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Progression of Epiphytic Microflora in Wheat and Alfalfa Silages as Observed by Scanning Electron Microscopy

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Wheat and alfalfa silages were examined by scanning electron microscopy and standard methods of microbial enumeration. Epiphytic microflora were present at levels of 10^6 to 10^8/g in the fresh-cut plants. This flora was initially observed microscopically primarily on the surfaces. After 4 days of fermentation, lactic acid bacteria were observed on the surface in high concentrations near open stomata and throughout the interior mesophyll air sac spaces. At 4 days, populations on interior surfaces were restricted to the exterior surfaces of the air sacs. After 8 days the mesophyllic cells showed marked deterioration, and bacteria were observed on their inner surfaces. At 32 days, the end of the fermentation, vascular bundles and epidermal cells remained intact whereas stomata and mesophyllic cells were collapsed and often contained microorganisms. It is concluded that the interior of the leaves offers substantial nutritional and environmental advantages to epiphytic flora and is an important if not major deterioration site in fermented products. Since little deterioration of exterior surfaces was observed, these sites may play a minor role in supplying nutrients for microbial growth.

The fermentation of forage crops to produce a storage-stable product is an important method of feed preservation (21). The forage is harvested, chopped, and placed in an enclosed environment to restrict oxygen transfer. The environment quickly becomes reduced because of continued removal of oxygen by residual plant respiration and bacterial metabolism. Within 1 to 3 days, lactic acid bacteria dominate the fermentation, producing lactic and acetic acids (4). The pH declines to generally <4.5, and the preserved silage will retain the maximum energy and nutritive value.

The speed and extent of the fermentation process depend on the presence of a lactic acid bacterial population that can rapidly grow and dominate the fermentation (12, 13). The characterization and isolation of the bacteria predominating during silage fermentation have been studied extensively (14, 20) and reviewed (13). The predominant lactic acid bacteria during fermentation are streptococci and lactobacilli, with *Lactobacillus plantarum* often being the most frequently recovered (18). Less is known about the source of the lactic acid bacterial population of forage plants before ensiling. The numbers of lactic acid bacteria are often quite low on plants and are usually a relatively minor portion of the total epiphytic microflora. The actual number of bacteria present on plants depends on the plant variety, climate, maturity, and plant part (11, 17).

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The actual site of bacterial growth in and on plants has not been clear (20). However, recently the interaction of bacteria and the leaf microenvironment has received more research emphasis. Bacteria can occur "on" as well as "in" growing plant parts (6). The surface of the leaf would be readily inoculated with bacteria from dust and airborne microflora, and these bacteria may be able to survive on leaf surfaces. However, survival would be determined by resistance to harsh environmental factors including ultraviolet light and limited nutrient supply (2). Bacteria can achieve entrance into the interior of leaves, where they might be better protected from harsh environmental factors, through stomatal openings and trichomes (7). Bacterial survival in the interior of leaves and other plant parts has been demonstrated (6, 11, 19). Microenvironmental considerations suggest that the interior of leaves may offer a richer source of nutrients and be the major site of deterioration during fermentation. There have been no sequential time studies to date on the fermentation process, so that the actual sites of bacterial action have not been identified.

The purpose of this investigation was to determine the sites and extent of growth of the microflora of wheat and alfalfa during silage fermentation. Results indicate that the interior

surfaces of leaves are a major site of bacterial colonization. A portion of this work has been presented (Proceedings of the Southern and South Carolina Branches of the American Society for Microbiology, Atlanta, Ga., 1979). This study is part of a larger research effort in defining the microbiology and chemistry of wheat, alfalfa, corn, and sorghum silages, which is being published elsewhere (N. J. Moon, L. O. Ely, and E. M. Sudweeks, manuscripts in preparation).

