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# Lactobacilli on Plants

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The distribution, enumeration, and identification of lactobacilli on vegetable plants were studied in an area described geographically as being subtropical and moist. The lactobacilli were obtained, by means of quantitative enrichment procedures in Rogosa's SL broth, from 35.3% of all samples incubated at 32 C, and from 15.4% of the samples incubated at 45 C. Less than 10 lactobacilli/g of plant material were enumerated in 54% of all positive samples. The lactobacilli were found much less frequently and in lower numbers than were streptococci or *Leuconostoc mesenteroides*. The most frequently isolated lactobacillus was very simular to, but not identical with, *Lactobacillus fermenti*. It was aerogenic, grew well at both 15 and 45 C, fermented arabinose, lactose, and sucrose, and liberated ammonia from arginine. Of the identified species, *L. plantarum*, *L. fermenti*, and *L. brevis* were the most frequently isolated, whereas *L. casei*, *L. viridescens*, *L. cellobiosus*, *L. salivarius*, and *L. buchneri* were obtained from small numbers of samples. The widespread but sporadic distribution of lactobacilli in low numbers seems to indicate that these organisms do not normally thrive on plant surfaces. A ternary cycle, beginning with intestinal waste, followed by mechanical distribution to and among plants, and return to the host via the oral cavity, is suggested.

Few systematic studies of the distribution, enumeration, and identity of lactobacilli on plants have been carried out. Some knowledge has been gained by studying the species which were most prevalent during fermentations or by studying plant materials under essentially artificial conditions which enabled the most adaptable species to increase in numbers (3). Studies by Kroulik, Burkey, and Wiseman (15) and by Stirling and Whittenbury (24) indicate that, in a natural plant environment, lactobacilli are present in low numbers. Nilsson and Nilsson (20), Allen et al. (4), and Woeller (26) found that lactobacilli may be absent under certain conditions, or that they may be present on plants in very large numbers under other conditions. When identification has been made, the main, if not the only, species reported has been *Lactobacillus plantarum*.

In this paper, we report studies of the distribution, enumeration, and identification of lactobacilli occurring on plants; these studies are comparable to those which have been done on the oral cavity (8, 22) and on intestinal contents (16). The climate of the area in which our studies were conducted is described geographically as being subtropical to moist, with 100 to 130 cm of rainfall per year. The rainfall is inequitably distributed, with the greater quantity falling during the late winter and spring.

## MATERIALS AND METHODS

*Samples.* Samples were obtained from fields, gardens, and processing plants. With the exception of squash and cucumber, from which surface portions were removed for use with a slotted paring knife, all samples were used as received, without washing or trimming. Raw processing samples, which received partial treatment, were obtained prior to blanching, and processed samples were obtained prior to or after freezing.

*Primary medium.* Rogosa's SL broth was prepared with 0.04% cycloheximide and with 10% less water than specified, for use at 45 C. To restrict overgrowth by *Leuconostoc mesenteroides* at 32 C, 95% ethyl alcohol was added to comprise 7% of the same medium after boiling, acidification, and cooling (19). The media were tubed into sterile screw-capped tubes in 9.0- and 4.5-ml quantities.

*Modified MRS medium (MMRS).* The medium of De Man, Rogosa, and Sharpe (17) was prepared with di- rather than tri-ammonium phosphate, 1.5% agar, and 0.01% 2,3,5-triphenyltetrazolium-HCl (TTC). When used as a liquid, the beef extract and the TTC were omitted. Other modifications are described in the text.

*Anaerobiosis.* All plate media were incubated after inoculation in large kettles fitted with gas taps. A mixture of 95% $N_2$ and 5% $CO_2$ replaced the air of the vessels after a single evacuation with a mechanical pump.

*Enumeration and isolation.* A 1-ml amount of 10% aqueous homogenate of plant material was added to 9 ml of SL media in triplicate for each temperature.

Internal, decimal dilutions were continually made in tubes of the respective media prepared in 4.5-ml quantities. The simple SL medium was incubated at 45 C in a water bath, whereas the medium containing ethyl alcohol was incubated in air at 32 C. Upon development of minimal turbidity, streaks were made on MMRS agar from those tubes in which lactobacilli were observed in stained preparations. Incubation of the broth was then continued for several days to ascertain the most probable numbers (11).

