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Growth and Survival of Genetically Manipulated *Lactobacillus plantarum* in Silage

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The growth and persistence of two genetically manipulated forms of *Lactobacillus plantarum* NCDO (National Collection of Dairy Organisms) 1193 have been monitored in grass silage. Both recombinants contained pSA3, a shuttle vector for gram-positive organisms that encodes erythromycin resistance. In one of the recombinants, pSA3 was integrated onto the chromosome, whereas in the other, a pSA3 derivative designated pM25, which contains a *Clostridium thermocellum* cellulase gene cloned into pSA3, was maintained as an extrachromosomal element. This extrachromosomal element is a plasmid. Rifampin-resistant mutants were selected for the recombinants and the parent strain. When applied to minisilos at a rate of 10^6 CFU/g of grass, both the recombinants and the parent strain proliferated to dominate the epiphytic microflora and induced an increase in the decline in pH compared with that of the noninoculated silos. The presence of extra genetic material did not appear to disadvantage the bacterium in comparison with the parent strain. The selective recovery of both strains by using rifampin and erythromycin was confirmed by Southern hybridization. Interestingly, the free plasmid (pM25) appeared more stable in silage than was expected from studies in MRS broth. The plasmid was retained by 85% of the rifampin-resistant *L. plantarum* colonies isolated from a day 30 silo. These data answer an important question by showing that genetically manipulated recombinants of *L. plantarum* can proliferate and compete with epiphytic lactic acid bacteria in silage.

Ensiling is a major method of forage conservation in temperate countries and is used to provide a feed of high nutritive value for ruminants. Silage is produced by the anaerobic fermentation of sugars in the forage by the indigenous microflora. The course of the silage fermentation is, therefore, directed by the type and number of the epiphytic microflora on the forage.

The principal natural fermenters are lactic acid bacteria (LAB), both heterofermentative and homofermentative strains (10). A natural fermentation can produce considerable quantities of acetic acid, butyric acid, and ethanol (9), which in comparison with lactic acid are undesirable in achieving a rapid reduction in pH and maximizing nutrient conservation (4). Furthermore, the number of LAB on both alfalfa (14) and grass (18) is highly variable, thereby influencing the onset of fermentation. To better control the ensilage process, homofermentative lactic acid bacteria are being increasingly used as silage inoculants (22). Provided that these inoculants are added at a rate sufficient to dominate the epiphytic microflora and are given an adequate supply of substrate, they should ensure a rapid, desirable fermentation.

Since strains of *Lactobacillus plantarum* are frequently used as silage additives, there is considerable potential in genetically manipulating this bacterium to enhance its fermentation profile (1). A limiting factor in the fermentation of grass silages is the availability of soluble sugars as a substrate for a rapid fermentation (15). A recent development to alleviate this problem has been the addition of commercially produced cellulases and hemicellulases during ensilage to release soluble sugars from the degradable fiber components of the ensiled grass (25). An alternative approach to the addition of extracellular enzymes is the genetic manipulation of *L. plantarum* such that cellulase activity is expressed by the organism itself (2, 21). These recombinants could potentially be used as silage inoculants.

However, given current concern about the risks associated with the use of genetically modified microorganisms in open ecosystems, it is important for control and legislative purposes that the survival and dispersion of such bacteria be monitored. A frequent criticism of genetically manipulated microorganisms and a limit to their commercial use has been their predicted inability to survive in a competitive environment, a consequence of their extra metabolic load. Experiments in soil examining the survival of genetically manipulated bacteria have shown a rapid decline in the number of bacteria with time (9, 27) and reflect the differing biotic pressures in each environment.

In this study, we monitored the growth of two engineered strains of *L. plantarum* NCDO (National Collection of Dairy Organisms) 1193 and compared their persistence with that of the parent strain. To facilitate recovery of the target organisms against a diverse microbiological background, spontaneous rifampin resistance was used throughout as a chromosomal marker. The genetically engineered strains retained the capacity to dominate the proliferation of the microflora during the ensilage process.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Cultures were all derived from *L. plantarum* NCDO 1193. The recombinant carrying plasmid pM25 was prepared by the electroporation of *L. plantarum* with the free plasmid pM25 (2). The recombinant with the plasmid vector pSA3 integrated onto the

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chromosome was prepared by the method of Rixon et al. (17). Both of these recombinant strains were resistant to erythromycin (10 μg/ml).