MATERIALS AND METHODS

Materials. Wheat (*Triticum aestivum*) and alfalfa (*Medico sativa*) were mechanically harvested (in April and June, respectively) from pastures at the University of Georgia Experiment Station (Experiment, Ga.). The crops were chopped into pieces about 5 cm in length and packed tightly into flexible polyethylene bags. Each bag was sealed to exclude air and then placed in a 0.21-m^3 steel drum. Drums were stored in an unheated barn (average ambient temperature of 17°C for wheat and 24°C for alfalfa), and the microbiological progress of the fermentation was assessed during the 32-day fermentation period. At 0, 1, 2, 4, 8, 16, and 32 days, one drum was selected at random and its bag was opened. Samples were aseptically removed from the center of the bag, placed in sterile oxygen-impermeable bags, and immediately examined for viable microorganisms. Subsamples were frozen at −15°C for up to 1 month before microscopic examination.

Sample preparation for scanning electron microscopy. Sample bags were removed from the freezer and opened, and several leaf blades were removed and allowed to thaw (suspended by forceps) at room temperature (about 2 min at 21°C). Smaller subsamples (~5 mm) were then immediately excised with sterile surgical scissors, taking care not to damage leaf surfaces. Excised samples were placed in sterile 30-ml screw-cap glass sample vials and further treated before microscopic examination. Samples for examination of specific plant parts (at each sample period) were taken from several different plants or silage pieces to help minimize sampling effect. For example, several alfalfa center leaf sections were examined from different leaves on different plants or silage pieces. At least four specimens from each fermentation time period were prepared for microscopic examination. Extensive observations were made of all excised samples at several magnifications. For example, when leaf stomata were examined for exterior surface characteristics, about 30 stomata on each of four different leaf samples were examined at low (×1,000) and high (×5,000) magnification. This resulted in about 120 observations at two magnifications on each sample for each structural detail.

To determine the possible role of freeze-thaw damage to leaf structure, duplicate samples of unfrozen silage at 0, 8, and 32 days were compared with those that had been frozen at −15°C. The possible effect of translocation of bacteria during sample preparation was evaluated by comparing adjacent leaf blade sam ples prepared either by standard fixation in glutaraldehyde and critical-point drying or by freeze-drying. Ethanol cryofracture was used to expose interior surfaces of leaves, and these samples were compared with a freeze-dried specimen to indicate the translocation of surface bacteria during sample preparation.

Fixation and dehydration. Leaf blade subsamples were fixed by immersion in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for at least 1 h. The samples were drained and placed in two consecutive 1-h washes of 0.1 M cacodylate buffer containing 5% sucrose. Samples were then stored in fresh cold cacodylate-sucrose buffer for transport to the electron microscopy laboratory. Samples were dehydrated in a series of ethanol-water washes (25% through 100% [vol/vol]) for 15 min each and critical-point dried from liquid CO_2. Finally, samples were sputter coated (Technics, Inc.) with a thin layer of gold-palladium and observed using a Cambridge Mark 2A or a Cambridge S150 scanning electron microscope.

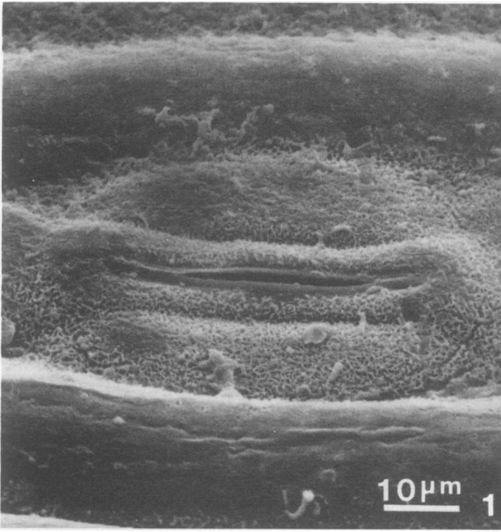
Cryofracture. Fixed samples were ethanol cryofractured as described by Humphreys et al. (8, 9). The thawed specimens were then dried by the critical-point method and coated for scanning electron microscope study as described above.

Freeze-drying. Leaf blades were frozen at −20°C and then dried under high-vacuum sublimation in a freeze dryer (Virtis Co., Gardiner, N.Y.). Samples were then coated with gold-palladium and observed by scanning electron microscope.