Selections of each type of colony appearing on the MMRS agar were made after 3 days of incubation at 32 C. Outgrowths in MMRS broth which were bacillary, gram-positive, and catalase-negative or weakly positive were assumed to be lactobacilli and were stored for later study. Cultures were restreaked for purity upon reactivation.

*Storage.* Heavy suspensions of young, mature cultures were made in Trypticase soy-0.1% yeast extract (TGYE) containing 16% glycerol (25). These suspensions were promptly frozen and maintained at −18 C. They were reactivated by introduction of 0.1 ml of thawed liquid into MMRS broth.

*Identification.* The criteria used and the keys given by Abo-Elnaga and Kandler (1, 2), Rogosa and Sharpe (21), and Sharpe (23) were used in the identification of the lactobacilli.

MMRS broth was the base medium used to determine the ability of the organisms to ferment carbohydrates and to determine tolerance to 6.5% NaCl. Bile tolerance was determined on MMRS agar containing 4% sodium taurocholate. TGYE replaced the nutritive portion of the medium of Ball and Sellers (5) for detection of motility at 24 hr and digestion of gelatin at 7 days. Tests for production of catalase were performed on the restricted carbohydrate medium of Evans and Niven (10), according to the method of Dacre and Sharpe (7). Presence of indole and nitrite was determined with Kovac's reagent and with $\alpha$-naphthylamine and sulfanilic acid, after growth in the standard indole-nitrate medium. The principle of the procedure of Gibson and Abdel-Malek (13) was used for detection of aerogenesis. To determine the release of ammonia, ammonium citrate was replaced by arginine in MMRS medium. Hydrolysis of esculin was detected by the formation of white, coral-like crystals in the medium of Gemmell and Hodgkiss (12).

Cell walls of cultures identified as or suspected of being similar to *L. plantarum* were hydrolyzed according to the method of Cummins and Harris (6), and chromatograms were made according to the procedure of Abo-Elnaga and Kandler (1) for the detection of diaminopimelic acid.

Inocula consisted of single drops of 1-day-old broth cultures in liquid media, shaken loop streaks on agar media, and needle stabs. Water baths were used for incubations at 15 and 45 C.

## RESULTS AND DISCUSSION

*Distribution.* Lactobacilli were obtained from 60 (35.3%) of 160 samples incubated with SL-ethyl alcohol broth at 32 C, and from 25 (15.4%) of the samples incubated at 45 C (Table 1). These organisms were recovered most frequently from corn, and were not prevalent on grasses, cereal plants, peppers, or leaves.

Stirling and Whittenbury (24) reported an increase in per cent recovery of lactobacilli from plants which had been in contact with harvesting equipment. The processing of harvested vegetables also resulted in an increase in the number of samples from which lactobacilli were recovered (Table 2), both prior to blanching for freezing and in the finished product. The presence of lactobacilli in the finished product, either immediately prior to or after freezing, is probably the result of airborne transfer from raw product areas and subsequent growth on processing equipment (18).