Spontaneous rifampin-resistant mutants of the recombinant and parent bacteria were selected on MRS agar containing rifampin at 100 μg/ml. Lactobacilli were grown in MRS (5) broth or agar (MRS broth plus 2% agar; Oxoid Ltd., Basingstoke, United Kingdom). Inoculants for the minisilo experiment were prepared by growing the respective organism to stationary phase in MRS broth and harvesting the cells by centrifugation at 8,000 × *g* for 10 min. After two washes in sterile distilled water to remove residual medium and antibiotic, the cells were diluted to a volume 100 times that of the original culture.

Silo studies. The ensiling study was performed by using as microcosms sterile 100-ml polypropylene tubes sealed with rubber bungs and silica gel. A valve system was placed in the bung to permit the release of excess fermentation gases (20). In this experiment, however, no excessive fermentation gases were produced, although fermentation was apparent, as indicated by the rise in temperature of the silos and the reduction in pH. Four treatments were studied: an uninoculated control (U), the *L. plantarum* 1193 parent strain (L), *L. plantarum* 1193 transformed with plasmid pM25 (P), and *L. plantarum* 1193 with pSA3 integrated into the chromosome (S). A sample of grass (5 kg [fresh weight] with dry matter of 265 g/kg of fresh weight) consisting predominantly of perennial ryegrass (*Lolium perenne*) was obtained from Cockle Park Experimental Farm (Morpeth, Northumberland, United Kingdom). The sample was divided into four equal portions in large plastic bags and inoculated with 50 ml of inoculant, to give 10^6 CFU/g (fresh weight) of grass. To the uninoculated material, 50 ml of sterile distilled water was added. After shaking for 10 min, 50-g samples were packed tightly into the minisilos. Silos were prepared in triplicate for each treatment and for each sampling times (1, 3, 6, 9, 12, 15, and 30 days after filling). Day 1 samples refer to those silos that were filled and sampled immediately to determine the efficiency of extraction of the added bacteria (24). The uninoculated (day 0) samples provided an indication of the background rifampin-resistant bacteria. Silos were sealed and maintained at 18°C. At the appropriate opening times, 5-g (fresh weight) subsamples were taken from each silo, and the bacteria were extracted by a repeated differential centrifugation technique (8, 24).

L. plantarum in the inoculum was enumerated on MRS agar. LAB in the silage extracts were counted on MRS agar and on MRS agar plus rifampin (100 μg/ml). For each treatment at each sampling interval, three silos were sampled once, and the extracts were enumerated in triplicate. To determine the proportion of rifampin-resistant LAB that were erythromycin resistant, 100 colonies from each treatment at each sampling time were plated onto MRS agar containing erythromycin. Erythromycin resistance would, in the case of treatments P and S, indicate the presence of the plasmid sequences conferring erythromycin resistance, whereas treatments U and L would provide an indication of any background erythromycin resistance. Plates were incubated at 37°C and counted after 48 h.

To confirm that the antibiotics had been an adequate means of detecting the bacterial strains in the silage extracts, 10 colonies were selected from the rifampin-containing plates, for treatments P and S at day 9, and restreaked on MRS containing erythromycin (10 μg/ml). These isolates were then cultured in 10 ml of MRS broth without antibiotics. For Southern hybridization, total DNA was then extracted from each culture (28). Total bacterial DNA was restricted with *Bam*HI and resolved by electrophoresis on an 0.8% agarose gel in Tris-acetate buffer (13). DNA fragment size standards (lambda DNA digested with *Hin*dIII) were obtained from GIBCO BRL (Life Technologies, Paisley, United Kingdom). The gel DNA was transferred to nylon membranes (Hybond N blotting membranes; Amersham Life Sciences, Amersham, United Kingdom), using standard procedures (10). DNA was fixed to the filters by heating at 80°C for 2 h. Hybridization was carried out in 10 ml of hybridization fluid (50% formamide, 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.5% sodium dodecyl sulfate [SDS], 5× Denhardt's solution [10], 0.01% salmon sperm DNA). Filters were hybridized overnight at 42°C. Plasmid pSA3 was used as a probe for the cultures derived from both the S and P treatment plates. The plasmid (1 to 2 μg) was linearized with *Bam*HI and labelled with 50 μCi of [α-^{32}P]dCTP (Amersham Corp.) by the random primer method (10). The labelled probe was added to the hybridization fluid and hybridized for a further 16 h at 42°C. The filters were washed at room temperature twice for 10 min with 2× SSC in 0.1% SDS and then twice for 20 min at 65°C with 0.1× SSC and 0.1% SDS. Filters were washed in 1× Tris-acetate buffer (33 mM Trizma base, 1 mM EDTA) and exposed to X-ray film at −70°C for 48 h.