Microbiological analysis. A 10-g subsample was aseptically removed from the sterile sample plastic bag and blended with 90 ml of sterile phosphate buffer (0.3 mM) for 1 min in a sterile 200-ml blender jar on a Waring blender. Standard microbiological procedures were followed in the preparation of pour plates (5). Total aerobes were determined by using tryptic soy broth (Difco) plus agar (1.5%), and plates were incubated at 32°C for 7 days. Total counts of microaerophilic flora were determined by using this same agar, incubated at 32°C in a 15% CO_2 atmosphere (Hot Pack Incubators, Philadelphia, Pa.). This latter method has been demonstrated to give good recovery of several lactic acid bacteria (15). To partially identify the predominant aerobic and microaerophilic flora on the plates, representative colonies (about 80) were picked and inoculated into tryptic soy broth and litmus milk. After a suitable incubation period, the cultures were examined for their litmus milk reaction, Gram stain, presence or absence of motility, cell shape, growth habit, and catalase production.

RESULTS

Few bacteria were observed on the surfaces of either wheat (Fig. 1) or alfalfa (Fig. 2) in fresh samples regardless of whether samples were prepared by standard glutaraldehyde fixation and dehydration or by freeze drying. The surfaces of wheat and alfalfa leaves were covered by a protective waxy cuticle (Fig. 1 and 