^4 per g of peel.

L. mesenteroides and the yellow streptococci occurred most frequently on both raw and processed vegetables. Singly, *S. faecalis*, *S. faecalis* var. *liquefaciens*, and *S. faecium* occurred with relatively low frequency, with a marked reduction of *S. faecalis* var. *liquefaciens* on processed vegetables. Collectively, the recoveries compared favorably with earlier data obtained with more

TABLE 1. *Frequency in occurrence of lactic acid-producing (LAP) bacteria on raw vegetables*

Vegetable	No. of samples	No. of samples containing								Frequency ratio
		No LAP bacteria	*Streptococcus faecalis*	*S. faecalis* var. *liquefaciens*	*S. faecium*	Yellow enterococci	*Leuconostoc*	*Aerococcus*	Miscellaneous	
Squash	31	7	1	11	4	9	13	2	11	2.1
Greens	27	8	1	4	1	10	10	3	7	1.7
Green beans	14	4	0	1	0	2	9	0	4	1.6
Okra	10	0	1	1	3	1	9	1	1	1.7
Peas	30	0	5	17	11	8	21	5	7	2.5
Lima butter beans	9	0	4	3	2	1	9	1	1	2.3
Pepper	7	1	0	1	1	1	2	0	1	1.0
Corn	21	1[a]	2	4	1	11	12	0	5	1.8
Cucumber	7	2	0	2	1	1	0	0	0	0.8
Number	156	23	13	45	24	44	83	11	37	
Per cent		14.7	8.3	28.8	15.4	28.2	53.2	7.0	23.7	

[a] Enclosed silk.

widespread sampling (14, 17). *A. viridans* was recovered from 7% of the raw and 16% of the processed vegetable samples.

Pediococcus pentosaceus was isolated from raw greens, southern peas, and butter and lima beans. *S. lactis* and *S. cremoris* were seldom isolated. *S. equinus* was isolated from one processing series of southern peas. This bacterium had been obtained with some frequency during earlier studies of airborne distribution (16), but in the interim marked improvements in internal plant sanitation had been instituted. *S. bovis* was isolated from one sample of raw squash. No members of the pyogenic group, and none of the viridans group of streptococci, except those mentioned, was isolated from either raw or processed vegetables.

Of the cultures recovered from the vegetables, 12% were not identified. These have fallen into approximately 20 groups, with a wide range of properties. Some may be highly aberrant members of the enterococci; most cultures, however, failed to grow at 45 C, and they possessed properties which did not permit affiliation with any presently known species.

Frequency ratio. The frequency ratio expresses the average number of taxa or groups occurring per sample. The ratios are shown in the last columns of Tables 1 and 2. The data suggest that

TABLE 2. *Frequency in occurrence of lactic acid-producing bacteria on processed vegetables*

Vegetable	No. of samples	No. of samples containing								Frequency ratio
		None	*Streptococcus faecalis*	*S. faecalis* var. *liquefaciens*	*S. faecium*	Yellow enterococci	*Leuconostoc*	*Aerococcus*	Miscellaneous	
Squash	22	5	1	1	1	3	16	4	9	4.9
Greens	11	1	0	1	0	7	8	4	8	2.8
Green beans	17	1	2	0	2	0	14	0	4	1.3
Okra	6	2	1	0	1	1	4	1	2	2.5
Peas	16	1	1	1	1	3	11	7	6	2.0
Lima butter beans	8	1	2	0	1	1	6	0	2	1.3
Number	80	11	7	3	6	15	59	16	31	
Per cent		13.7	8.7	3.7	7.5	18.6	73.7	20.0	38.7	

TABLE 3. *Frequency in occurrence of the lactic acid-producing bacteria singly and in association on raw and on processed vegetables*

Lactic acid-producing bacteria	Frequency in occurrence singly or with						
	Streptococcus faecalis	*S. faecalis* var. *liquefaciens*	*S. faecium*	Yellow enterococci	*Leuconostoc*	*Aerococcus*	Miscellaneous
On raw vetegables							
S. faecalis	**2**[a]	10	7	4	9	2	4
S. faecalis var. *liquefaciens*	10	**13**	15	14	23	5	12
S. faecium	7	15	**2**	11	17	7	9
Yellow enterococci	4	14	11	**12**	23	8	20
Leuconostoc	9	22	17	23	**28**	17	24
Aerococcus	2	5	7	8	17	**0**	5
Miscellaneous	4	12	9	20	24	5	**4**
On processed vegetables							
S. faecalis	**0**	2	2	2	7	0	4
S. faecalis var. *liquefaciens*	2	**0**	0	3	3	0	3
S. faecium	2	0	**0**	2	5	1	2
Yellow enterococci	2	3	2	**0**	12	6	11
Leuconostoc	7	3	5	12	**16**	13	25
Aerococcus	0	0	1	6	13	**0**	11
Miscellaneous	4	3	2	11	25	11	**3**

[a] Figures in boldface type indicate that organisms occurred singly.