Statistics. Comparisons between microbial counts were performed at each time interval by using one-way analysis of variance. Significant differences between the treatments were detected by using Tukey's multiple-range test.

TABLE 1. Enumeration of bacteria in initial inocula and recovery immediately after inoculation

Treatment[a]	Log CFU/g of grass ± SEM	Recovery[a] ± SEM
L	6.16 ± 0.179	129 ± 35
P	6.14 ± 0.058	93 ± 12
S	6.27 ± 0.028	115 ± 10
U		

[a] Recovery = (inoculant bacteria extracted/inoculant bacteria added) × 100.

RESULTS

Inoculum potential and recovery from silage. The mean counts for the inoculant cultures (n = 9) and the recoveries of the added bacteria are shown in Table 1. The cultures were initially estimated by using previously prepared turbidity curves; the final counts, which were used for statistical purposes and shown here, were determined by plate counts, using MRS agar. The recoveries of the added cultures were between 93 and 129%.

Effect of inoculation on silage pH. The three inoculant-treated sets of minisilos had pH values lower than those of the untreated silos after 3 days of fermentation. The pH of the untreated herbage, however, did not start to decline until 6 days after ensilage. This difference was maintained up to 15 days postensiling and is clearly represented in Fig. 1. This observation indicates that the inoculants were successful in initiating a more rapid fermentation.

Growth of bacteria. Enumeration of LAB early in the experiment showed that the numbers of inoculum bacteria were greater than numbers of the indigenous LAB. The counts of LAB (Fig. 2) rapidly increased from log 6.2 to log 8.2 CFU/g of grass for the treated grass and from log 5.4 to

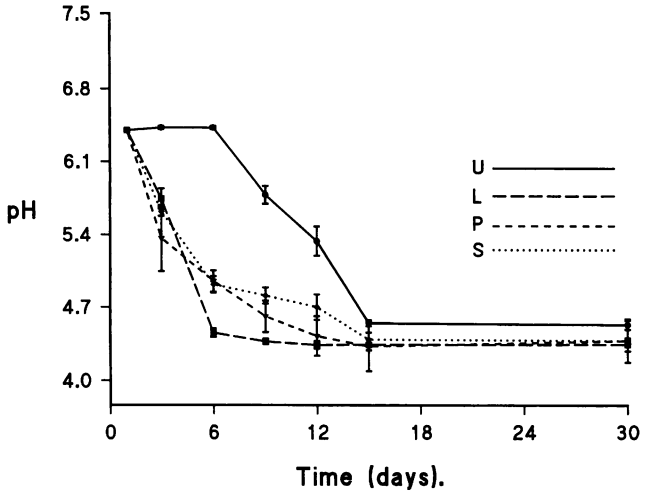

FIG. 1. Decline in pH of each silage treatment.

log 7.6 CFU/g of grass in the uninoculated grass within 3 days of ensilage. The values plotted are the means of single samples from triplicate silos, which were each enumerated in triplicate on MRS agar (uninoculated samples) and MRS agar with rifampin (100 μg/ml) added (inoculated samples). The day 1 inoculated samples were calculated from the counts obtained on MRS agar plus rifampin minus the counts obtained with the untreated samples, enumerated on MRS agar with rifampin added.

The counts of LAB were significantly greater ($P < 0.05$) for all of the treated silos than for the untreated silos on day 1 and day 3, after which time the epiphytic LAB in the untreated silos had increased to the level in the treated silos. Interestingly, on day 9 the LAB counts for treatment P were significantly lower ($P < 0.05$).

Generally, the counts of LAB enumerated on MRS agar were for all silos greater than those on MRS plus rifampin. That the rifampin-resistant strains constituted the major proportion of the total LAB in the treated silos is demonstrated in Fig. 3 for treatments L, P, and S. This finding further supports the suggestion that both of the genetically modified inoculants were successful in dominating the indigenous microflora throughout the fermentation.