2). The stomata on the lower surface of the leaves were often open, whereas those on the upper surface remained closed. Since few bacteria could be ob-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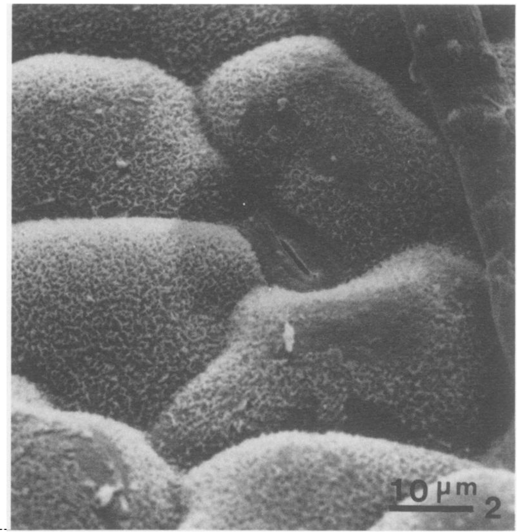
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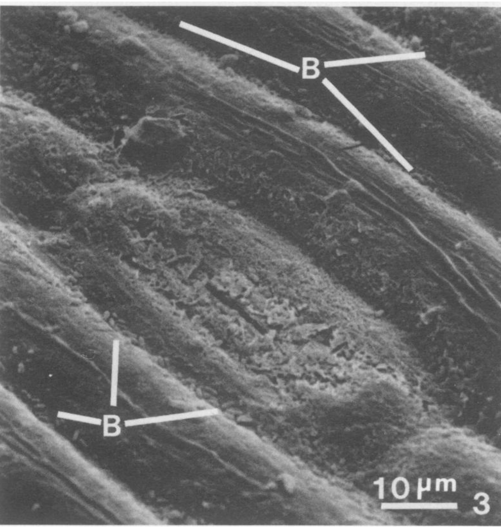
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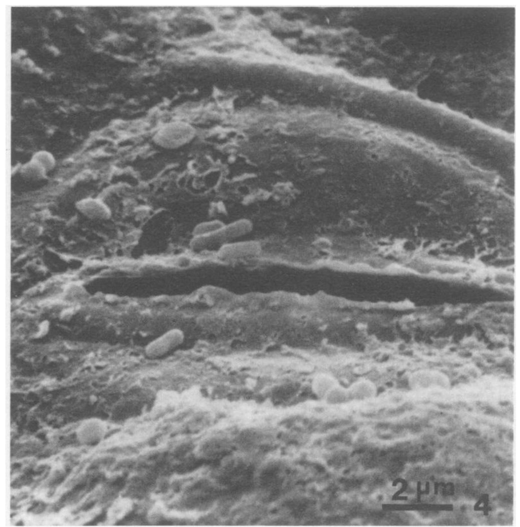
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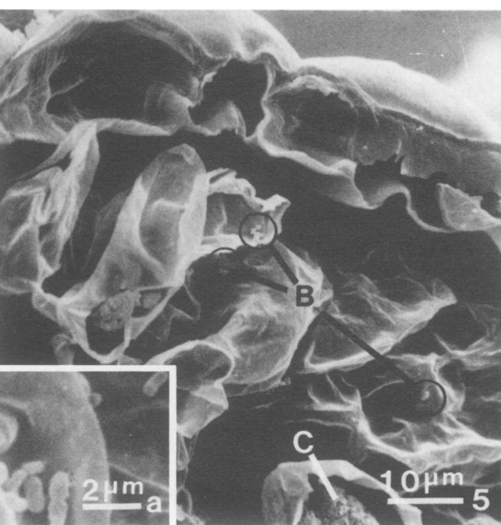
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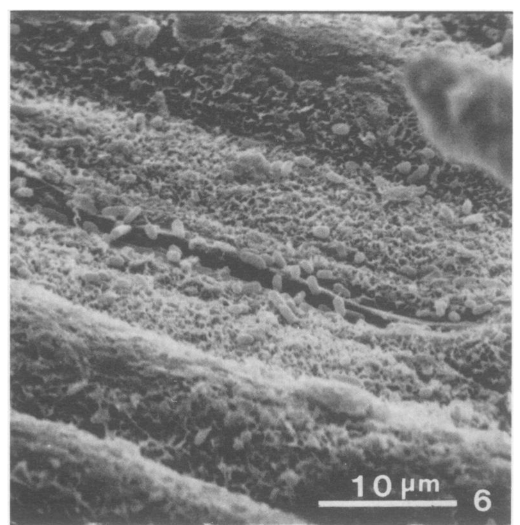
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served on the outer fresh-leaf surfaces, the interior of the leaves was examined for evidence of microcolonies that could serve as the inoculum for the fermentation. Observations of freeze-dried preparations with the epidermal cell layer carefully removed before coating for scanning electron microscopy did not indicate the presence of bacteria on the interior of the sample. Cryofractured preparations did not indicate the presence of bacteria within plant cells nor in the interior areas around the open stomata. These areas were examined with particular care, but no bacteria were observed. Apparently the few bacteria on the leaf surface can serve as a source of inoculum, or else bacteria are harbored at other sites on or in the plant.