TABLE 1. *Frequency of occurrence and numbers of lactobacilli on plants*

| Plant source | No. of samples | No. with growth | | MPN[a] per gram | |
|---|---|---|---|---|---|
| | | 32 C | 45 C | 32 C | 45 C |
| Cereal plants | 8 | 0 | 1 | 0 | 3.6 |
| Sorghum, Sudan | 11 | 2 | 0 | 23–43 | 0 |
| Corn, flowers and leaves | 16 | 13 | 2 | 3.6–460 | 23 |
| Greens, raw | 5 | 0 | 0 | | |
| Greens, processed | 5 | 2 | 2 | 3.6–7.3 | 3.6 |
| Okra, raw | 6 | 2 | 0 | 3.0–9.1 | 0 |
| Okra, processed | 2 | 2 | 2 | 240–460 | 23–93 |
| Green beans, raw | 5 | 1 | 1 | 23 | 3.6 |
| Green beans, processed | 2 | 1 | 1 | 23 | 9.1 |
| Squash, raw | 21 | 6 | 3 | 3.6–7.3 | 3.6 |
| Squash, processed | 15 | 12 | 8 | 3.6–7.3 | 3.6 |
| Lima beans, pod | 5 | 0 | 0 | | |
| Lima beans, hulled | 5 | 1 | 1 | 43 | over 460 |
| Lima beans, raw processed | 1 | 1 | 0 | 23 | 0 |
| Lima beans, frozen | 2 | 0 | 0 | | |
| Pepper, green | 4 | 0 | 0 | | |
| Peas (Southern) pods | 14 | 4 | 0 | 3.6–4.3 | 0 |
| Peas, hulled | 8 | 8 | 4 | 3.6–460 | 43–240 |
| Peas, processed | 2 | 0 | 0 | | |
| Cucumber | 7 | 3 | 0 | 3.6–7.3 | |
| Lettuce | 2 | 1 | 0 | 7.3 | |
| Leaves, miscellaneous[b] | 14 | 1 | 0 | 3.6 | |
| Total | 160 | 60 | 25 | | |

[a] Most probable numbers.

[b] Leaves include English pea, beet, radish, strawberry, rhubarb, turnip, and potato.

TABLE 2. *Frequency of occurrence of lactobacilli according to source of sample and temperature of incubation*

| Source of sample | No. of samples | Incubation temp (C) | Lactobacilli present | |
|---|---|---|---|---|
| | | | No. | Per cent |
| Field | 120 | 32 | 33 | 27.5 |
| | 120 | 45 | 7 | 5.8 |
| Raw product in process | 14 | 32 | 10 | 71.4 |
| | 14 | 45 | 5 | 35.7 |
| Processed for freezing | 28 | 32 | 17 | 60.7 |
| | 28 | 45 | 13 | 46.4 |

*Numbers.* The most probable numbers of lactobacilli per gram of sample are listed in Table 1. The numbers may have exceeded $4.6 \times 10^2$/g in only six samples, three of which were of corn. Less than 10 lactobacilli/g were recovered from 54% of the positive samples. The very low numbers account for the less extensive recovery when solid isolating media were used, since the maximal amount of material introduced to the plates was either 0.1 or 0.01 g. The low numbers suggest a mechanical distribution of the lactobacilli, without subsequent growth. Had there been any degree of colonization, the numbers per gram of sample would have been materially greater.

*Identification.* The lactobacilli were distributed among eight identified species and one other group (Table 3). *L. plantarum*, *L. brevis*, and *L. fermenti* were present most frequently and in the greatest numbers. *L. cellobiosus*, first isolated from saliva (22), was isolated from five samples of corn. *L. viridescens* and *L. salivarius*, which have not been reported previously to occur on plants, were isolated once. The latter species was the only member of the thermal subgroup of the genus to be isolated in this study. With the exception of cucumbers, which yielded only *L. plantarum*, more than one species occurred within each of the plant groups. *L. casei* was isolated once from each of three different plants, and *L. buchneri* was isolated once.

Nearly 100 of the unidentified cultures comprised a basically homogeneous, aerogenic group which grew well at both 15 and 45 C. These cultures fermented arabinose and sucrose, and most of them fermented lactose and liberated ammonia from arginine. Although these cultures are similar to *L. fermenti*, many of them fermented mannitol, which is a distinguishing feature of *L. salivarius*, melezitose, which is characteristic of *L. buchneri*, or cellobiose, a property of *L. cellobiosus*. More than 50% of the cultures produced nitrite from nitrate. The cultures were isolated from a variety of plants, including corn, greens, lima beans, okra, and squash.

*Comparison of occurrence of lactobacilli and streptococci.* To obtain a comparison in the frequency of these two groups, fruiting heads of grasses and cereals, representing all stages of maturity, were placed into tubes of MMRS-0.02% $NaN_3$ or into TGYE-0.02% $NaN_3$ broths. Other samples were plated after homogenization on MMRS agar containing $NaN_3$. Outgrowths were isolated and identified, with the results shown in Table 4.