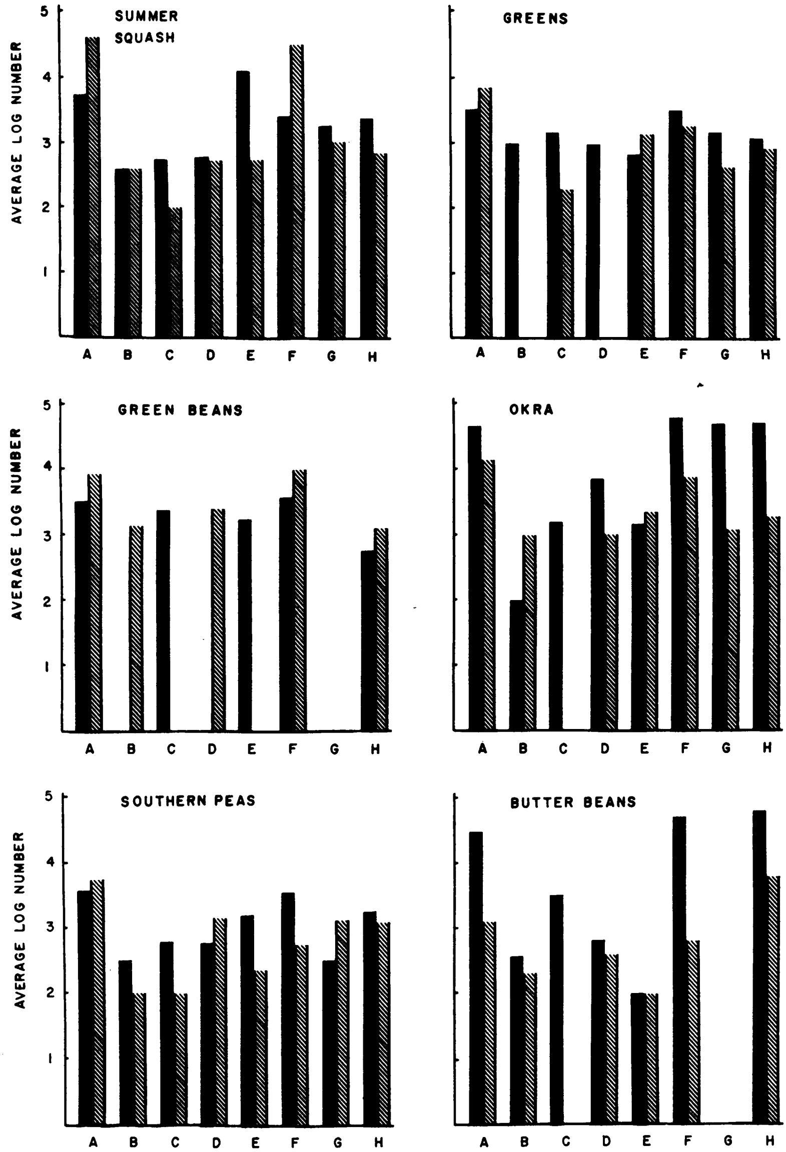

FIG. 1. *Average numbers of Streptococceae per gram of raw and processed vegetables. (A) total count, (B) Streptococcus faecalis, (C) S. faecalis var. liquefaciens, (D) S. faecium, (E) yellow streptococci, (F) Aerococcus viridans, (G) miscellaneous and unidentified streptococci.*

a mixture of flora existed more frequently than did a single taxon on either raw or processed vegetables. The flora of the processed vegetables was more diverse (average ratio of 2.47) than was that of the raw vegetables (average ratio of 1.98). The higher ratio for the processed vegetables is attributed to the influence of airborne distribution of bacteria arising during handling of the raw product.

Associations. The data of Table 3 confirm the conclusions which may be drawn from the frequency ratios. In all instances, identified members occurred more frequently in association than they did alone. The numbers of associates ranged from one through five. The enterococci collectively were present alone (figures in boldface) on 29 of 133 raw vegetable samples, but in no instance did they occur alone on processed vegetables. All taxa and groups occurred in random fashion, with no discernible pattern of relationship of one to another.

Population levels. The average populations

(Fig. 1) of *S. faecalis*, *S. faecalis* var. *liquefaciens*, and *S. faecium* on all raw vegetables was less than 10^3 per g, with a range from 1.5×10^2 to 2.6×10^4 per g when present. The yellow streptococci, while usually occurring in low numbers, were as numerous as 4.0×10^5 per g of squash surface and of corn flowers, occasionally exceeding the numbers of the ubiquitous *L. mesenteroides*. They also occurred more frequently than did the remaining enterococci on shelled southern peas.