The viable numbers of bacteria increased most rapidly for wheat after only 1 day (Table 1). Thereafter a second slower growth rate was observed, with maximum populations being reach after 4 days. Alfalfa showed a similar pattern, with maximum populations reached after 2 days. The initial population in wheat (10^6/g) was much lower than that in alfalfa (10^8/g) and may relate to actual differences in bacterial populations or plant nutrients or to some environmental factor (e.g. weather or season of harvest).

After 4 days of fermentation, wheat and alfalfa samples reached maximum population levels of 10^9 to 10^{10}/g (Table 1). Viable counts remained at this level for about 8 days, but then declined 10-fold by the end of the 32-day storage period. Examination of leaf surfaces at 4 days indicated bacteria between ridges of vascular bundles of wheat (Fig. 3), whereas alfalfa samples tended to have microorganisms more evenly distributed over the entire surface of the leaf. It appeared that in both plants there were larger numbers of bacteria on or near the stomata than elsewhere on the leaf surface (Fig. 3 and 4).

Observation of interior surfaces of samples taken at 4 days and prepared by cryofracture indicated the presence of bacteria in both wheat and alfalfa (shown on alfalfa, Fig. 5). The bacteria were mainly confined to the exterior of the walls of the air spaces of the leaves. Bacteria were observed neither in the vascular bundle region nor in epidermal cells. Few of the cells lining the air sacs were invaded by bacteria, but many cells appeared to be collapsed, indicating some deterioration of integrity. The bacteria observed on both interior and exterior surfaces were predominantly short rods. Very few yeasts or molds were observed, but cocci were often present on the surface, particularly in the case of alfalfa. Light microscopic observation of bacteria from colonies on tryptic soy agar incubated in a CO_2 (15%) environment indicated that gram-positive rods and cocci were predominant. Subsequent examination of selected colonies indicated that these organisms were members of the genera *Lactobacillus* and *Streptococcus*. The aerobic colonies tended to be primarily streptococci, but some yeast and gram-negative motile rods were present early in the fermentation.

After 8 days, the number of bacteria observed by scanning electron microscope on the surfaces of the leaves had increased (Fig. 6 and 9), although numbers of colony-forming units re-

TABLE 1. *Viable number of bacteria in wheat and alfalfa silages during fermentation*

Silage	Population[a]	No. of bacteria ($\log_{10}$ CFU/g)[b] after days of fermentation:						
		0	1	2	4	8	16	32
Wheat	Microaerophilic	6.25	8.90	9.75	9.90	9.60	9.05	8.75
	Aerobic	6.40	9.16	9.75	9.70	9.50	8.90	8.25
Alfalfa	Microaerophilic	8.18	9.00	9.30	9.25	9.25	8.90	8.35
	Aerobic	8.28	8.80	9.20	9.00	9.25	8.90	8.20

[a] Microaerophilic population as determined by plating on tryptic soy agar and incubation at 32°C in 15% CO_2 atmosphere. Aerobic population as determined by plating on tryptic soy agar and incubation at 32°C.

[b] CFU, Colony-forming units.

FIG. 1. *Leaf surface of freshly cut wheat. Note stoma in center and waxy surface.*

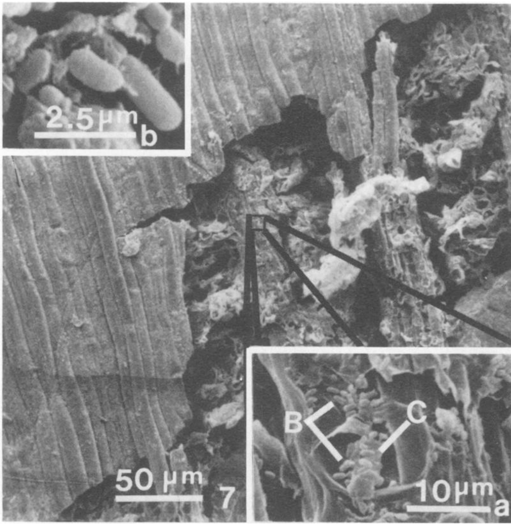
FIG. 2. *Leaf surface of freshly cut alfalfa. Note stoma in center, waxy surface, and leaf hair.*

FIG. 3. *Leaf surface of wheat ensiled for 4 days. Note bacteria (B) between ridges of epidermal cells and on stoma.*

FIG. 4. *Leaf surface of alfalfa ensiled for 4 days. Note open stoma and bacteria on surface.*

FIG. 5. *Ethanol cryofracture of alfalfa leaf after 4 days of ensiling. Note bacteria (B) on inner surface. (a) Enlargement of bacteria on mesophyllic cell surface.*