Detection of genetically manipulated *L. plantarum* by antibiotic resistance and Southern hybridization with extracted DNA. Numbers of indigenous epiphytic bacteria showing rifampin resistance (determined for the untreated silos) were less than 10^4 CFU/g of grass throughout the experiment (Fig. 2). This level was sufficiently low for us to assume that with use of rifampin, the bacteria detected in the extracts from the treated silos were derived from the inoculum. The proportions of rifampin-resistant colonies in both the control (U) and inoculated (L) treated extracts, which retained erythromycin resistance, were shown to be 4% ± 1.32% and 2.8% ± 1.3%, respectively. Isolates from treatment S were 100% erythromycin resistant. The proportion of colonies showing erythromycin resistance for the silos inoculated with treatment P is shown in Fig. 4; after 30 days, 85% of the isolates showed erythromycin resistance.

The presence of pSA3 and pM25 sequences in these isolates was confirmed by Southern hybridization. All of the colonies tested carried DNA sequences homologous to the probe pSA3 sequence and indicated that the antibiotic selection system had been successful, since antibiotic-resistant bacteria carried DNA sequences which showed homology to the probe pSA3. The presence of the *Clostridium thermocellum* endoglucanase gene in pM25 does not appear to interfere with hybridization of pSA3 to target DNA in treatment P, either in these or in similar experiments performed previously in this laboratory. The agarose gels of the extracted DNA and the corresponding bands which were labelled by the probe after hybridization are shown in Fig. 5.

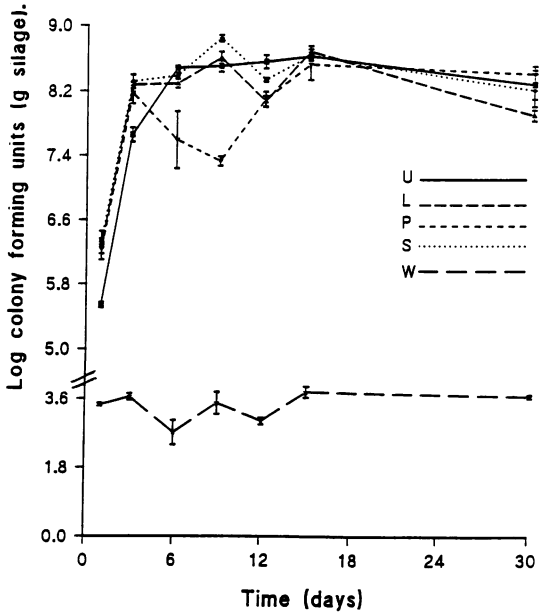

FIG. 2. Mean counts of LAB (per gram of silage) enumerated on MRS. Treatment U was enumerated on MRS agar only; treatments L, P, and S were enumerated on MRS agar with rifampin (100 μg/ml) added. W, rifampin-resistant endogenous bacteria.

DISCUSSION

This report shows that two genetically manipulated recombinants of *L. plantarum* 1193, an LAB frequently used as a silage additive, proliferate during the ensiling process. This result was particularly surprising in view of the fact that silage is an open ecosystem in which competitive pressures are in operation. One advantage for the genetically manipulated bacteria in this experiment was the level of inoculum. The numbers of epiphytic LAB (10^5 CFU/g of grass) were within the ranges previously reported (18), estimated by using herbage samples obtained from commercial farms. It has been recognized that to dominate any silage fermentation, an inoculant should be applied at a rate equal to or exceeding that of the LAB count on the grass to which it is to be applied (16, 18). In the current experiment, the levels of epiphytic LAB were approximately 10^5 (log 5.6) CFU/g of grass ensiled; thus, an inoculum of 10^6 CFU/g of grass provided an ideal level of inoculum. Prior to this study, inoculants of LAB in silage have been shown to be successful in dominating the fermentation only by experiments monitoring the monosaccharide and lactic acid content compared with that of untreated silages (19, 23). This experiment, however, demonstrates by a much more direct technique that all three inoculants added in treatments L, P, and S were dominant throughout the fermentation.