The average population of the enterococci on processed vegetables was 1.8×10^2 per g, with a range from 1.5×10^2 to 2.2×10^3 when present. The average population for the numerically superior *L. mesenteroides* was 2.5×10^4 per g on raw vegetables and 2.3×10^3 per g on processed vegetables, with individual counts in excess of 1.2×10^6 per g of raw okra, corn flowers, and shelled southern peas. The average population of *A. viridans*, when it was present, was approximately 2.0×10^3 per g on both raw and processed vegetables. The average of the total counts of all the LAP bacteria, as determined on the plating media, exceeded 10^4 per g only on raw okra and butter beans, and on processed okra and squash, corn flowers, and southern peas.

DISCUSSION

The majority of the samples of raw vegetables and plant parts were taken directly in the field, under conditions precluding introduction of bacteria from outside agencies. Few or no differences were noted in either the variety or the population of the LAP bacteria between field-sampled and delivery-sampled vegetables. Whole, unharvested pods of southern peas and of butter beans generally have a very low population of LAP bacteria. The edible portions of these vegetables used in this study were removed from the pods in the field by mobile harvesters. During continued operation, the surfaces of the equipment become seeded through contact with leaves, stems, and pods, and growth ensues to the extent that liberated plant juices permit, an observation made with other LAP bacteria by Stirling and Whittenbury (21). Consequently, a product which probably is free from bacteria in nature by virtue of its protection will acquire a marked load of bacteria during harvesting.

In the sense that a field of plants does not represent a fluid mixture, the frequency of a given species of bacterium and the level of population to which it ascends on surfaces of individual plants is dictated by its frequency in the environment, and by its ability to reproduce on that surface. Most of the plant parts employed in this study are extensions of growing tips. Bacteria are present either because at one time the growing tip has come into contact with the bacteria and they then accompany it (11), or because they have been introduced by some outside agency. The relative constancy in occurrence of the same species of the LAP bacteria on a variety of plants which have been subjected to differing methods of sampling and harvesting, despite seasonal progression, points very strongly to a natural residuum affecting the growing tip.

Insects have been studied as vectors of streptococci in nature by Eaves and Mundt (4), and of enterococci by Geldreich et al. (7), but their role may not be major in the distribution of the streptococci. The diversity of streptococcal flora observed by Eaves and Mundt has not been observed on the vegetables in this study. There is a subjectively observed dearth of winged insects in large, monolithic crop fields. In contrast to the grasses and woodlands, there is a marked absence of the motile vertebrate fauna, a feature which has been noted earlier (14) for cultivated fields. The conclusion, therefore, of a wholly independent existence of the LAP bacteria, with a distribution and a level of population compatible with the ability of each taxon to thrive under the conditions imposed by the environment, seems inescapable. The consistent association of the enterococci with other members of the LAP bacteria strengthens the contention that they are part of the natural microflora of nature.

Splittstoesser and Gadjo (19) suggested that geography plays a role in the pattern of the LAP bacteria present on vegetables. The distribution of *S. lactis* tends to confirm the observation. The bacterium occurs with some frequency on raw plant parts in New York State (20), and it is also dominant among the LAP bacteria on processed peas, beans, and corn (19). In neither Geldreich's study (17) nor in this one was the bacterium recovered with any frequency or in significant numbers.

A. viridans has been reported to occur in the atmosphere (24), diseased lobster (E. R. Hitchner and S. F. Sniezko, J. Bacteriol. **54**:48), and cured (2) and processed (12) meats. These would not seem to be natural habitats for it. Recovery during this study from vegetables such as squash, okra, greens, and southern peas suggests that it may be a natural resident on plants. Deibel and Niven (2) suggested that it is a member of the genus *Pediococcus*.

Approximately 70% of the isolates of *L. mesenteroides* produced slime on 4% sucrose-agar. The great majority of these were like type A, according to the description of McCleskey and Faville (13), and the remainder were like type B. Only four of the cultures not producing dextran were identified as other than *L. mesenteroides*. Apparently, species such as those described by Garvie (6) either are rare in this geographic area, or else they do not occur on the plants included in this study.

The yellow streptococci produce a pale, lemon-yellow pigment on clear media. Graubal (9) considered them identical to *S. faecium*. There are, however, differences in physiology between the taxa, and between the yellow bacteria and the classical enterococci in distribution. They occurred most frequently, and were most numerous, during the spring and early summer months. They tended to disappear as the season progressed, when the enterococci were in the ascendancy.

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