FIG. 6. *Surface of wheat leaf ensiled for 8 days. Note open stoma and bacteria around and in opening and abundance of wax.*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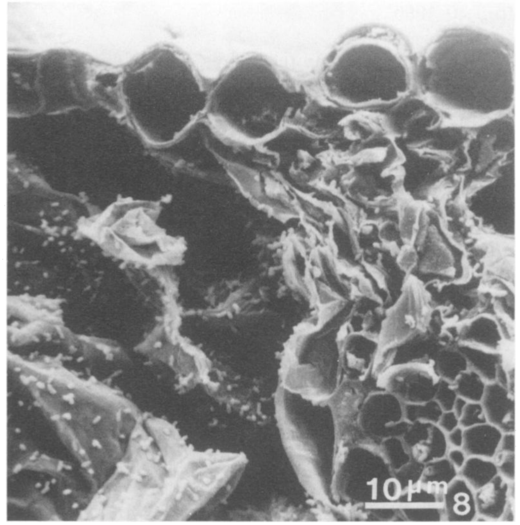
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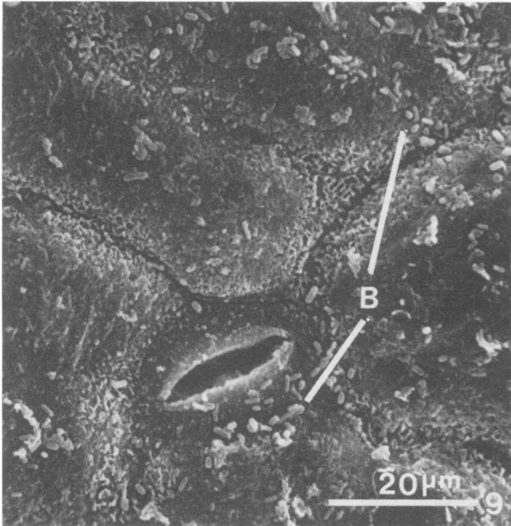
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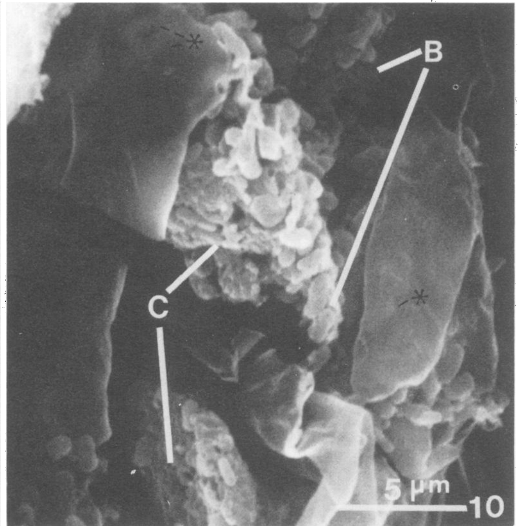
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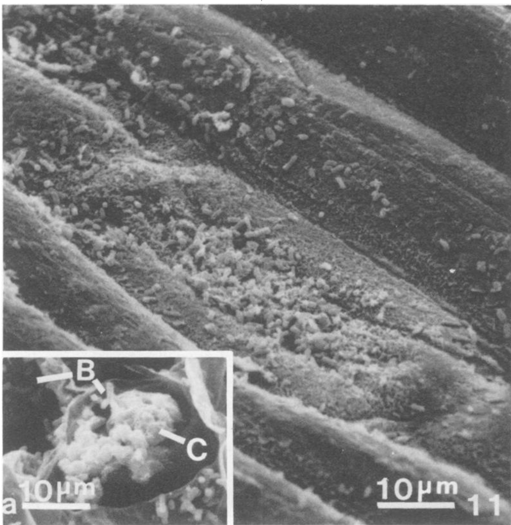
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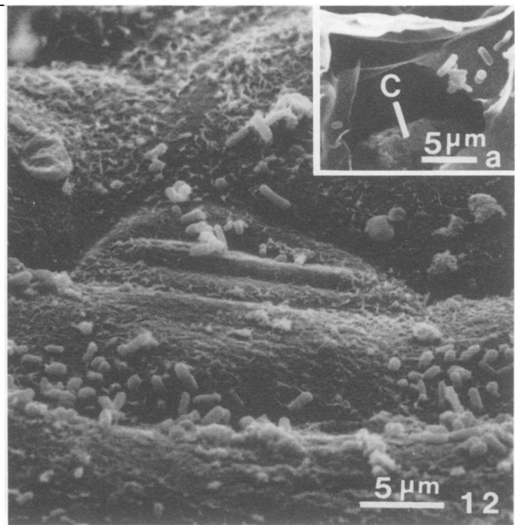
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mained about the same. Apparently bacterial growth continued but not all cells were viable, or viable cells were not recovered by the enumeration procedure. Surface bacteria were found more often near or on the stomata and in the ridges of leaves. The stomata were more collapsed than at earlier times and were likely to be open on both the upper and lower surfaces. The interior of the leaves contained large numbers of bacteria regardless of whether samples were prepared by freeze-drying (Fig. 7) or ethanol cryofracture (Fig. 8 and 10). Since the interior area is much larger than the exterior surface area, relatively larger total numbers of bacteria were observed on the leaves' interior. Again, as observed in samples taken at 4 days, the primary area of observation of bacteria was in the air spaces around the mesophyllic cells and vascular bundles. The mesophyllic cells showed further deterioration (comparing Fig. 5 and Fig. 10) which was apparently not due to freezing and thawing effects, as was evidenced by the presence of bacteria in some cell interiors (Fig. 7a and 10).