Recovery of the bacterial amendment was between 93 and 129% for treatments L, P, and S. The variation associated

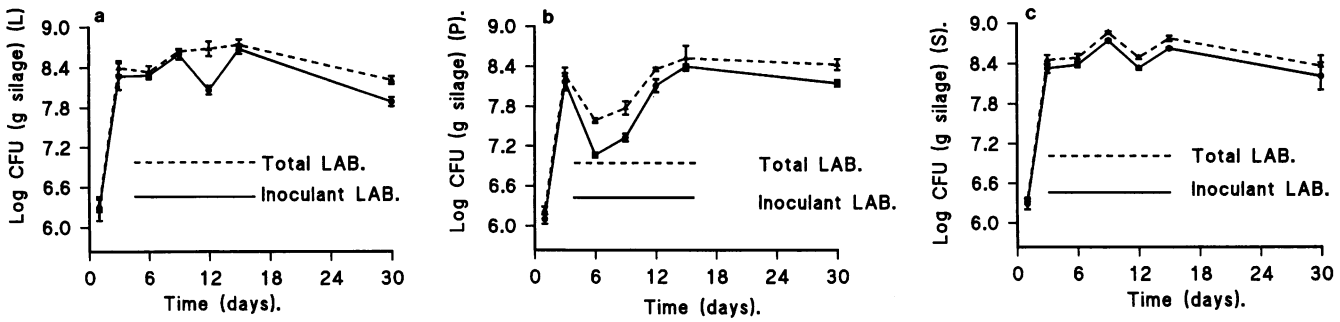

FIG. 3. Comparison of log mean counts of LAB (per gram of silage) for each treatment enumerated on MRS and on MRS plus rifampin (100 μg/ml). (a) Treatment L; (b) treatment P; (c) treatment S.

with these values was quite high; however, the values correspond to those previously observed (24). Such high recovery values may not be maintained throughout the experiment, as bacteria form a stronger association with the grass as the fermentation progresses.

A measure of pH in silage is an accepted indication of the extent and type of fermentation that has occurred in silage (22). A rapid reduction in pH to 3.5 to 4.5 indicates a predominantly lactate fermentation, dominated by homofermentative LAB. A reduction in pH to approximately 4.5 was achieved in the treated silos; although the final pH of the untreated silos was the same, it was attained at a slower rate, indicating that the epiphytic LAB were predominantly heterofermentative.

By comparing the growth curve (Fig. 2) and the pH curve (Fig. 1), it is apparent that little or no reduction in pH was achieved during growth of LAB in the untreated silos, providing further evidence that inefficient heterofermentative LAB dominated these silos or indicating that the enumeration of LAB by using MRS includes bacteria that are not LAB. Previous estimates of the selectivity of MRS for LAB have shown that only 61% of isolates recovered from MRS agar were in fact LAB (3). However, this was not the case for the treated silos, in which the proliferation of LAB was associated with a rapid reduction in silage pH.

The emphasis of the study was to elucidate the survival of the genetically manipulated bacteria rather than to study the efficiency of the constructs used. The presence of a cellulase-producing plasmid in treatment P silos could be seen as a factor affecting the survival of treatment P bacteria; however, it should be recognized that the construct contains a poor promoter and that pM25 is a low-copy-number plasmid. Thus, it is unlikely that expression of the protein will be an excessive metabolic load for the bacteria or, in contrast, confer any advantage to the bacteria in terms of release of substrate from cellulose. The reduction in the numbers of treatment P bacteria at day 9 is of interest, especially in view of the fact that the numbers increase again in parallel with the other treatments. The proportion of colonies that are derived from the inoculant is shown in Fig. 3c; it is evident that it was not just the number of inoculant bacteria that declined but the number of total LAB. Thus, it may be that the environment of the silos varied in some manner, although it should be noted that the silos were placed randomly in a constant-temperature environment.