Members of the genera *Streptococcus* and *Leuconostoc* were obtained from 52.3% of 108 samples, whereas lactobacilli were obtained from 4, or 3.7%, of the samples. Spherical bacteria were only obtained from samples from which the plate counts ranged between $5 \times 10^5$ to $3.4 \times 10^7$/g of material. Woeller (26) reported very large populations of lactobacilli on cereals; however, in reaching this conclusion, he appears to have relied upon morphology of random observations, rather than upon numerical distinction between the lactobacilli and streptococci. Thus, it seems likely that, as in this study, most of the lactic acid-producing bacteria which he reported belong to the spherical, rather than the bacillary, genera.

The cumulative literature on the subject of lactobacilli on plants is not in accord with the concept that plants are a natural reservoir of the lactobacilli. Not all species of lactobacilli considered to be typical "plant types" (14) were isolated in our study, nor were the identified species restricted to "plant types." The lactobacillary flora do not appear to be any more characteristic for plants than for the oral cavity (8, 22) or for the intestinal tract (16). The "plant types" are found in all environments, and they are quite susceptible to implantation into the intestinal tract (16).

Davis (9) has suggested that the intestinal tract is the natural habitat of the lactobacilli, and that these organisms escape through wastes and thrive in nature under unique environmental conditions. The studies of Nilsson and Nilsson in Sweden (20) implicate temperature, since lactobacilli were recovered from cereals, clovers, and grasses after the beginning of July. Woeller's studies in Germany (26) suggest the influence of rainfall and humidity; he reported that the presence of lactobacilli on cereals was infrequent or in low numbers during a relatively dry year, but generally present in high numbers during a year of greater rainfall. Woeller's observation appears to be most applicable to the southeastern part of the United States. The infrequent occurrence of lactobacilli in low numbers throughout the growing season

TABLE 3. *Frequency of occurrence of species of Lactobacillus on plants*

| Plant source | No. of samples | No. of samples containing various species of *Lactobacillus* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *plantarum* | *brevis* | *fermenti* | *viridescens* | *cellobiosus* | *buchneri* | *casei* | *salivarius* | Unidentified |
| Cereal plants | 8 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sorghum, sudan | 11 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Corn | 16 | 5 | 7 | 0 | 1 | 5 | 0 | 0 | 0 | 4 |
| Greens | 10 | 2 | 0 | 2 | 0 | 0 | 1 | 0 | 0 | 2 |
| Okra | 8 | 2 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| Green beans | 7 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 |
| Squash | 36 | 10 | 3 | 13 | 0 | 0 | 0 | 0 | 0 | 12 |
| Lima beans | 13 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| Pepper, green | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Peas, Southern | 24 | 11 | 6 | 4 | 0 | 0 | 0 | 1 | 0 | 7 |
| Cucumber | 7 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Leaves | 16 | 1 | 3 | 2 | 0 | 0 | 0 | 0 | 0 | 2 |
| Total | 160 | 35 | 21 | 23 | 1 | 5 | 1 | 3 | 1 | 33 |
| Per cent | | 21.9 | 13.1 | 14.4 | 0.6 | 3.1 | 0.6 | 1.9 | 0.6 | 20.6 |

TABLE 4. *Occurrence of spherical lactic acid-producing bacteria as compared with occurrence of lactobacilli on plants*

| Type of sample | No. of samples | No. of spherical bacteria obtained | No. of lactobacilli obtained |
|---|---|---|---|
| Grasses[a] | 13 | 7 | 1 |
| Fodders[b] | 36 | 25 | 1 |
| Legumes | 6 | 3 | 1 |
| Vegetables | 13 | 5 | 0 |
| Cereals[c] | 41 | 17 | 1 |
| Totals | 109 | 57 | 4 |
| Per cent | | 52.3 | 3.7 |

[a] Fescue, orchard, and rye grasses.
[b] Corn and sorghum sudan.
[c] Barley, oats, rye, and wheat.

makes the concept of a ternary cycle, from intestinal waste to plants to reimplantation via the oral cavity, tenable.

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