Some bacteria in these interior surfaces appeared to be attached to each other and to the surface by fibers (Fig. 7b). However, it is possible that the observed phenomenon here is an artifact of sample preparation and is due to dried cytoplasm or cellular debris. Observation by transmission electron microscopy could help define the significance of these fibers.

Further observations at later times of fermentation indicated continued deterioration of leaves (16 days, Fig. 11 and 12; 32 days, Fig. 13 and 14). Leaf stomata were slightly more collapsed. Many were open, and bacteria could be observed in the stomata openings (Fig. 13, 14). The surfaces of the samples at the end of the fermentation had considerably more debris than fresh plants, probably from bacterial as well as plant cell decomposition (Fig. 1 and 2 versus Fig. 13 and 14). The waxy surface of the leaves remained intact, as did the physical integrity of the epidermal cell wall. Inner surfaces of leaves at later fermentation times again had bacteria in and on cells (Fig. 11a and 12a).

DISCUSSION

The epiphytic microflora present in the fresh forage was located primarily on the surface. Few bacteria were observed in the interior of the leaves. Others (6, 7, 11) have suggested that bacteria may survive on the leaf's interior after achieving entry through open stoma. Saprophytic bacteria have been observed on internal surfaces but are believed to be inactive through lack of nutrients or inhibition by host defenses (19). This study suggests that the primary inoculum for fermentation is on the leaf surface or possibly harbored at other sites on the plant.

The population of bacteria increased soon after the plants were harvested, chopped, and ensiled. The nutrients for the bacterial fermentation and relatively more rapid growth rate initially (Table 1) are probably primarily obtained from the plant cells sheared during the harvesting and chopping of the fresh forage, as others (3) have observed that finely chopped plant material undergoes fermentation more rapidly than whole plants. A second but probably minor source of nutrients initially may be from leakage of plant cells, which is often observed in normal healthy plants (16). Nutrients are probably available to the bacteria in solution as a surface water layer, since the moisture content of these silages was about 70%.

Within a few days the population of bacteria increased greatly within and on the leaves. The first site of bacterial increase within the plant was in the air sac spaces near stoma openings. Some bacteria may be present here initially, or they could migrate in surface water through

FIG. 7. *Leaf surface of wheat ensiled for 8 days and then freeze-dried. Note stoma on surface, vascular bundles, mesophyllic area, and air spaces revealed by removal of epidermal cell layer. (a) Enlarged view of area within rectangle. Note bacteria (B) on exposed inner leaf surface and ruptured plant cell with exposed cytoplasm (C). (b) Enlarged view of bacteria on exposed inner surface shown in Fig. 7. Note presence of fibers.*

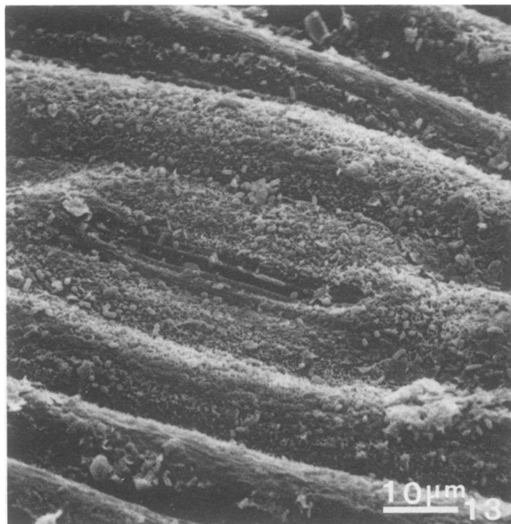
FIG. 8. *Ethanol cryofracture of wheat leaf after ensiling for 8 days. Note vascular bundle in center, surrounded by mesophyllic layer with many bacteria in cells and spaces.*