This report also describes a method for detecting genetically manipulated *L. plantarum* in silage by using a dual-antibiotic selection system. The efficiency of this approach was confirmed by Southern hybridization. Unfortunately, this technique only indicates the presence of specific DNA sequences having homology with the probes used. Southern hybridization does, however, provide an indication of the size of the DNA fragment showing homology with the probe. For treatment S, in which pSA3 was inte-

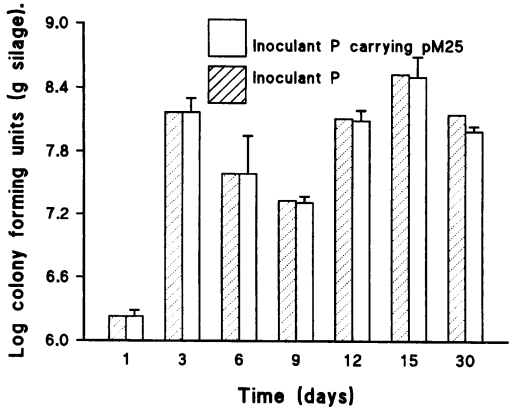

FIG. 4. Log mean counts of LAB (per gram of silage) for treatment P, enumerated on MRS and on MRS plus rifampin (100 μg/ml) and then restreaked on MRS plus erythromycin (10 μg/ml). The data give putative indication of the survival of plasmid pM25 in the silage ecosystem.

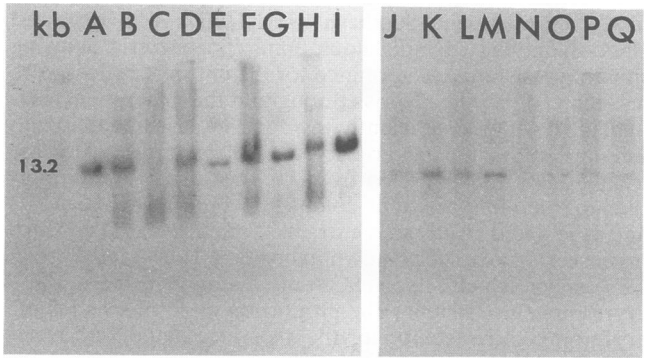

FIG. 5. Confirmation of the identity of the extracted bacteria. *L. plantarum* DNA for treatments P and S was digested with *Bam*HI and fractionated on a 0.8% agarose gel. The restriction fragments were transferred to a nylon membrane and probed with radiolabelled linearized pSA3 DNA. Size markers were from *Hin*dIII-digested lambda DNA. Lanes: A, linearized probe pSA3; B to I, treatment P DNA; J to Q treatment S DNA.

grated onto the genome, the size of the chromosomal DNA fragment digested with *Bam*HI and carrying the pSA3 sequence was identical to that of the inoculant and confirms that the colonies detected on the isolation plates were the same as those added. For treatment P, there was no means of detecting which strains of bacteria were carrying pM25, and transfer of the plasmid DNA could have occurred. However, the probability of the erythromycin resistance sequence being transferred by conjugation in the silage ecosystem to a rifampin-resistant bacterium must be very low, considering the low numbers of endogenous rifampin-resistant bacteria. Conjugation frequencies in *Lactobacillus* spp. are generally very low, even during agar or filter matings, when frequencies of transconjugants per donor or recipient are usually in the range of $1:10^{-7}$ to $1:10^{-5}$ (7).

For silos with treatment L (the parental strain), we relied solely on rifampin resistance as a means of detection, with no additional method used to confirm that the bacteria extracted and detected were the same as those added. By comparing the numbers of endogenous rifampin-resistant bacteria and inoculant rifampin-resistant bacteria (Fig. 2), it is obvious that there was a low probability of contamination.

This study has also provided a direct comparison of the survival rates of a free plasmid and a chromosomally integrated plasmid in the silage. The 100% survival rate of the chromosomally integrated plasmid (treatment S) could have been anticipated, as could the slow rate of loss of plasmid pM25. The plasmid has been shown to have a loss rate of 3% per generation in nonselective culture media (2), with only 1% of bacteria retaining the plasmid after 2 days. The slow rate of loss of the plasmid in minisilos may be explained by the difference in nutrient availability in the two environments; hence, the number of generations is likely to be much lower in the silage than in MRS broth. Similarly, the reduction in pH in the silage is likely to provide conditions in which bacteriostasis will occur, unlike in subculturing experiments (2). In this light, it is interesting that it was bacteriostatic and not bactericidal conditions that existed up to day 30.

This report clearly demonstrates that genetically manipulated recombinants of *L. plantarum* proliferate during the silage fermentation and remain viable for 30 days after a stable pH has been attained. This viability paralleled that of the epiphytic LAB. It would be of interest to determine how long this viability can be maintained in silage and in harsher environments such as soil and the rumen.

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