FIG. 9. *Surface of alfalfa leaf after 8 days of ensiling. Note bacteria (B) on surface.*

FIG. 10. *Interior of alfalfa leaf after 8 days of ensiling and ethanol cryofracture. Note ruptured plant cell with exposed cytoplasm (C) and bacteria (B). Bacteria are visible within the cell still covered by cell wall (*) due to the use of a high accelerating voltage (20 kV) and a very thin coating of gold palladium.*

FIG. 11. *Surface of wheat leaf after 16 days of ensiling. Note concentration of bacteria around stoma. (a) Interior of same leaf exposed by removal of the epidermis, showing exposed cytoplasm of a ruptured plant cell (C) and bacteria (B).*

FIG. 12. *Surface of alfalfa leaf after 16 days of ensiling. Note presence of wax on surface and large numbers of bacteria. (a) Interior of same leaf exposed by removal of epidermis, with bacteria and exposed cytoplasm (C) of a ruptured plant cell visible.*



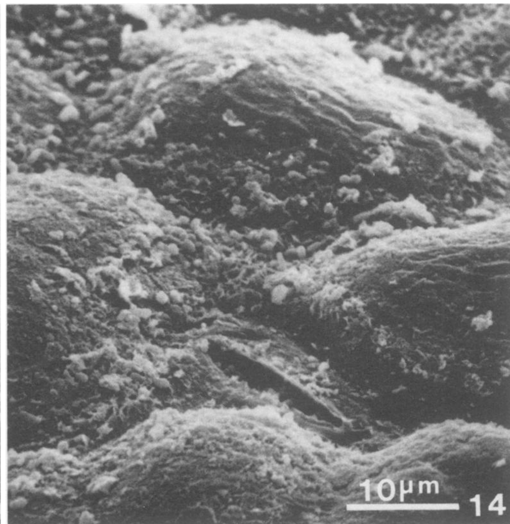


FIG. 13. *Surface of wheat leaf ensiled for 32 days. Note open stoma and bacteria in opening.*
FIG. 14. *Surface of alfalfa leaf ensiled for 32 days. Note large open stoma and bacteria on surface.*

open stoma during the early fermentation period. The relatively vast interior area of the leaf continued to harbor high populations of bacteria, and mesophyll cells appeared to become more deteriorated with time and ultimately were invaded by bacteria. The epidermal surface remained relatively more intact. The nutrients during later periods of fermentation, when rates of increase of viable counts were slower, were obtained, probably, mainly from deteriorating mesophyll cells. Those cells most resistant to digestion, namely vascular bundles and epidermal cells (9), remained intact throughout the fermentation.

The discrepancy observed here between visual populations and viable bacterial counts at later fermentation times may be because of the inability of the plating techniques used to recover the bacteria observed. The lactic acid bacteria growing later may not be recovered, or many observed bacteria may be nonviable. There may also be other bacteria which would not be recovered by the plating techniques used. For example, anaerobic flora such as clostridia often cause a secondary fermentation of lactate to produce butyric and other acids. These bacteria would probably not be recovered on the tryptic soy agar incubated in a 15% CO_2 atmosphere used in this study.

A similar progression of lactic acid bacterial populations and plant decomposition may occur in the interior of other fermented food products such as pickles or sauerkraut. The fermentation process presented here for silage is visually similar to that described by Akin (1) for forage digestion in the rumen. However, digestion in the rumen is a much more complete deterioration of plant parts than is silage fermentation, where the supporting structural integrity of the plant is maintained.

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